Sample Size Determination: Statistical Methods

In the initial plan, the total target number of patients was set at 600, given a statistical power of 80%, an enrollment period of 3 years, and a follow-up period of 2 years. However, because patient enrollment was faster than expected, the target number of patients was revised to 750 to provide the study with a statistical power of 90%. Consequently, the final analysis was performed after the occurrence of 680 events had been confirmed. An interim analysis was not performed. Although the actual median OS in the gemcitabine group was better than initially expected, because an adequate number of patients had been enrolled, a power of $\geq$ 90% was maintained on recalculation of the power on the basis of the actual results.

Quality of Life

To assess the quality of life, the health status of patients on the EQ-5D questionnaire was converted into a single simple utility index ranging from 0 for death to 1 for complete health. Quality-adjusted life-years (QALYs) for individual patients were estimated as the product of the utility index during follow-up and survival time and were compared between the groups, using the generalized Wilcoxon test.

As a result, median QALYs were 0.401 in the gemcitabine group, 0.420 in the S-1 group, and 0.525 in the GS group. The QALY value in the S-1 group was similar to that in the gemcitabine group, and there was no statistically significant difference between the two groups ($P = .56$). The QALY value in the GS group was significantly better than that in the gemcitabine group ($P < .001$). The details of quality-of-life assessments will be reported elsewhere.

FULL PAPER

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Keywords: pentraxin family; pancreatic carcinoma; cancer inflammation

Clinical impact of pentraxin family expression on prognosis of pancreatic carcinoma

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Background: Inflammatory mediators may have decisive roles at different stages of tumour development. Mediators within the pentraxin family may be used as strong biomarkers in prognosis of advanced pancreatic carcinoma patients.

Methods: Using pancreatic carcinoma cell lines and gene transfectant, we measured long pentraxin (PTX3) level in culture solution and carried out cellular migration assay *in vitro*. *In vivo* study of the treatment-naive patients with advanced pancreatic carcinoma assigned to undergo gemcitabine therapy was prospectively conducted to measure and investigate the role of plasma PTX3, C-reactive protein (CRP), and eight inflammatory mediators by using collected clinical data.

Results: Elevated PTX3 production was observed in several cell lines, and a direct relationship between migratory activity and PTX3 level was identified *in vitro*. High PTX3 level (117 days) was significantly less than that of patients with low PTX3 level (357 days, $P<0.001$). Multivariate analysis of the pancreatic carcinoma revealed a strong correlation between pentraxin family member expression and prognosis of pancreatic carcinoma. The relationship between PTX3 expression and the expression of other pro-inflammatory mediators indicated that PTX3 level is positively correlated with levels of CRP, interleukin-6, and macrophage-inhibitory factor.

Conclusion: Pentraxin family members, especially PTX3, may be used as promising biomarkers in the prognosis of pancreatic carcinoma patients.

Pancreatic carcinoma is one of the most lethal cancers and is the fourth leading cause of cancer-related death in developed nations (Jemal *et al*, 2011). As pancreatic carcinoma has a high propensity for both local invasion and distant metastasis, surgical treatment is precluded for most patients who present with an advanced stage of the disease. Despite many treatment advances that have improved the outcomes of some pancreatic carcinoma patients, standard therapy has been found to have only a modest beneficial impact on advanced-stage patients (Vincent *et al*, 2011), as reflected in their 5-year overall survival (OS) of <5% (Jemal *et al*, 2011).

Identification of biomarkers that accurately predict disease recurrence, response to chemotherapy, and/or prognosis would be of substantial aid in individual risk assessment and treatment selection. Identification of targets for molecular intervention in specific subsets of patients may even lead to the development of novel therapies. There is thus a need to identify a superior marker of prognosis to enable the improved survival of advanced pancreatic carcinoma patients.

Inflammatory responses have decisive roles at different stages of tumour development, including initiation, promotion, malignant conversion, invasion, and metastasis, and affect immune surveillance and response to therapy. The invasive capacity of malignant cells has been observed to increase in the presence of inflammatory cytokines, including TNF-alpha, interleukin (IL)-1beta, and IL-6, as well as transcription factors, including AP-1, NF-κB, and STAT3 (Mantovani *et al*, 2008). In a previous study, we identified C-reactive protein (CRP), which is produced via IL-6 and TNF-alpha stimulation in the liver, as an important factor in the prognosis of pancreatic carcinoma (Morizane *et al*, 2011). In other studies, long pentraxin (PTX3), a member of the pentraxin family, which includes CRP and whose members may have a significant role in tumour inflammatory and malignant behaviours, was

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reported to be overexpressed in several malignancies, including liposarcomas (Germano *et al*, 2010) and lung cancer (Diamandis *et al*, 2011). These findings indicate that reduction of the key inflammatory mediators may be an important means of promoting antitumour activity.

In a previous study, we had observed direct secretion of PTX3 from pancreatic carcinoma cell lines *in vitro*. Building on this finding, we aimed to determine the biological significance of PTX3 in pancreatic cancer via further *in vitro* study of several pancreatic carcinoma cells lines, as well as prospective clinical investigation of the clinical significance of plasma PTX3 expression in chemotherapy-naive pancreatic carcinoma patients. We found that PTX3 expression might be a promising biomarker for pancreatic carcinoma prognosis.

MATERIALS AND METHODS

Cells, plasmids, and transfection procedures. The PANC-1 (ATCC number: CRL-1469), MIA PaCa-2 (ATCC number: CRL-1420), BxPC-3 (ATCC number: CRL-1687), and AsPC-1 (ATCC number: CRL-1682) pancreatic carcinoma cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum (FBS) was used as the base medium for the PANC-1 and MIA PaCa-2 lines, and RPMI with 10% FBS was used as the growth medium for the BxPC-3 and AsPC-1 lines. We used the transfectant pCMV6-entry PTX3 open reading frame (ORF) clones (OriGene Technologies, Inc., Rockville, MD, USA; cat. no. RC207922) to investigate the cellular activity induced by intracellular PTX3 overexpression. The plasmids were transfected into cells using Lipofectamine 2000 (Life Technologies Corp., Carlsbad, CA, USA). In experiments using neomycin-resistant vectors, transfected cells were selected using 0.5 mg ml^{-1} of G-418 to establish stable transformants.

