as 79.6%.[16] Moreover, the cost of these high-quality methods cannot be disregarded; DIA costs approximately twice that of PJC and about 3 to 4 times that of FISH. The costs of these tests add up and they require very special methodology and equipment. The technique for a routine clinical diagnosis should be simple, safe, rapidly performed, and relatively inexpensive to be widely used. From this viewpoint, the routine use of DIA and FISH in clinical practice remains impractical.

The sensitivity of BC seems to be better than that of pure PJC because, through brushing of the stricture, it is possible to get more cells than by simply collecting PJ.[18,19] However, the diameter of the brushing device is usually larger than that of the pancreatic duct; furthermore, the pancreatic duct is often tortuous and offers resistance to the stiffness of the instrument, leading to insufficient brushing of the stricture site. To overcome these disadvantages guide wire–aided brushing cytology has been performed.[20] According to our experience, the guide wire could be passed through the stricture in all the cases, but the brushing catheter could not be passed through in some of the strictures. In general, routine cytology is ended regardless of the quality of the sample, and PJC after brushing is less frequently performed routinely. In this study, we speculated that, after brushing, PJ contained many more cancer cells than before brushing, increasing the sensitivity of PJC for pancreatic cancer.

Thus, mechanical stimulation of the pancreatic duct stricture led to the release of cancer cells into the PJ. In fact, in the diagnosis of bile duct stricture, Mohandas et al[21] found that the cancer detection rate of bile cytology increased from 27% to 63% after dilation of the stricture. Also, Uehara et al[17] reported almost the same method to increase the sensitivity of cytology for pancreatic cancer. They used a 0.025-in hydrophilic guide wire to scrape the stricture site of the pancreatic duct before collecting PJ and showed an incredibly higher sensitivity of 93%. In our study, the sensitivity of PJC after brushing was 41% and significantly higher than the 21% sensitivity of PJC before brushing. Furthermore, of the 65 patients whose lesion was diagnosed as benign both by PJC before brushing and by BC, PJC after brushing showed malignancy in 16 patients.

There were no statistically significant differences in nondiagnostic value and sensitivity with regard to the location of the tumor. However, the diagnostic value for tumors in the head of the pancreas tends to be lower compared with that for tumors in the body and/or the tail.[22] This lower diagnostic value for tumors in the head is thought to be due to the short length of the pancreatic duct between the papilla and the tumor, which does not allow the collection of a sample with a sufficient number of cells. And the sensitivity of BC for pancreatic cancer tends to be lower in case of tumors located in the body/tail compared with that for tumors in the head, probably because the longer distance from the papilla to the stricture site often prevents the proper introduction of the brushing instrument or because of failure of the brushing procedure.

Secretin is conventionally administered before collecting PJ for cytological examination.[6,18] However, secretin has not been commercially available in Japan since 2005. Under such situation, cytology was nondiagnostic for 29 patients with PDAC in whom PJ collection before BC was inadequate. But we obtained PJ containing a sufficient number of tumor cells without secretin after brushing, although the volume of PJ was not as much as that obtained subsequent to secretin administration. Nonetheless, the volume of PJ was not enough for cytological examination in some patients, and it was usually necessary to inject 1 to 3 mL of sterile saline solution to flush the pancreatic duct.

Brush cytology combined with PJC after brushing showed 61% sensitivity for pancreatic cancer; this percentage was equal or higher than that obtained with most ancillary methods. It is true that a sensitivity of 61% is not satisfactory; yet diagnosis of BC plus PJC after brushing ads on reported ancillary methods such as DIA or FISH will much improve the sensitivity for pancreatic cancer.

Complications associated with PJ collection or brushing of the stricture were rare, but the rate of pancreatitis was higher than after ERCP alone.[23] Careful attention must be paid to avoid violation of the procedure protocol or excessive increase in the pancreatic duct pressure so that pancreatitis due to iatrogenic causes does not occur.

In conclusion, we recommend BC plus PJC after brushing as routine cytology during ERCP for the diagnosis of pancreatic cancer to increase the sensitivity of the test.

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The cellular level of histone H3 lysine 4 dimethylation correlates with response to adjuvant gemcitabine in Japanese pancreatic cancer patients treated with surgery

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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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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.

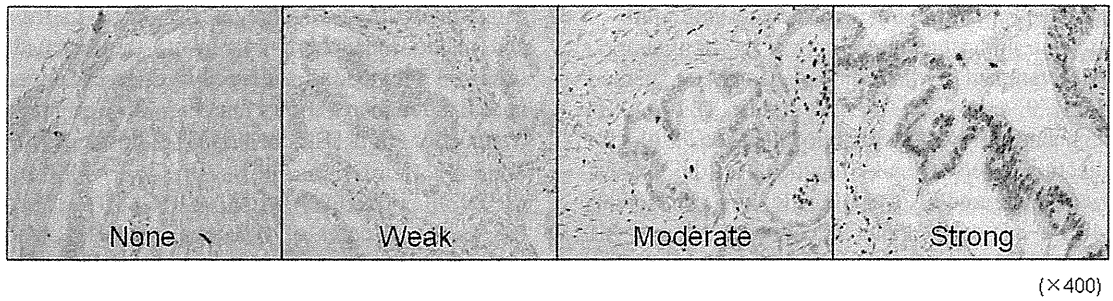

Figure 1. Representative examples of H3K4me2 immunohistochemical staining in pancreatic cancer tissues. Demonstrative images for each criterion are shown. Scale-bars: 100 μm.