Cell migration and invasion assay. Cell migration assay was performed as described previously using Transwell inserts (5-μM pore size; Corning Inc., Corning, NY, USA). Cells were placed in 100 μl of 0.6% FBS medium in the upper chamber at 1×10^5 cells ml^{-1}, and the bottom chamber contained VEGF (Cat. no. PHC9391; Life Technologies Corp.), PTX3 (Cat. no. 1826-TS-025; R&D System, Minneapolis, MN, USA), and CRP (Cat. no. 1707-CR-200; R&D System) in a medium containing 0.6 or 20% FCS. After 12 h, the inserts were fixed and stained with Diff-Quick (Symex International Reagents Co. Ltd., Kobe, Japan). After the cells that had not migrated were removed from the upper surface of the inserts using cotton swabs, images from three different high-power fields were captured from each insert and the number of migratory cells was counted (Kondo *et al*, 2012a).

Cytokine measurement. Pancreatic carcinoma cells at 75–80% confluence were growth-arrested by FBS deprivation for 24 h and cultured in fresh FBS-free medium. Supernatants were collected at 6 h. Plasma samples collected from patients before initiation of gemcitabine treatment were stored at −80 °C until being subjected to enzyme-linked immunosorbent assay (ELISA) and antibody-suspension bead array analysis. Serum CRP levels were measured according to the manufacturer's directions by using a commercially available kit (Nanopia CRP; Sekisui Medical Co., Ltd., Tokyo, Japan) capable of assaying a wide range of values (0.01–100 mg dl^{-1}) without the prozone phenomenon. Plasma-PTX3 levels were measured according to the manufacturer's directions by using a commercially available ELISA kit (Cat. no. PP-PD03-E0; Perseus Proteomics, Inc, Tokyo, Japan) capable of measuring a wide range of values (0.1–20 ng ml^{-1}) linearly. After being drawn into prechilled tubes containing ethylenediaminetetraacetic acid (EDTA), peripheral blood was immediately subjected to centrifugation at 1000 g and 4 °C for 15 min. The plasma was then transferred into microtubes and subjected to further centrifugation at 10 000 g and 4 °C for 10 min to remove contaminating platelets.

The plasma concentrations of the pro-inflammatory mediators IL-1beta, IL-6, C-C motif ligand (CCL) 2, CCL3, CCL4, CCL7, C-X-C motif ligand 9, and macrophage-inhibitory factor (MIF) were assayed in a subgroup of patients and control individuals using the Bio-Plex suspension array system (Bio-Rad, Hercules, CA, USA), which allows for simultaneous detection of cytokines in a 96-well filter plate. In brief, the appropriate cytokine standards and diluted plasma samples were added to a 96-well filter plate and incubated at room temperature for 30 min with antibodies chemically attached to fluorescent-labelled micro beads. After three filter washes, premixed detection antibodies were added to each well and incubated for 30 min. After three additional washes, premixed streptavidin–phycoerythrin was added to each well for 10 min of incubation. Subsequent to three more washes, the beads were resuspended in 125 μl of assay buffer and the reaction mixture was quantified by using the Bio-Plex protein array reader. Data were automatically collected and analysed using Bio-Plex Manager Software 4.1, and the standard curve was obtained using a recombinant cytokine standard (Kondo *et al*, 2012b).

Study approval. Prior to initiation, this prospective study had been approved by the Institutional Review Board of the National Cancer Center, and written informed consent had been obtained from all patients. This study is registered with the University Hospital Medical Information Network in Japan (UMIN; number UMIN000002323) and has been completed.

Patient selection and blood sample collection. A total of 78 chemotherapy-naive patients with histologically or cytologically confirmed advanced or recurrent invasive ductal pancreatic carcinoma were prospectively enroled in this study between April 2009 and March 2010 for treatment with gemcitabine chemotherapy. Patients with coexisting infections and/or cardiovascular illness were excluded from participation. Prior to initiation of gemcitabine treatment, each patient had undergone collection of a detailed history; physical examination; assessment of pretreatment baseline laboratory parameters; and determination of baseline tumour status by computed tomography (CT) of the chest, abdomen, and pelvis. Baseline and post-treatment laboratory parameters were evaluated by performing peripheral blood sampling prior to treatment initiation and on day 28 ± 7 after treatment initiation, respectively. Gemcitabine at a dosage of 1000 mg m^{-2} was administered intravenously for 30 min on days 1, 8, and 15 of a 28-day cycle until disease progression, unacceptable toxicity, or patient refusal to continue treatment. The data collected included those pertaining to standard demographics; disease characteristics; and disease chronology, including the dates of initial treatment, best response to treatment, progression, and death or final follow-up. Tumours were evaluated every 6–8 weeks after initiation of each course of gemcitabine, and the best responses were documented according to the Response Evaluation Criteria in Solid Tumours version 1.1 (RECIST ver1.1).

Statistical analyses. The cutoff points used in the assessment of high and low expression levels of each mediator were based on the mean values of these mediators, whereas those of the CRP and CA19-9 levels were based on previous reports (Morizane *et al*, 2011). Associations between patient characteristics were assessed by χ^2 statistics. Survival in terms of both progression-free survival (PFS) and OS; demographic factors, including age and gender; and clinical factors, including Eastern Cooperative Oncology Group (ECOG) performance status (PS) and clinical stage was examined using the Cox proportional hazards model. The survival curves for PFS and OS were estimated using the Kaplan–Meier method, with the resulting Kaplan–Meier curves used only to identify trends in the associations between the inflammatory mediators and PFS and

OS, as determination of the optimal cutoff point for the mediators relative to PFS and OS was beyond the scope of this study. All statistical analyses were performed using IBM SPSS Statistics 18 software (IBM Corp, Somers, NY, USA).

RESULTS

PTX3 expression in pancreatic carcinoma cell lines. Measurement of expression of elevated levels of PTX3 by pancreatic carcinoma cells in culture solution, considered an indication of direct PTX3 secretion by these cells, revealed that the PANC-1 and MIA PaCa-2 cell lines expressed higher levels of PTX3 compared with the AsPC-1 and BxPC-3 lines (Figure 1A).