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

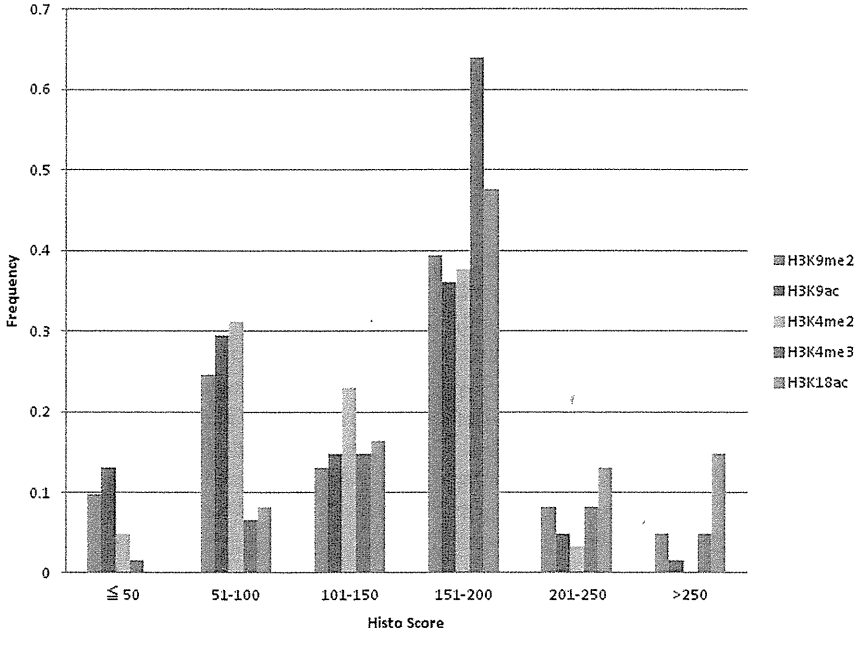

Figure 2. Histograms showing the distribution of H-scores plotted against the number of cases exhibiting the histone modifications.

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

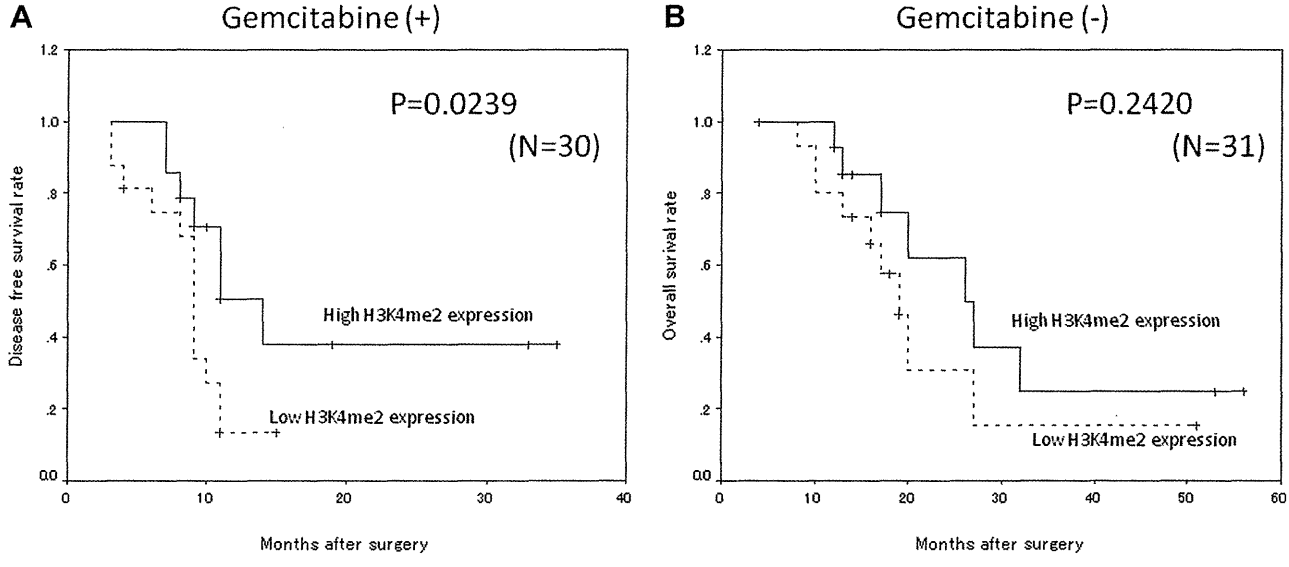

Figure 3. Disease-free survival according to postoperative chemotherapy. The low H3K4me2 expression group was significantly associated with the worse DFS in group A.

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.

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ORIGINAL ARTICLE

Factors affecting the diagnostic accuracy of endoscopic ultrasonography-guided fine-needle aspiration (EUS-FNA) for upper gastrointestinal submucosal or extraluminal solid mass lesions

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Aim: A number of potential variables are associated with the diagnostic accuracy of endoscopic ultrasonography-guided fine-needle aspiration (EUS-FNA). The aim of this study was to evaluate factors affecting the diagnostic accuracy of EUS-FNA for upper gastrointestinal submucosal or extraluminal solid lesions.

Methods: Patients with such lesions who underwent EUS-FNA between January 2009 and December 2010 were studied retrospectively. Needles of 22, 25 and 19 gauge were used. The associations between the EUS-FNA results and factors such as mass location, mass size, needle size, number of needle passes, combined histologic-cytologic analysis and final diagnosis were analyzed.

Results: A total of 170 EUS-FNA procedures were performed in 158 patients with upper gastrointestinal submucosal or extraluminal solid lesions. The overall accuracy of EUS-FNA was 86.5% (147/170). The diagnostic accuracy with three or more needle passes was higher than with less than 3.0 needle passes (90.0%, 108/120 *vs* 78.0%, 39/50; $P < 0.05$). Mass location, mass size, and final diagnosis were not associated with EUS-FNA accuracy. Combined cytologic-histologic analysis had significantly higher diagnostic accuracy than either cytologic or histologic analysis alone ($P < 0.001$). In a subgroup of 90 patients, both 22 and 25 gauge needles were used for EUS-FNA. The overall diagnostic accuracy was similar for 25 gauge needles and 22 gauge needles (80.0% *vs* 78.9% $P = 1.000$) in this subgroup.

Conclusion: Overall, 25 and 22 gauge needles have a similar diagnostic accuracy. Our results suggest that 3.0 or more needle passes and combined cytologic-histologic analysis enhance the diagnostic accuracy of EUS-FNA.

Key words: endoscopic ultrasonography (EUS), EUS-guided fine-needle aspiration (EUS-FNA), pancreatic mass, submucosal tumor.