Effect of PTX3 on migratory and invasive potential of pancreatic carcinoma cell lines. To determine the association between the intra- and extracellular levels of PTX3 and the migratory or invasive behaviour of pancreatic carcinoma cells, pCMV6-entry PTX3 ORF clones were used to establish stable transfectants. As shown in Figure 1B, introduction of PANC-1 and AsPC-1 cells increased recombinant human (rh) PTX3-induced cell migration, with the level of migration activity depending on the concentration of extracellular PTX3. On the other hand, CRP was

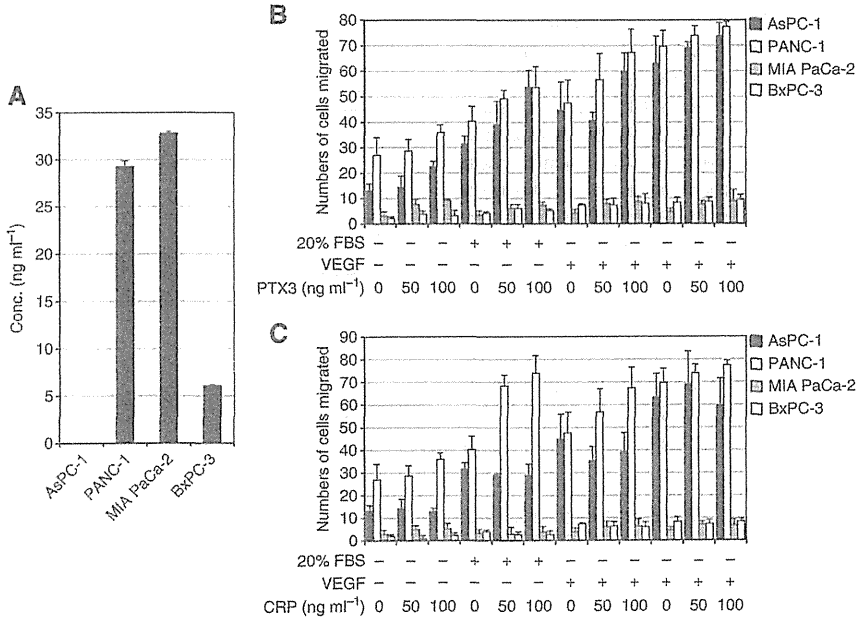

Figure 1. (A) Pentraxin 3 production levels of supernatants used for culturing of pancreatic carcinoma cell lines. (B, C) Cell migration assay in the presence or absence of the indicated reagents. NT (non-treated), FBS, 20% in the lower chamber; VEGF, 50 ng ml^{-1} in the lower chamber; PTX3, 50–100 ng ml^{-1}; CRP, 50–100 ng ml^{-1}. Statistical significance was evaluated by comparison with or without the presence of PTX3 and CRP.

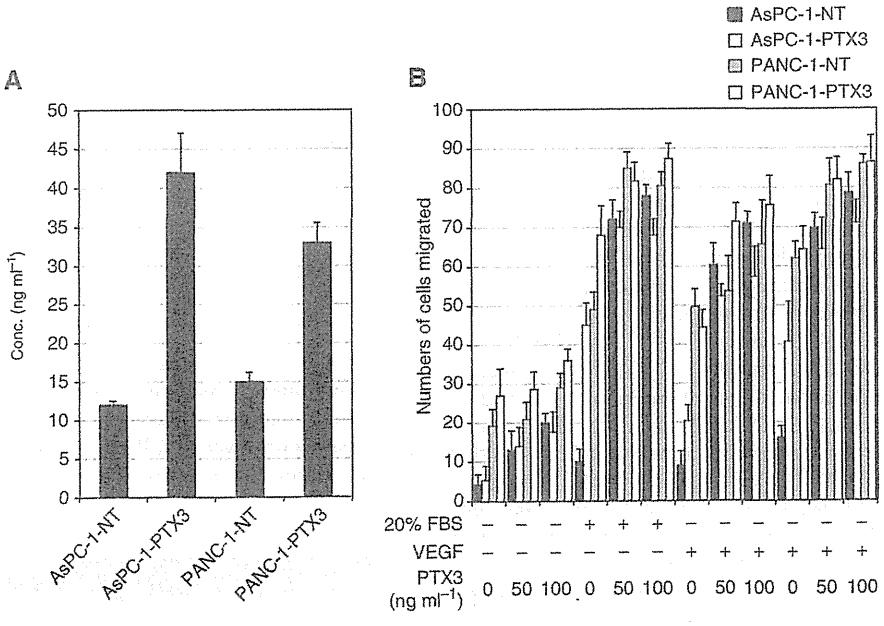

Figure 2. (A) Pentraxin 3 production levels of supernatants used for culturing of PTX3 and NT-control transfectant of pancreatic carcinoma cell lines. (B) Cell migration assay using pancreatic carcinoma cell lines (AsPC-1 and PANC-1) transfected with pCMV6-entry PTX3 ORF in the presence or absence of the indicated reagents. NT (non-treated), FBS, 20% in the lower chamber; VEGF, 50 ng ml^{-1} in the lower chamber; PTX3, 50–100 ng ml^{-1}; CRP, 50–100 ng ml^{-1}. Stable transformants were subjected to cell migration assay.

observed to have a significant role in the regulation of migration activity in pancreatic carcinoma cell lines (Figure 2C). Transformants of PTX3 clone was not observed to increase migration activity significantly (Figure 2B). These results indicate that the extracellular PTX3 in the pancreatic carcinoma cell lines had promoted migratory activity.

Patient characteristics. Of the 78 pancreatic carcinoma patients prospectively enroled in this study, 42 (54%) were male and the median age was 68 years (range, 44–79 years). Regarding prior diagnosis and treatment, 25 patients (32%) presented with locally advanced pancreatic carcinoma, 47 (60%) presented with metastases, and 6 (8%) had been enroled following recurrence after surgery. Regarding the ECOG PS score, 44 patients (56%) had an ECOG PS of PS0, 27 (35%) of PS1, and 7 (9%) of PS2. Histologically, 31 patients (40%) had a poorly differentiated adenocarcinoma, 27 (35%) had a moderately differentiated adenocarcinoma, 1 (1%) had a well-differentiated adenocarcinoma, 2 (2%) had an adenosquamous carcinoma, and 17 (22%) had a cytological adenocarcinoma. Regarding response to treatment, 0 patients (0%) experienced complete response to treatment, 5 (6%) experienced partial response (PR) to treatment, 43 (55%) experienced stable disease (SD) after treatment, 27 (35%) experienced progressive disease, and 3 (4%) patients were not evaluable after treatment (Table 1). The mean PTX3 level of all patients was $4.94 \pm 3.63\,ng\,ml^{-1}$ (range, $0.9–17.7\,ng\,ml^{-1}$; median, $3.76\,ng\,ml^{-1}$). Regarding the values of other inflammatory mediators, the mean IL-1beta, IL-6, CCL2, CCL3, CCL4, CCL7, CXCL9, and MIF levels were found to be 95.48 ± 37.62, 294.38 ± 516.6, 746.58 ± 518.12, 177.8 ± 58.4, 2886.28 ± 1974.73, 80.81 ± 13.72, 3161.89 ± 2146.07, and $7240.52 \pm 5461.4\,pg\,ml^{-1}$, respectively.

Relationship between PTX3 level and treatment outcome. For comparison of the clinical parameters associated with PTX3 level, the patients were divided into two groups: a group of patients with a PTX3 level $\geqslant 4.94\,ng\,ml^{-1}$, referred to as the $PTX3^{high}$ group ($n=22$), and a group with a PTX3 level $<4.94\,ng\,ml^{-1}$, referred to as the $PTX3^{low}$ group ($n=56$). The median PFS of the $PTX3^{high}$ group was 76 days (95% confidence interval (CI), 43–109) and that of the $PTX3^{low}$ group was 150 days (95% CI, 131–197; log-rank test, $P=0.002$; Figure 3A). The median OS of the $PTX3^{high}$ group was 117 days (95% CI, 82–152) and that of the $PTX3^{low}$ group was 357 days (95% CI, 239–475; log-rank test, $P<0.001$; Figure 3B).

Placement in the $PTX3^{high}$ group was found to be significantly associated with advanced clinical stage ($P<0.01$), poor PS (PS2, $P=0.01$), and elevated CRP level (over $1.0\,mg\,dl^{-1}$, $P<0.01$). The results of the univariate Cox regression analysis indicated that high PTX3 level and/or high CRP level is significantly associated with poor OS (hazard ratio (HR), 4.80; 95% CI, 2.62–8.78; $P<0.001$ and HR, 6.56; 95% CI, 3.32–12.96; $P<0.001$, respectively). The results of univariate analysis indicated that clinical stage (IV + recurrence *vs* III), PS (2 *vs* 0 + 1), histological differentiation (poorly differentiated *vs* not poorly differentiated), CA19-9 level (>3000 *vs* $<3000\,U\,ml^{-1}$), and IL-6 level ($IL\text{-}6^{high}$ *vs* $IL\text{-}6^{low}$) are significantly associated with improved OS (Table 2), whereas age, gender, and levels of several pro-inflammatory mediators (IL-1beta, CCL2, CCL3, CCL4, CCL7, CXCL9, and MIF) are not. Subsequent multivariate Cox regression analysis indicated that CRP level (CRP^{high} *vs* CRP^{low}; HR 2.59, 95% CI 1.05–6.36, $P=0.04$), and PTX3 level ($PTX3^{high}$ *vs* $PTX3^{low}$; HR 3.00, 95% CI 1.47–6.14, $P=0.003$) are independent prognostic variables (Table 2). Taken together, these results suggest that members of the pentraxin family, particularly PTX3, may be predictive biomarkers in the prognosis of human pancreatic carcinoma in the clinical setting.

Relationship between PTX3 level and expression of pro-inflammatory mediators. Examination of the relationship between PTX3 expression and the expression of other pro-inflammatory mediators using Spearman's rank correlation coefficient analysis indicated that PTX3 level is positively correlated with level of CRP ($r=0.56$, $P<0.001$), IL-6 ($r=0.59$, $P<0.001$), and MIF ($r=0.38$, $P=0.001$; Table 3).

Table 1. Patient demographic and clinical characteristics

	PTX3 group (number of patients)			
	$PTX3^{high}$ group	$PTX3^{low}$ group	Total	*P*-value*
Age (years)				
Over 70	7	21	28	0.79
Below 70	15	35	50	
Sex				
Male	15	27	42	0.14
Female	7	29	36	
Stage				
III	1	24	25	0.005
IV	19	28	47	
Recurrence	2	4	6	
ECOG PS score				
0	7	37	44	0.01
1	13	14	27	
2	2	5	7	
Histology				
Well differentiated	0	1	1	0.12
Poorly differentiated	7	24	31	
Moderately differentiated	12	15	27	
Adenosquamous	1	1	2	
NE (cytology only)	2	15	17	
Tumour response				
Partial response	2	3	5	0.29
Stable disease	9	34	43	
Progressive disease	10	17	27	
NE	0	3	3	
CA19-9 ($U\,ml^{-1}$)				
Over 10 000	11	24	35	0.62
Below 10 000	11	32	43	
CRP ($mg\,dl^{-1}$)				
Over 1.0	12	10	22	0.002
Below 1.0	10	46	56	

Abbreviations: CRP = C-reactive protein; ECOG PS = Eastern Cooperative Oncology Group Performance Status; NE = not evaluable; PTX3 = pentraxin 3. *P-values calculated using χ^2 statistics.

DISCUSSION

The findings of the present study provide the first evidence of the clinical importance of PTX3 expression as a prognostic factor in pancreatic carcinoma due to its involvement in cancer cell behaviour. Specifically, several pancreatic carcinoma cell lines

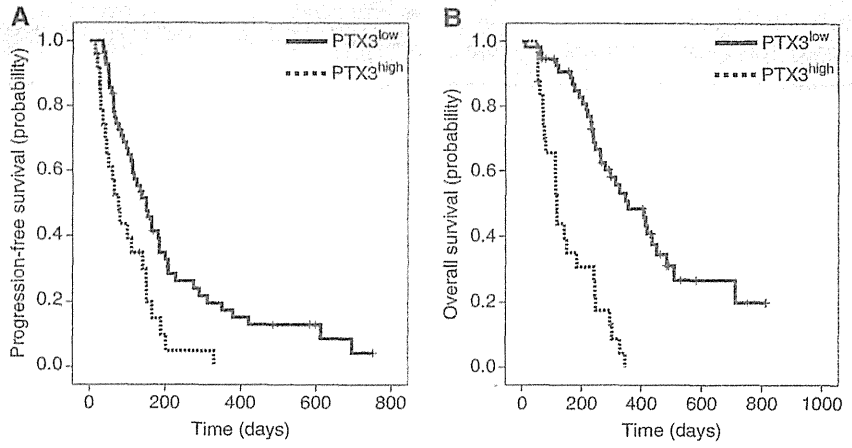

Figure 3. Kaplan–Meier curves for (A) progression-free survival according to blood-PTX3 level and (B) overall survival according to blood-PTX3 level. Cutoff points for PTX3 level were based on mean PTX3 level.