INTRODUCTION

Endoscopic ultrasonography-guided fine-needle aspiration (EUS-FNA) has been established as a minimally invasive technique for sampling lesions, such as gastrointestinal submucosal tumors, pancreatic tumors, enlarged abdominal and mediastinal lymph nodes, and mediastinal masses, arising in the gastrointestinal tract and adjacent organs. The accuracy of EUS-FNA for the cytologic or histologic diagnosis of such lesions ranges from 80% to 90%.[1–5] A number of variables including target lesion location, the endoscopist's skill, needle size, FNA technique and the technical preparation of cytologic specimens are associated with the diagnostic accuracy of EUS-FNA.[6] However, the association between these factors and the accuracy of EUS-FNA for diagnosis is poorly understood. For example, the results of several studies comparing diagnostic accuracy between 22 and 25 gauge needles remain inconclusive.[7–9] Furthermore, most of these studies on needle size focused on solid pancreatic masses. Many endoscopists are now attempting to further enhance the diagnostic accuracy of EUS-FNA, but it remains unclear whether FNA should be performed with a specific technique according to the location and expected type of tumors. We retrospectively reviewed the results of EUS-FNA in patients not only with pancreatic masses, but also in those with upper gastrointestinal submucosal tumors. Our main aim was to identify factors affecting the diagnostic accuracy of EUS-FNA. In addition, in a subgroup of 90 patients, both 22 and 25 gauge needles were used for EUS-FNA to perform a cross-over study. Our secondary aim was to compare the diagnostic accuracy of 22 and 25 gauge needles.

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METHODS

Patients

Patients referred for EUS-FNA of unconfirmed upper gastrointestinal submucosal or extraluminal solid lesions from January 2009 through December 2010 were studied retrospectively. All procedures were performed by four physicians and were supervised by one experienced endoscopist.

Patients who received EUS-FNA to treat cystic lesions were excluded from this study. Data were collected on patient demographics, radiographic findings (including ultrasonography, computed tomography, magnetic resonance imaging and endoscopic retrograde cholangiopancreatography), EUS-FNA procedural details (e.g. tumor characteristics, needle size and number of needle passes), pathological results (including cytologic and histologic results) and follow-up.

EUS technique

EUS-FNA was performed using a curved-linear echoendoscope (GF-UC2000P, GF-UCT240-AL5 or GF-UCT260; Olympus Medical Systems, Tokyo, Japan). Patients were sedated with intravenous midazolam and pethidine, sometimes in combination with propofol.

Puncture was performed with the use of a 22 gauge (Olympus Medical Systems), 25 gauge (Echotip; Wilson-Cook, Winston-Salem, NC, USA) or 19 gauge needles (Echotip; Wilson-Cook). Both 22 and 25 gauge needles were used in 90 patients.

During each puncture, the needle was inserted into the lesion about 20 times with a prefixed suction syringe. Twenty milliliter syringes were used for 22 gauge needles, and 10 mL syringes were used for 25 gauge needles. During all EUS-FNA procedures, tissue acquisition was confirmed macroscopically. On-site cytopathologic assessment was not performed in any patient. The procedure was completed when the endoscopist deemed that adequate material was obtained macroscopically.

Histologic and cytologic preparation

The aspirated specimens were pushed out into a plastic tube filled with normal saline. Saline containing the aspirated material was transferred to a Petri dish and examined macroscopically. Reddish masses (coagula with tumor tissue) and whitish masses (tumor tissue) were picked up with forceps, fixed in 20% buffered formalin and embedded in paraffin for histologic preparation. The remaining saline was collected. After adding one drop of 1% albumin solution, the saline solution was processed to obtain cytospin preparations of cells. The cells were coated on slides, fixed in 95% ethanol overnight and stained with Papanicolaou stain. If the cells were of unclear histogenesis (e.g. suspected neuroendocrine differentiation, gastrointestinal stromal tumor [GIST] or lymphoma), immunocytochemical and immunohistochemical studies were performed.[10]

Diagnostic interpretation

Each aspirated specimen was considered adequate for cytologic examination if it contained cells from the target organ or lesions and for histologic examination if it contained a coherent tissue specimen from the target organ. Specimens that contained inadequate material were not excluded from our analysis of diagnostic accuracy, but were classified as negative results because they could not provide an accurate diagnosis.

The cytologic specimens were classified as malignancy, suspected malignancy, atypical or negative for malignancy. A classification of malignancy or suspected malignancy was considered to indicate malignant disease. A classification of atypical or negative for malignancy was considered to indicate benign or inflammatory disease. Immunocytochemical and immunohistochemical studies were performed if lesions were suspected of being neuroendocrine tumors, leiomyomas, GIST or lymphomas.

The final diagnosis was based on the unequivocal results of EUS-FNA (including repeat EUS-FNA), histologic diagnosis from other sources such as the histopathological examination of surgical specimens, cytologic examinations during endoscopic retrograde cholangiopancreatography, and the results of follow-up radiologic studies. Inoperable pancreatic cancers, which were treated by chemotherapy, were diagnosed mainly during the six or more follow-up visits throughout the clinical course and radiologic studies. Furthermore, in lesions thought to be benign (e.g. focal pancreatitis, benign lymphadenopathy), clinical follow-up of at least 6 months with negative repeated imaging and/or a clinical course compatible with benign disease was necessary.

Patients in whom final diagnoses could not be obtained were excluded. In our study, accuracy was defined as the concordance between the diagnosis on EUS-FNA and the final diagnosis.

Statistical analysis

Multivariate logistic regression analysis was performed to identify variables affecting diagnostic accuracy. The McNemar χ^2 test was used for pair-wise comparison of 22 and 25 gauge needles. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

A total of 158 patients who underwent 170 EUS-FNA procedures were studied. Six patients were excluded because the final diagnosis could not be confirmed. The baseline characteristics of the patients and lesion characteristics are summarized in Table 1. The mean age of the 158 patients was 63.4 years (range: 28–88), and the sex ratio was 1.1 men: 1 women. The mean number of needle passes per procedure was 3.0 (range: 1.0–6.0). Repeat EUS-FNA procedures were performed in 12 patients. Ninety procedures were performed with both 22 and 25 gauge fine needles, including three procedures in which 19 gauge fine needles were also used. Of the remaining procedures, 21 procedures were performed with 25 gauge needles, 35 with 22 gauge needles, 8 with 19 gauge fine needles, and 16 with both 22 and 19 gauge fine needles. There were no clinically significant complications.

The mean lesion size was 3.91 ± 2.59 cm (range: 1.0–18.0 cm). The lesions consisted of 101 pancreas masses, 46 submucosal tumors (including 8 cases arising in the esophagus, 33 cases in the gastric submucosal layer and 5 cases in duodenum) and 23 abdominal or mediastinal enlarged lymph nodes or abdominal masses. In the 90 procedures performed with both 22 and 25 gauge needles, the mean lesion size was 3.59 ± 1.63 cm (range: 1.1–12.0 cm). The lesions consisted of 54 pancreatic masses, 27 submucosal tumors (including 3 cases in the esophagus, 23 cases in the gastric submucosal layer and 1 case in duodenum) and 9 abdominal or

Table 1. Baseline and procedural factors and multivariate analysis of the effects of these factors on the diagnostic accuracy of EUS-FNA

	Baseline and procedural factors	Accurate diagnosis FNA procedures (*n*)	*P*-value
Sex (men : women)	1.1:1		
Mean age, year (range)	63.4 (28–88)		
Location of mass (*n*)			
Pancreas	101	87 (86.1%)	0.485
SMT	46	37 (80.4%)	
Abdominal LN	23	23 (100%)	
Size, mean ± SD (cm)	3.91 ± 2.59		0.486
≥3.0 (*n*)	111	95 (85.6%)	
<3.0 (*n*)	59	52 (88.1%)	
Number of passes, mean ± SD (*n*)	3.0 ± 0.94		0.024
≥3.0	120	108 (90.0%)	
<3.0	50	39 (78.0%)	
Combined histologic-cytologic analysis (*n*)	170	147 (86.5%)	
Cytologic analysis (*n*)	170	124 (72.9%)	<0.001*
Histologic analysis (*n*)	170	97 (57.1%)	<0.001*
Diagnosis (*n*)			
Pancreatic carcinoma	79	67 (84.8%)	0.854
Pancreatic neuroendocrine tumor	9	7 (77.8%)	
Focal pancreatitis†	13	13 (100%)	
GIST	24	20 (83.3%)	
Leiomyoma	14	11 (78.6%)	
Ectopic pancreas	2	2 (100%)	
Glomus tumor	1	1 (100%)	
Hamartoma	1	0	
Neurilemmoma	1	0	
Lymphoma	5	5 (100%)	
Carcinoid	1	1 (100%)	
Lipoma	1	1 (100%)	
Lung adenocarcinoma	1	1 (100%)	
Malignant lymphadenopathy	13	13 (100%)	
Benign lymphadenopathy	5	5 (100%)	
Total (*n*)	170	147 (86.5%)	

*McNemar χ^2test.
†Includes two cases of autoimmune pancreatitis.
EUS-FNA, endoscopic ultrasonography-guided fine-needle aspiration; GIST, gastrointestinal stromal tumor; LN, ; SMT, .

mediastinal enlarged lymph nodes or abdominal masses. The final diagnoses are shown in Table 1.

The numbers of FNA procedures providing accurate diagnoses and the results of multivariate logistic regression analysis of the association of baseline and procedural factors with the diagnostic accuracy of EUS-FNA are shown in Table 1. The overall diagnostic accuracy rate was 86.5% (147/170). Diagnostic accuracy with 3.0 or more needle passes was higher than with less than 3.0 needle passes (90.0%, 108/120 *vs* 78.0%, 39/50; $P < 0.05$). Mass location, mass size and final diagnosis were unrelated to the diagnostic accuracy of EUS-FNA. Combined cytologic-histologic analysis provided significantly better diagnostic accuracy than either cytologic or histologic analysis alone ($P < 0.001$).

A total of 90 EUS-FNA procedures were performed using both 22 and 25 gauge needles, and each needle performed 1.0 or 2.0 passes. The cytologic and histologic results of these procedures are shown in Table 2. The overall diagnostic accuracy of these 90 procedures was 88.9% (80/90). The overall diagnostic accuracy of EUS-FNA using 25 gauge needles was similar to that of EUS-FNA using 22 gauge needles (80.0% *vs* 78.9%; $P = 1.000$).

Table 2. Comparison of EUS-FNA between 25 gauge and 22 gauge needles

	25 gauge	22 gauge	*P*-value
Cytologic adequacy	98.9% (89/90)	100% (90/90)	n.s.
Cytologic accuracy	75.6% (68/90)	68.9% (62/90)	0.109
Histologic adequacy	61.1% (55/90)	73.3% (66/90)	0.026
Histologic accuracy	33.3% (30/90)	41.1% (37/90)	0.265
Combined cytologic–histologic accuracy	80.0% (72/90)	78.9% (71/90)	1.000
Total diagnosis accuracy	88.9% (80/90)		–

EUS-FNA, endoscopic ultrasonography-guided fine-needle aspiration; n.s., not significant.

The rate of acquiring adequate material for histologic evaluation was significantly higher for 22 gauge needles than for 25 gauge needles (73.3% *vs* 61.1%; $P < 0.05$). Overall, cytologic adequacy, cytologic accuracy and histologic accuracy did not differ significantly between 22 gauge needles and

Table 3. Comparison of EUS-FNA of pancreatic solid masses between 25 gauge and 22 gauge needles

	25 gauge	22 gauge	*P*-value
Cytologic adequacy	100% (54/54)	100% (54/54)	n.s.
Cytologic accuracy	81.5% (44/54)	74.1% (40/54)	0.219
Histologic adequacy	61.1% (33/54)	70.4% (38/54)	0.332
Histologic accuracy	27.8% (15/54)	33.3% (18/54)	0.629
Combined cytologic-histologic analysis accuracy	83.3% (45/54)	79.6% (43/54)	0.727
Total diagnosis accuracy	88.9% (48/54)		–

EUS-FNA, endoscopic ultrasonography-guided fine-needle aspiration; n.s., not significant.