Table 2. Results of univariate and multivariate analyses

	HR	95% CI	*P*-value*
Univariate analysis			
Age: over 70 *vs* below 70	1.16	0.66–2.05	0.61
Gender: male *vs* female	1.12	0.85–1.47	0.41
Stage: IV + recurrence *vs* III	1.93	1.05–3.56	0.03
ECOG PS score: 0 + 1 *vs* 2	3.42	1.40–8.37	0.007
Histology: poorly differentiated *vs* not poorly differentiated	2.34	1.34–4.12	0.003
CA19-9 ($U\,ml^{-1}$): over 10000 *vs* below 10000	1.96	1.12–3.44	0.02
CRP ($mg\,dl^{-1}$): over 1.0 *vs* below 1.0	6.56	3.32–12.96	<0.001
PTX3: $PTX3^{high}$ *vs* $PTX3^{low}$	4.80	2.62–8.78	<0.001
IL-6: $IL\text{-}6^{high}$ *vs* $IL\text{-}6^{low}$	7.72	3.88–15.35	<0.001
IL-1beta: $IL\text{-}1beta^{high}$ *vs* $IL\text{-}1beta^{low}$	0.84	0.48–1.47	0.84
CCL2: $CCL2^{high}$ *vs* $CCL2^{low}$	1.40	0.79–2.45	0.25
CCL3: $CCL3^{high}$ *vs* $CCL3^{low}$	1.57	0.92–2.68	0.10
CCL4: $CCL4^{high}$ *vs* $CCL4^{low}$	1.15	0.64–2.06	0.65
CCL7: $CCL7^{high}$ *vs* $CCL7^{low}$	1.08	0.62–1.88	0.79
CXCL9: $CXCL9^{high}$ *vs* $CXCL9^{low}$	1.51	0.87–2.63	0.15
MIF: MIF^{high} *vs* MIF^{low}	1.21	0.70–2.09	0.50
Multivariate analysis			
Stage: IV + recurrence *vs* III	1.14	0.42–2.37	0.72
ECOG PS: 0 + 1 *vs* 2	2.11	0.71–6.25	0.18
Histology: poorly differentiated *vs* not poorly differentiated	1.19	0.60–2.38	0.62
CA19-9 ($U\,ml^{-1}$): over 10000 *vs* below 10000	1.52	0.84–2.74	0.16
CRP ($mg\,dl^{-1}$): over 1.0 *vs* below 1.0	2.59	1.06–6.36	0.04
PTX3: $PTX3^{high}$ *vs* $PTX3^{low}$	3.00	1.4–6.14	0.003
IL-6: $IL\text{-}6^{high}$ *vs* $IL\text{-}6^{low}$	2.57	1.00–6.59	>0.05

Abbreviations: CCL = chemokine (C-C motif) ligand; CI = confidence interval; CRP = C-reactive protein; CXCL = chemokine (C-X-C motif) ligand; ECOG PS = Eastern Cooperative Oncology Group Performance Status; HR = hazard ratio; IL = interleukin; MIF = macrophage-migration-inhibitory factor; PTX3 = pentraxin 3; *P-values calculated using the Cox proportional hazards model.

were observed to secrete PTX3 to a remarkable extent, with expression of extracellular PTX3 promoting pancreatic carcinoma cell migration in a concentration-dependent, highly efficient manner and expression of transformants of PTX3 promoting migration in a less-efficient manner. Supporting this observation, evaluation of clinical blood samples of pancreatic carcinoma patients revealed a strong correlation between blood-PTX3 level and prognosis of the disease.

PTX3 is known to be produced by a variety of cells at the site of infection or inflammation, including macrophages, dendritic cells (Doni *et al*, 2003), neutrophils (Jaillon *et al*, 2007), endothelial cells (Norata *et al*, 2008), epithelial cells (Han *et al*, 2005), fibroblasts (Doni *et al*, 2008), and vascular smooth muscle cells (Klouche *et al*, 2004). In human liposarcomas, increased levels of PTX3 RNA and intracellular PTX3 have been detected (Willeke *et al*, 2006), whereas elevated levels of serum PTX3 have been observed in patients with lung cancer (Diamandis *et al*, 2011) and prostate cancer (Sardana *et al*, 2008). In the present study, detection of the direct secretion of extracellular PTX3 by several pancreatic carcinoma cell lines indicated the existence of a PTX3 autocrine–paracrine loop that regulates angiogenesis and stromal cell activity.

Table 3. Relationship between PTX3 level and levels of other immunological factors

	Average (SD)	CRP	PTX 3	IL-6	IL-1beta	CCL 2	CCL 3	CCL 4	CCL 7	CXCL 9	MIF
CRP (mg dl^{-1})	1.57 (3.06)		*r= 0.56 P<0.001	0.71 <0.001	0.25 0.03	0.13 0.26	0.10 0.40	0.04 0.73	0.03 0.77	0.31 <0.01	0.28 0.01
PTX3 (ng ml^{-1})	4.94 (3.63)	0.56 <0.001		0.59 <0.001	0.19 0.09	0.17 0.15	−0.04 0.71	0.07 0.56	0.15 0.21	0.16 0.17	0.38 0.001
IL-6 (pg ml^{-1})	294.38 (516.6)	0.71 <0.001	0.59 <0.001		0.44 <0.001	0.31 <0.01	0.27 0.02	0.31 <0.01	0.18 0.12	0.29 0.01	0.43 <0.001
IL-1beta (pg ml^{-1})	95.48 (37.62)	0.25 0.03	0.19 0.09	0.44 <0.001		0.44 <0.001	0.11 0.35	0.50 <0.001	0.25 0.03	0.01 0.91	0.70 <0.001
CCL2 (pg ml^{-1})	746.58 (518.12)	0.13 0.26	0.17 0.15	0.31 <0.01	0.44 <0.001		0.21 0.06	0.43 <0.001	0.10 0.37	0.24 0.03	0.08 0.47
CCL3 (pg ml^{-1})	177.80 (58.40)	0.10 0.40	−0.04 0.71	0.27 0.02	0.11 0.35	0.21 0.06		0.29 <0.01	0.04 0.73	0.06 0.60	−0.11 0.36
CCL4 (pg ml^{-1})	2886.28 (1974.73)	0.04 0.73	0.07 0.56	0.31 <0.01	0.50 <0.001	0.43 <0.001	0.29 <0.01		−0.005 0.96	0.03 0.80	0.34 <0.01
CCL7 (pg ml^{-1})	80.81 (13.72)	0.03 0.77	0.15 0.21	0.18 0.12	0.25 0.03	0.10 0.37	0.04 0.73	−0.005 0.96		0.15 0.20	0.40 <0.001
CXCL9 (pg ml^{-1})	3161.89 (2146.07)	0.31 <0.01	0.16 0.17	0.29 0.01	0.01 0.91	0.24 0.03	0.06 0.60	0.03 0.80	0.15 0.20		003 0.77
MIF (pg ml^{-1})	7240.52 (5461.40)	0.28 0.01	0.38 0.001	0.43 <0.001	0.70 <0.001	0.08 0.47	−0.11 0.36	0.34 <0.01	003 0.77	0.40 <0.001	