Table 4. Comparison of EUS-FNA of SMT between 25 gauge and 22 gauge needles

	25 gauge	22 gauge	*P*-value
Cytologic adequacy	96.3% (26/27)	100% (27/27)	n.s.
Cytologic accuracy	55.6% (15/27)	48.1% (13/27)	0.625
Histologic adequacy	55.6% (15/27)	74.1% (20/27)	0.180
Histologic accuracy	48.1% (13/27)	59.3% (16/27)	0.549
Combined cytologic-histologic analysis accuracy	66.7% (18/27)	70.4% (19/27)	1.000
Total diagnosis accuracy	85.2% (23/27)		–

EUS-FNA, endoscopic ultrasonography-guided fine-needle aspiration; n.s., not significant; SMT, submucosal tumors.

25 gauge needles. Moreover, the cytologic adequacy, cytologic accuracy, histologic adequacy and histologic accuracy were similar for 22 and 25 gauge needles not only for solid pancreatic mass lesions, but also for submucosal tumors (Tables 3, 4).

DISCUSSION

In the present study, the results of 170 EUS-FNA procedures in patients with upper gastrointestinal submucosal or extraluminal solid mass lesions were analyzed retrospectively. The overall accuracy was 86.5%, consistent with the results of previous studies.[1–5]

A number of factors are associated with the diagnostic accuracy of EUS-FNA. Many previous studies have analyzed such factors and discussed how to further enhance the accuracy of EUS-FNA. Determinants of diagnostic accuracy include the number of needle passes, needle size (higher accuracy with 19, 22 and 25 gauge fine needles), combined histologic-cytologic analysis,[11] on-site cytologic evaluation,[12] and repeat EUS-FNA.[13]

In our study, multivariate analysis showed that 3.0 or more needle passes resulted in better diagnostic accuracy. Indeed, the impact of the number of needle passes on the diagnostic accuracy of EUS-FNA remains somewhat controversial. Turner *et al.* reported that only a small number of needle passes (i.e. 2.0–3.0) were required to obtain relatively good accuracy for pancreatic neoplasia, even without on-site cytopathologic evaluation.[2] LeBlanc *et al.* recommended that a median number of 3.4 passes should be performed for all indications.[14] In our study, we did not have an on-site pathologist, so the procedure was completed when the endoscopist deemed that adequate material was obtained macroscopically. An average 3.0 (range: 1.0–6.0) needle passes per procedure were performed. Difficult lesions tend to need more needle passes. For the lesions located on the pancreatic head or small lesions, 5.0 or even 6.0 needle passes were performed to get adequate material. As a result, the diagnostic accuracy with 3.0 or more needle passes was significantly higher than that with less than 3.0 needle passes. Therefore, we concluded that 3.0 or more needle passes are necessary to ensure the diagnostic accuracy of EUS-FNA for upper gastrointestinal submucosal or extraluminal solid lesions.

Most previous studies of EUS-FNA have focused on cytologic analysis and discriminating between malignant and benign lesions. However, cytologic analysis of EUS-FNA specimens may be associated with specific disadvantages, such as a limited yield, especially when the objective is to distinguish between different tumor types. Only a few reports have assessed the diagnostic value of combined cytologic-histologic analysis. Moller *et al.* reported that combined histologic-cytologic analysis achieved a sensitivity of 82.9% and that histologic analysis alone had a lower sensitivity than cytologic analysis alone (60% *vs* 68.1%) in patients who underwent EUS-FNA for pancreatic masses.[11] The results of our study similarly showed that combined cytologic-histologic analysis had a diagnostic accuracy of 86.5%, which was significantly higher than that of either cytologic analysis (73.5%) or histologic analysis (57.1%) (both $P < 0.001$). Moreover, histologic analysis provided specific information that assisted diagnosis, especially for rare tumors such as pancreatic endocrine tumors, submucosal glomus tumors, hamartomas and neurilemmomas. Therefore, we recommend histologic-cytologic analysis for EUS-FNA.

In our study, repeat EUS-FNA procedures were performed in 12 patients. The final diagnosis was pancreatic carcinoma in eight patients, a pancreatic neuroendocrine tumor in one, a duodenal GIST in one, and B-cell lymphomas in two. In the eight patients with pancreatic carcinoma, repeat EUS-FNA was performed using the same gauge needle as the prior procedure, and final diagnoses were obtained in six patients. For the other four patients receiving repeat EUS-FNA, the repeat procedure was performed with 19 gauge fine needles, and histologic diagnoses were obtained. Our results suggest that repeat EUS-FNA may be worthwhile in patients with suspected pancreatic malignancy who have negative results on prior EUS-FNA or who require a detailed histologic diagnosis. This is supported by the findings of Nicaud *et al.*,[13] who reported that repeat EUS-FNA provides a reasonable diagnostic accuracy of 61% in patients with suspected pancreatic cancer who had negative results on prior EUS-FNA.

Needle size is one of the important determinants of the accuracy of EUS-FNA. At present, 19, 22 and 25 gauge fine needles are mainly used for EUS-FNA. Diagnostic FNA is most commonly performed with the use of 22 and/or 25 gauge needles.[6] Several previous studies compared these three gauges of EUS-FNA fine needles.[7,9,15,16] However, most previous studies concentrated on pancreatic masses, and

enrolled patients were usually divided into two groups and randomly assigned to one of the two gauges of needles. Although there were no statistically significant differences in baseline patient or tumor characteristics between the two groups, a diverse range of factors might still have affected the results. Furthermore, most previous studies focused solely on cytologic analysis. To avoid the drawbacks of prior studies, we performed a paired comparison analysis of the 90 procedures done with both 22 and 25 gauge needles, all of which were accompanied by combined cytologic-histologic analysis. Some experts have suggested that hemorrhage or tissue injury caused by passage of the first needle may influence the results of EUS-FNA performed with the second needle when different needles are used to aspirate tissue from the same lesion. We therefore randomly assigned the order of needle usage to minimize such order-related effects.

Our results showed that overall diagnostic accuracy was similar for 25 and 22 gauge needles (80.0% *vs* 78.9%; $P = 1.000$). Our results are similar to those of Siddiqui *et al.* and Imazu *et al.*[7,8] Although Sakamoto *et al.* reported that the overall diagnostic accuracy of 25 gauge needles was superior to that of 22 gauge needles,[15] and Yusuf *et al.* reported 25 gauge needles had higher sensitivity and negative predictive values than 22 gauge needles.[16] The former study included only 24 consecutive patients, and the latter did not conduct statistical analysis.

Additionally, our results showed cytologic adequacy, cytologic accuracy and histologic accuracy did not differ significantly between 25 and 22 gauge needles. However, the rate of acquiring adequate material for histologic evaluation was significantly higher for 22 gauge needles than for 25 gauge needles, suggesting that 22 gauge needles may have the advantage of acquiring more tissue. Perhaps a larger diameter needle can acquire more tissue and afford a more correct histologic diagnosis. Furthermore, 19 gauge needles were used to perform 27 procedures in twelve patients with submucosal tumors, two with neuroendocrine tumors, three with pancreatic carcinoma, four with lymphoma and six with focal pancreatitis. Correct histologic diagnoses were obtained in 23 procedures (85.2%) performed with 19 gauge needles.

Some investigators have recommended that needle size should be based on multiple factors, such as the location and expected type of tumor. Imazu *et al.* reported that specimen quantity was greater with 25 gauge needles for pancreatic lesions and with 22 gauge needles for submucosal tumors.[8] In addition, 25 gauge needles were found to be better suited for puncturing pancreatic masses, which tend to be hard. When lesions located in the head and uncinate process of the pancreas were punctured, smaller needles were easier to maneuver in angulated positions.[17] Sakamoto *et al.* reported that overall diagnostic accuracy was higher when EUS-FNA was performed with 25 gauge needles than with 22 gauge needles for lesions located in the head and uncinate process of the pancreas.[15] In contrast, large-diameter needles have the advantage of acquiring more tissue, which was more evident in patients with submucosal tumors, for which a large tissue specimen is often necessary for diagnosis by histologic evaluation with immunohistochemical analysis.

Unlike previous reports, our study showed that the cytologic adequacy, cytologic accuracy, histologic adequacy and histologic accuracy did not differ significantly between 22 and 25 gauge needles in the subgroups of patients with pancreatic masses and those with submucosal tumors. The limitations of our study, such as the small number of patients, retrospective design and performance at a single center, may account for the lack of a difference in these variables. Further studies are needed to clarify the rationale for the optimal use of EUS-FNA needles according to lesion characteristics.

In conclusion, our study showed that 22 and 25 gauge needles have similar overall diagnostic accuracy when used to perform EUS-FNA of upper gastrointestinal submucosal or extraluminal solid lesions. The diagnostic accuracy of EUS-FNA is likely to be enhanced by 3.0 or more needle passes in the lesion and the performance of combined cytologic-histologic analysis.

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Clinical impact of *K-ras* mutation analysis in EUS-guided FNA specimens from pancreatic masses (CME)

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Background: EUS-guided FNA (EUS-FNA) is considered optimal for differentially diagnosing pancreatic masses. However, the sensitivity of EUS-FNA ranges from 65% to 95%, respectively, which requires improvement.

Objective: To evaluate clinical impact of *K-ras* mutation analysis in EUS-FNA specimens from pancreatic masses.

Design: Prospective registration, single-center study.

Setting: Tertiary referral center.

Patients: This study involved 394 consecutive patients with pancreatic masses (307 pancreatic ductal adenocarcinomas [PDACs], 47 pancreatic inflammatory lesions, and 40 other types of tumors) who underwent EUS-FNA and analysis of *K-ras* mutations.

Intervention: EUS-FNA, Cycleave polymerase chain reaction.

Main Outcome Measurements: Improvement of the diagnostic accuracy by *K-ras* mutation analysis; absence of *K-ras* mutations in non-PDAC masses.

Results: *K-ras* mutations were detected in 266 of 307 PDAC aspirates (87%) and in 3 of 87 non-PDAC masses (3%). *K-ras* mutations were detected in 18 of 39 patients (46%) who remained cytohistopathologically undiagnosed. The sensitivity, specificity, positive and negative predictive values, and accuracy of cytohistopathological and *K-ras* mutation analyses alone were 87%, 100%, 100%, 54%, and 89%, respectively, and, when combined, were 93%, 100%, 100%, 68%, and 94%, respectively. Adding *K-ras* mutation analysis to standard cytohistopathological assessment increased the sensitivity and accuracy of EUS-FNA by 6% ($P < .001$) and 5% ($P < .001$), respectively.

Limitations: Single-center study.

Conclusions: *K-ras* mutation analysis may be helpful in patients with suspected PDAC yet inconclusive EUS-FNA findings. *K-ras* mutations were extremely rare in pancreatic inflammation and other pancreatic tumors. (Gastrointest Endosc 2012;75:769-74.)

The prognosis of pancreatic ductal adenocarcinoma (PDAC) is one of the poorest among malignant tumors. The 5-year survival rate among all patients with PDAC is less than 3.5%.[1,2] On the other hand, the prognosis of pancreatic inflammatory lesions such as chronic pancreatitis (CP), autoimmune pancreatitis (AIP), and other rare

Abbreviations: AIP, autoimmune pancreatitis; CP, chronic pancreatitis; EUS-FNA, EUS-guided FNA; NPV, negative predictive value; PanIN, pancreatic intraepithelial neoplasia; PCR, polymerase chain reaction; PDAC, pancreatic ductal adenocarcinoma; PNET, pancreatic neuroendocrine tumor.

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tumors is much better. To differentiate PDAC from these inflammatory conditions is critical because treatment strategies and prognoses differ.

However, despite advances in imaging techniques, differentiating PDAC from pancreatic inflammation lesions and other tumors remains challenging.[3-6] EUS-guided FNA (EUS-FNA) is considered the best method for establishing a differential diagnosis of pancreatic masses. However, the overall accuracy of EUS-FNA in this setting widely varies, with sensitivity ranging from 65% to 95% and with negative predictive values (NPVs) ranging from 50% to 70%.[7-10] One limitation of EUS-FNA is the small volume of acquired specimens, which can render differentiating between malignancy and benign lesions difficult.[7-10] The value of techniques such as core needle biopsy,[11] elastography,[12] and harmonic contrast EUS[13] for patients with inconclusive diagnoses has been reported, but other modalities are still required to further improve diagnostic accuracy.

The rates of various genetic abnormalities, particularly *K-ras* mutations in PDAC, are high.[14,15] We previously suggested that a combination of *K-ras* mutation analysis and cytohistopathological assessment of EUS-FNA aspirates increases the accuracy of a diagnosis of PDAC and allows its differentiation from CP.[16] This study extends our previous findings by prospectively investigating the clinical benefit of *K-ras* analysis in pancreatic EUS-FNA samples from a large number of consecutive patients.