Abbreviations: CCL = chemokine (C-C motif) ligand; CRP = C-reactive protein; CXCL = chemokine (C-X-C motif) ligand; IL = interleukin; MIF = macrophage-migration-inhibitory factor; PTX3 = pentraxin 3. *r and P-values calculated using Spearman's rank correlation coefficient analysis.

Known to be a soluble recognition receptor, PTX3 binds to selected pathogens and has a non-redundant protective role against these pathogens as part of an innate immunological response. PTX3 also interacts with other biologically active molecules, such as fibroblast growth factor-2 (FGF2; Camozzi *et al*, 2006), to suppress FGF2-mediated cell-migration angiogenesis (Basile *et al.*, 2013). In androgen-regulated breast cancer cells, PTX3 inhibits FGF8-driven cell proliferation (Leali *et al*, 2011). In contrast, PTX3 promotes VEGF-stimulated migration of pancreatic carcinoma cells, although exerting no influence on VEGF-dependent angiogenesis and cell migration and not interacting with VEGF (Basile *et al*, 2013).

Invasion of malignant cells was found to result in increased levels of inflammatory mediators, such as TNF-alpha, IL-1beta, and IL-6, that increase the ability of malignant cells to infiltrate, migrate, and metastasise, possibly as a result of the upregulation of chemokine-receptor expression elicited by these mediators (Mantovani *et al*, 2008). The results of this study indicate that direct PTX3 stimulation promotes pancreatic carcinoma cell-migration activity. This finding and that of the present study, specifically that high PTX3 level is significantly correlated with a more advanced stage of pancreatic cancer, indicates that PTX3 acts as a mediator of inflammation that has a tumour-promoting effect in pancreatic carcinoma cells. In a previous study, PTX3 expression was found to be higher in high-Gleason-grade prostate tumour tissue compared with contralateral prostate lobes, possibly in association with pro-inflammatory and repair-process activation (Ravenna *et al*, 2009). Similar results have been reported for

human mammary-invasive carcinoma in association with hypoxia-driven HIF-1α and NF-κB activation (Tafani *et al*, 2010). These findings, along with the knowledge that pro-inflammatory signals upregulate PTX3 expression in different mesenchymal and epithelial cell types (Garlanda *et al*, 2005), suggest that PTX3 has a role in tumour activation, invasion, and migration in some types of cancer. On the other hand, some results suggest that PTX3 expression is silenced in cancer cells at a relatively early stage of tumour progression in parallel with hypoxia- or inflammation-driven cytokine production by stromal components and inflammatory cell infiltrates. To clarify these contradictory findings and determine the true nature of the effect of PTX3 expression on cancer cells, further studies are required.

Previous studies of advanced pancreatic carcinoma patients have found median OS to be 8–12 months for patients who present with locally advanced unresectable disease but only 3–6 months for those who present with metastases or recurrent pancreatic carcinoma. Several studies of advanced pancreatic carcinoma patients who have undergone gemcitabine monotherapy reported that median OS after treatment ranged from 5.4 to 7.2 months (Burris *et al*, 1997; Berlin *et al*, 2002; Herrmann *et al*, 2007). In the present study, the median OS of patients with high PTX3 levels was remarkably brief, found to be only 117 days. Other well-known prognostic factors in pancreatic carcinoma patients are elevated serum level of CA19-9, a widely used prognostic marker and indicator of disease activity (Ueno *et al*, 2000; Berger *et al*, 2008; Tanaka *et al*, 2008; Morizane *et al*, 2011), and elevated CRP level, which is likely to be part of the systemic inflammatory response to tumour development. Activation of CRP has also been linked to cancer cachexia, characterised by malnutrition and an accelerated starvation state, and shorter OS (Ebrahimi *et al*, 2004). Despite the importance of these factors, the findings of this study indicate that elevated PTX3 level is associated with more advanced disease and poorer PS, and is therefore a more useful neo-factor in prognosis than CA19-9 and CRP in pancreatic carcinoma patients.

Pro-inflammatory mediators are frequently expressed in the tumour microenvironment after they have infiltrated leukocyte, stromal, and cancer cells (DeNardo *et al*, 2008; Mantovani *et al*, 2008). Several mediators, such as IL-1beta, enhance the production of PTX3 elicited by inflammatory signalling (Polentarutti *et al*, 1998), whereas others, such as IL-6, CCL2, and interferon-gamma, have negligible effects on PTX3 expression (Alles *et al*, 1994; Polentarutti *et al*, 1998). Previous studies have found PTX3 levels to be correlated with CRP, IL-6, and elevated levels of MIF, which are known to control autonomous properties of tumour cells, such as proliferation, apoptosis, DNA-damage response, senescence, and invasion (Dessein *et al*, 2010), in the tumour and serum of pancreatic cancer patients (Winner *et al*, 2007).

In consideration with these findings, the present study aimed to clarify the profile of these mediators in pancreatic carcinoma patients and the correlations among them. Although this study yielded significant findings, it was limited by several phenomena, for instance the insufficient insolubility of the mechanism between each mediator and the inability to examine the intricate interrelations among the inflammatory mediators of cancer patients, each of which has multiple roles in various tumorigenic behaviours.

In conclusion, the results of this study provide strong evidence that elevated levels of pentraxin family members, especially PTX3, are associated with poor prognosis in pancreatic carcinoma patients. Expression of PTX3 appears to be a promising biomarker for pancreatic carcinoma prognosis. However, the mechanism of tumour inflammation and exact nature of the role of PTX3 expression remains unclear, calling for investigation of the mechanisms underlying PTX3 activity in carcinoma cells and the tumour environment.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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RESEARCH ARTICLE Open Access

Association between variations in the fat mass and obesity-associated gene and pancreatic cancer risk: a case–control study in Japan

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Abstract

Background: It is clear that genetic variations in the fat mass and obesity-associated (FTO) gene affect body mass index and the risk of obesity. Given the mounting evidence showing a positive association between obesity and pancreatic cancer, this study aimed to investigate the relation between variants in the FTO gene, obesity and pancreatic cancer risk.

Methods: We conducted a hospital-based case–control study in Japan to investigate whether genetic variations in the FTO gene were associated with pancreatic cancer risk. We genotyped rs9939609 in the FTO gene of 360 cases and 400 control subjects. An unconditional logistic model was used to estimate the odds ratio (OR) and 95% confidence interval (CI) for the association between rs9939609 and pancreatic cancer risk.

Results: The minor allele frequency of rs9939609 was 0.18 among control subjects. BMI was not associated with pancreatic cancer risk. Compared with individuals with the common homozygous TT genotype, those with the heterozygous TA genotype and the minor homozygous AA genotype had a 48% (OR=1.48; 95%CI: 1.07–2.04), and 66% increased risk (OR=1.66; 95%CI: 0.70–3.90), respectively, of pancreatic cancer after adjustment for sex, age, body mass index, cigarette smoking and history of diabetes. The per-allele OR was 1.41 (95%CI: 1.07–1.85). There were no significant interactions between TA/AA genotypes and body mass index.

Conclusions: Our findings indicate that rs9939609 in the FTO gene is associated with pancreatic cancer risk in Japanese subjects, possibly through a mechanism that is independent of obesity. Further investigation and replication of our results is required in other independent samples.

Keywords: The fat mass and obesity-associated gene, Pancreatic cancer, rs9939609, Case–control study

Background

In 2010, approximately 28,000 Japanese subjects died from pancreatic cancer, making it the fifth leading cause of cancer deaths in Japan [1]. Despite extensive research efforts, the etiology of pancreatic cancer remains poorly understood. Cigarette smoking and long-standing type II diabetes are two well-established risk factors, based on consistent findings from epidemiologic studies [2,3]. In addition, being overweight and obese have been implicated in the development of pancreatic cancer [4], with statistically significant, positive associations observed in large cohort studies conducted in Western countries [5-7], and corroborated in at least four meta-analyses [8-11] and three pooled analyses [12-14]. The positive association between body mass index (BMI) and pancreatic cancer, however, has not been clearly observed in Asian populations. To date, four cohort studies have examined the association between BMI and pancreatic cancer in Asians, but the results have been inconsistent and inconclusive [15-18].

Recently, genome-wide association (GWA) studies have identified at least 30 loci that affect BMI and the

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risk of obesity [19]. Among these loci, the fat mass and obesity-associated (FTO) gene, which was first identified in a GWA study of diabetes in 2007 [20], has the strongest influence on BMI and obesity. Rs9939609, located in the first intron of the FTO gene, was found to be associated with both BMI and type II diabetes in subsequent GWA studies in diverse populations [21-23]. The association of rs9939609 with various traits, including hip circumference, energy intake and total mortality has also been studied [24-26]. In addition, rs9939609 genotypes have been linked with the risk of prostate, breast and endometrial cancers [27-29]. The association between genetic variations in the FTO gene and the risk of pancreatic cancer, however, is not clear. Of the three studies that examined this association, only one case–control study, conducted at the MD Anderson Cancer Center in the United States, reported that the minor A allele of FTO, rs9939609, was associated with an increased risk of pancreatic cancer among overweight subjects [30]. Another two studies examined rs8050136 of the FTO gene, with one study reporting a positive association [31], and the other no association [32].

Given the mounting evidence showing a positive association between obesity and pancreatic cancer, we hypothesized that variants in the FTO gene may be associated with pancreatic cancer risk through effects on obesity or other mechanisms. In a search of the literature for obesity-related genetic variants, we found that FTO rs9939609 was the most widely studied single nucleotide polymorphism (SNP), and has been found to exert strong effects on BMI, as well as diabetes. Furthermore, it showed strong linkage disequilibrium with other SNPs in the FTO gene, such as rs8050135 and rs17817449 [22]. We therefore investigated the association between FTO rs9939609 and pancreatic cancer risk in a case–control study in Japan.

Methods

Study subjects

Our study is an ongoing hospital-based case–control study focusing on the role of genetic polymorphisms and gene-environment interaction in pancreatic cancer. For the present analysis, eligible cases were patients aged older than 20 years, who were newly diagnosed with pancreatic cancer in five hospitals located in central, north and Tokyo metropolitan areas from April 1, 2010 through May 15, 2012. The diagnosis of pancreatic cancer was based on imaging modalities or pathologic reports. The response rate among cases was 85% (441/516) as of July 1, 2012. Almost all of the cases were approached within a week after the diagnosis of pancreatic cancer, and very few cases died before they were invited to participate in our study. During the same period, we recruited control subjects with no diagnosis of cancer from inpatients and outpatients from the participating hospitals where the cases were enrolled, as well as relatives of inpatients, and individuals undergoing a medical checkup in one of the participating hospitals. Control subjects were eligible if they were more than 20 years old and had no prior cancer diagnoses. Recruitment of controls was accomplished by approaching eligible participants in the hospitals who satisfied the study requirements, and the response rate was 98% (525/534). Control subjects had a variety of diseases, such as anemia, gastric ulcer, and irritable bowel syndrome. Control subjects were matched with case patients according to sex and age (within 10-year categories). As a result, data from 360 case patients and 400 control subjects were included in the present analysis.

All subjects provided written, informed consent. This study was approved by the ethical board of Aichi Medical University (Nagakute, Japan), the Institutional Review Board (IRB) of Cancer Institute Hospital (Tokyo, Japan), the IRB of Kanagawa Cancer Center Hospital (Kanagawa, Japan), the IRB of Tokyo Metropolitan Komagome Hospital (Tokyo, Japan), and the IRB of Sapporo Medical University (Sapporo, Japan).

Data collection

Study subjects were asked to fill out a self-administered questionnaire including information on demographic characteristics, medical history, and lifestyle factors, such as cigarette smoking, alcohol consumption and dietary intake. For body weight, data on usual weight over the year prior to study entry as well as weight at age 20 were reported by the study participants. For current or former smokers, we collected detailed data on smoking exposure, including smoking status (never, former, or current smokers), average number of cigarettes smoked per day, age at starting and quitting, and duration of smoking. For subjects with type II diabetes, we recorded the age at diagnosis. In addition to the questionnaire survey, all consenting participants provided a 7-mL venous blood sample. Genomic DNA was extracted from peripheral lymphocytes at SRL Hachioji Laboratory and then stored at -30°C at the Department of Public Health, Aichi Medical University.