Take-home Message

- The sensitivity of EUS-guided FNA for diagnosing pancreatic ductal adenocarcinoma (PDAC) was improved by adding *K-ras* mutation analysis.
- *K-ras* mutations were absent in all pancreatic inflammatory lesions and almost all tumors other than PDAC.

PATIENTS AND METHODS

Patients

The Aichi Cancer Center Hospital Institutional Review Board approved this study of *K-ras* mutation analysis in 394 consecutive patients who underwent EUS-FNA of pancreatic masses between March 2004 and September 2009 at the Aichi Cancer Center Hospital. All patients provided written informed consent for all procedures associated with the study.

EUS-FNA technique

We performed EUS as described[9,17] at 7.5-MHz frequency by using a convex linear-array echoendoscope (GF-UGT240; Olympus Optical Co Ltd, Tokyo, Japan) connected to a US device (SSD5500; Aloka, Tokyo, Japan) and a 22-gauge needle (NA-10J or NA-11J-KB; Olympus Optical Co Ltd or EchoTip-Ultra Needle; Cook Medical, Limerick, Ireland). Aspirated material was separated into 1 part each for cytopathological evaluation, cell-block preparation, and *K-ras* point mutation analysis. The material aspirated from all 394 patients was immediately evaluated (by using Diff Quick staining) by a cytopathologist and/or cytotechnologist for rapid diagnosis.[9] Material was immediately fixed in 10% formalin in a standard specimen bottle, centrifuged, and then embedded in paraffin for cell-block analysis. Sections then were visualized by hematoxylin and eosin as well as by immunohistochemical staining if necessary.

Analysis of *K-ras* mutations

Material for the genetic study was based on either the fresh specimens or the paraffin-embedded sections of the cell blocks obtained by EUS-FNA. Total RNA was extracted from the fresh specimens, and mutational analysis was performed by using reverse transcriptase polymerase chain reaction (PCR) coupled with direct sequencing methods, as described previously.[18] When the direct sequencing displayed no mutational signal when the cytological diagnosis was atypical cells, suspicious, or adenocarcinoma, we further investigated the *K-ras* mutation by using the corresponding cell block slides by a Cycleave PCR assay.[19] This is a highly sensitive assay that can detect as little as 5% of tumor cells mixed with normal tissues.

Final diagnosis

The final diagnosis was based on pathological examinations of specimens obtained by surgical resection and/or EUS-FNA. If signs of malignancy were absent at the end of follow-up (disease regression or no evidence of disease progression), PDAC was ruled out. These patients were considered to have other pancreatic diseases according to their clinical course and/or cytohistopathological diagnosis obtained by EUS-FNA. The final diagnosis was a benign disorder if the clinical course was consistent with EUS-FNA findings and the patient had been followed for at least 1 year.

Statistical analysis

Continuous variables are expressed as medians and ranges. Incidences and concordance between groups were compared by using the Fisher exact test or McNemar test where appropriate. All statistical analysis were performed by using StatMate IV (ATMS Co Ltd, Tokyo, Japan). A P value of $\leq .05$ was considered statistically significant.

RESULTS

Patient's characteristics

Tables 1 and 2 show patient's characteristics. Among 394 patients, 307 had PDAC (185 male, 122 female; mean age, 64.7 years; mean size of mass, 31.3 mm), 47 had

TABLE 1. Patient characteristics (N = 394)

	PDAC	Pancreatic inflammatory lesions	Other tumors
No. of patients (M/F)	307 (185/122)	47 (38/9)	40 (20/20)
Mean age, y (range)	64.7 (35-84)	64.2 (41-84)	53.9 (23-81)
Mean no. of needle passes (range)	2.3 (1-4)	2.3 (1-4)	2.3 (1-4)
Mean size of mass, mm (range)	31.3 (7.0-50.0)	20.4 (10.0-58.5)	25.6 (8.0-90.0)

PDAC, Pancreatic ductal adenocarcinoma; *M*, male; *F*, female.

TABLE 2. Final diagnosis (N = 394)

	No. of patients
PDAC	307
Pancreatic inflammatory lesions	
Focal chronic pancreatitis	24
AIP	23
Other tumors	
PNET	20
Metastatic tumor*	8
Acinar cell carcinoma	3
Malignant lymphoma	3
Solid pseudopapillary tumor	2
Serous cystic tumor, lymphoepithelial cyst	1

PDAC, Pancreatic ductal adenocarcinoma; *AIP*, autoimmune pancreatitis; *PNET*, pancreatic neuroendocrine tumor.
*Metastatic tumors 8 (2 from lung, 2 from the stomach, 1 from the ovary, 1 from the esophagus, 1 from the breast, 1 from the kidney).

TABLE 3. Detection of *K-ras* mutations

	K-ras mutations, no. (%)		
	Positive	Negative	*P* value
PDAC (n = 307)	267 (87)	40 (13)*	
Pancreatic inflammatory lesions and other tumors (n = 87)	3 (3)†	84 (97)	<.001

PDAC, Pancreatic ductal adenocarcinoma.
*Including patient in whom *K-ras* mutation was not possible.
†Comprising 1 each of poorly differentiated pancreatic neuroendocrine tumor and metastasis from the stomach and ovary.

pancreatic inflammatory lesions (38 male; mean age, 64.2 years; mean size of mass, 20.4 mm), and 40 had other tumors (20 male; mean age, 53.9 years; mean size of mass, 25.6 mm). The mean number of needle passes in all patients was 2.3. Pancreatic inflammatory lesions constituted AIP in 24 patients and focal CP in 23 patients. The miscellaneous tumors included pancreatic neuroendocrine tumors ([PNETs] n = 20), metastatic tumors (n = 8), acinar cell carcinomas (n = 3), malignant lymphomas (n = 3), solid pseudopapillary tumors (n = 3), serous cystic tumors (n = 2), and a lymphoepithelial cyst (n = 1). The primary sites for metastasis to the pancreas were lung (n = 2), stomach (n = 2), and ovary, esophagus, breast, and kidney (n = 1 each). The average follow-up period was 341 ± 313 days for PDAC, 1090 ± 493 days for pancreatic inflammatory lesions, and 672 ± 371 days for other tumors.

Detection of *K-ras* mutations

Table 3 shows the results of *K-ras* analysis of the 394 patients. *K-ras* analysis was successfully performed in 99.7% of patients (393/394). The aspirate of 1 patient did not contain any cell components in PDAC. *K-ras* mutations were detected in 266 of 307 aspirates from PDAC (87%) and in only 3 of 87 non-PDAC masses (3%), including 1 poorly differentiated PNET and 2 metastatic tumors (1 each in the stomach and ovary). *K-ras* mutations were significantly more frequent in PDAC ($P < .001$). Among 307 patients with PDAC, 68 were found to be negative for the *K-ras* mutation by direct sequencing, and 27 were found positive for the *K-ras* mutation by Cycleave PCR. As a result, 78% of PDAC patients were positive for the *K-ras* mutation by direct sequencing alone, but this improved to 87% with the additional use of Cycleave PCR. The 3 patients with conditions other than PDAC who were positive for the *K-ras* mutation were also positive by direct sequencing.

Diagnostic flow of PDAC

Figure 1 shows the diagnostic flow of 307 patients with a final diagnosis of PDAC. Cytological evaluation alone diagnosed PDAC in 255 of them, malignancy was suspected in 31, and 21 were negative for malignancy. In addition, histological assessment diagnosed malignancy in 13 of the total of 52 patients with inconclusive or negative cytological results. Of the remaining 39 patients who re-

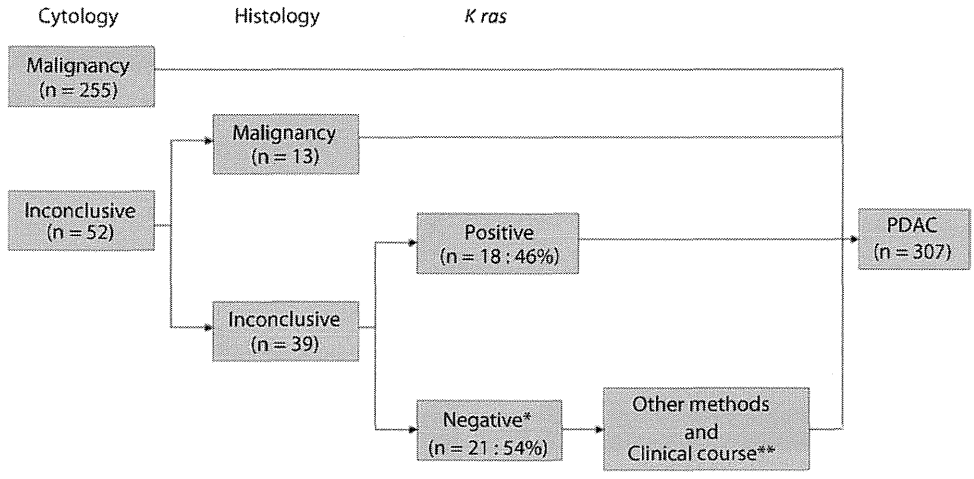

Figure 1. Diagnostic flow of pancreatic ductal adenocarcinoma. Of the remaining 39 patients who remained undiagnosed by cytological and histological assessment, *K-ras* mutations were detected in 18 (46%) of them. *Including the patient in whom the *K-ras* mutation was not possible. **Malignancy was diagnosed by ERCP, surgery, and aspiration from metastasis foci.

TABLE 4. Cytohistopathological and *K-ras* mutation analysis alone and combined with EUS-FNA material in the differential diagnosis of PDAC (n = 307) and pancreatic inflammatory lesions (n = 47)

	Sensitivity, %	Specificity, %	PPV, %	NPV, %	Accuracy, %
K-ras mutation analysis alone	87	100	100	54	89
Cato-/histopathology alone	87	100	100	54	89
Combined cytohistopathological and *K-ras* mutation analysis	93	100	100	68	94

PDAC, Pancreatic ductal adenocarcinoma; *PPV*, positive predictive value; *NPV*, negative predictive value.

mained undiagnosed by cytological and histological assessment, *K-ras* mutations were detected in 18 (46%) of them.

Finally, 3 of the 18 who were positive for the *K-ras* mutation were diagnosed with PDAC by a second EUS-FNA. Of the remaining 15, PDAC was diagnosed by surgical resection (n = 9) and by biopsy specimens from metastatic foci (n = 6). Among the 21 who were negative for the *K-ras* mutation, 3 were diagnosed with PDAC by a second EUS-FNA. Of the remaining 18, PDAC was diagnosed by surgical resection (n = 3), pancreatic juice cytology (n = 2), and biopsy specimens from metastatic foci (n = 13). For the purposes of this study, a cytology result was considered negative for malignancy if identified as either atypical or suspicious for malignancy.

Differential diagnosis of PDAC and pancreatic inflammations

Table 4 shows that the sensitivity, specificity, positive predictive value, NPV, and accuracy of the cytohistopathological examination alone and of the *K-ras* analysis alone were each 87%, 100%, 100%, 54%, and 89%, respectively, whereas these values of the 2 analyses combined were 93%, 100%, 100%, 68%, and 94%, respectively. The sensitivity and accuracy of EUS-FNA increased by 6% ($P < .001$) and 5% ($P < .001$), respectively, when *K-ras* analysis was added to standard cytohistopathological assessment.

Complications

No complications were associated with EUS-FNA in any of the 394 patients.

DISCUSSION

Various genetic abnormalities, such as *K-ras*, *p53*, *p16*, and *DPC4*, have been demonstrated in PDAC.[14,15,20-22] *K-ras* mutations are frequent and are found in 75% to 90% of cases of PDAC. Several authors suggested that *K-ras* mutation analysis of EUS-FNA specimens is valuable.[16,23-28] However, published reports have included relatively small numbers of patients with PDAC and other pancreatic tumors for comparison (Table 5). Here, we prospectively investigated the most patients in a single study to date and examined other pancreatic diseases for a differential diagnosis.

K-ras analysis was informative for all of our patients except 1, whose aspirate did not contain any cellular compo-