Genotyping assays

Genotyping was performed using the Taqman SNP Genotyping Assay (Applied Biosystems, Foster City, CA, USA) at the laboratory of Aichi Cancer Center Research Institute, Nagoya, Japan. Laboratory staff were blinded to case or control status. Four quality control samples were included in each assay, and the successful genotyping rate was 100%.

Statistical analysis

Case–control differences in selected demographic characteristics and risk factors were evaluated using t tests (for continuous variables) and Chi-square tests (for categorical variables). A chi-square test was used to test genotype frequencies in control subjects for Hardy-Weinberg equilibrium (HWE) by comparing observed genotype frequencies with those expected under HWE. A co-dominant genomic model was assumed for SNP effects. Unconditional logistic regression methods were used to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for the association between rs9939609 genotypes and pancreatic cancer risk. Homozygous carriers of the common FTO rs9939609 T allele served as the reference group. All analyses were adjusted for age (continuous), sex (male or female), BMI (<20, 20–22.4, 22.5-24.9, ≥25.0), history of diabetes (yes or no), and cigarette smoking (current, former, never smokers). ORs were also estimated for the variant allele on the basis of a log-additive model. The interaction of genotype-BMI and genotype-history of diabetes with respect to pancreatic cancer risk was assessed using the likelihood ratio test. Because recent-onset diabetes may result from pancreatic cancer, we performed an analysis excluding cases who had onset of diabetes within 2 years prior to the diagnosis of pancreatic cancer.

All P-values were two-sided, with $P<0.05$ indicating statistical significance. All statistical analyses were performed using SAS 9.2 (SAS Institute, Inc., Cary, NC, USA).

Results

The distribution of genotypes among control subjects did not deviate from the Hardy-Weinberg equilibrium (P=0.94). The minor allele frequency (MAF) was 0.18 among control subjects. Table 1 summarizes the characteristics of cases and controls. Both groups had a similar distribution of sex and 10-year age groups. The mean age was 65.1±8.1 years for cases, and 58.5±9.1 years for controls. Cases were more likely to be current smokers and have a history of diabetes compared with controls. Current smokers had an approximately 2.9-fold increased risk of pancreatic cancer compared with nonsmokers, after adjustment for age, sex, BMI, and history of diabetes (OR=2.86; 95%CI: 1.79-4.57). Individuals who had a BMI of 30 or more had a 1.21-fold increased risk, but the association was not statistically significant. Similar results were obtained in an additional analysis in which BMI at age 20 was used (data not shown). Risk of pancreatic cancer was significantly increased among subjects reporting a history of diabetes (OR=2.94; 95%CI: 1.90-4.57). The significant, positive association remained after excluding pancreatic cancer cases with recent-onset diabetes (OR=1.92; 95%CI: 1.20–3.08). Among control subjects, the mean BMI was 22.7±3.1 for the TT genotype, 23.2±3.3 for the TA genotype, and 21.1±2.9 for the AA genotype.

Table 1 Association between variations in the fat mass and obesity-associated gene and pancreatic cancer risk: a case–control study in Japan

Characteristics	Case patients (N=360)	Control subjects (N=400)	OR (95% CI)
Age group			Matching factor
<50	12 (3.3)	19 (4.8)	
50-59	44 (12.2)	79 (19.8)	
60-69	141 (39.2)	170 (42.5)	
70-79	138 (38.3)	115 (28.8)	
≥80	25 (6.9)	17 (4.3)	
Sex			Matching factor
Female	215 (59.7)	226 (56.5)	
Male	145 (40.3)	174 (43.5)	
Body mass index (kg/m^2)			
<25	278 (77.2)	312 (78.0)	1.00
25.0-29.9	64 (17.8)	75 (18.7)	0.96 (0.65-1.43)
≥30	16 (4.4)	12 (3.0)	1.21 (0.53-2.77)
Unknown	2 (0.6)	1(0.3)	-
Smoking status			
Non-smokers	145 (40.2)	202 (50.5)	1.00
Former smokers	119 (33.1)	140 (35.0)	1.23 (0.82-1.85)
Current Smokers	96 (26.7)	58 (14.5)	2.86 (1.79-4.57)
History of diabetes			
No	269 (74.7)	362 (90.5)	1.00
Yes	87 (24.2)	35 (8.7)	2.94 (1.90-4.57)
Unknown	4 (1.1)	3 (0.8)	-

OR: odds ratio; CI: confidence interval.
OR was adjusted for sex, age, smoking status and history of diabetes.

Table 2 shows the association between variants in the FTO gene (rs9939609) and pancreatic cancer risk. Compared with individuals with the TT genotype, the multivariate adjusted OR for developing pancreatic cancer was 1.48 (95%CI: 1.07–2.04) among those with the TA

Table 2 Association between the FTO rs9939609 and pancreatic cancer risk

FTO rs9939609	Cases	Control subjects	Age- and sex-adjusted OR	Multivariable-adjusted OR
TT	213	271	1.00	1.00
TA	133	116	1.49 (1.09-2.03)	1.48 (1.07-2.04)
AA	14	13	1.49 (0.67-3.29)	1.66 (0.70-3.90)

OR: odds ratio ; CI: confidence interval.
Multivariable adjusted OR: adjusted for age, sex, body mass index, cigarette smoking and history of diabetes.

genotype, and 1.66 (95%CI: 0.70–3.90) among those with the AA genotype. Under the dominant model, the OR was 1.49 (95%CI: 1.09–2.05) among carriers of the TA/AA genotype. Under the log-additive model, each additional copy of minor allele A was associated with a 1.4-fold increased risk of pancreatic cancer (OR=1.41, 95% CI: 1.07–1.85).

We found no significant interaction between FTO rs9939609 and BMI (Table 3). Individuals with both a TA/AA genotype and a history of diabetes had a 3.7-fold increased risk of pancreatic cancer compared with those with a TT genotype and no history of diabetes (Table 4), but a test for the interaction was not statistically significant.

Discussion

This was a hospital-based case–control study in Japan to investigate whether genetic variations in the FTO gene were associated with pancreatic cancer risk. The main findings of our study were: 1) individuals with the FTO rs9939609 TA genotype had a significant 1.5-fold increased risk of pancreatic cancer compared with those with the TT genotype; and 2) a combination of the FTO rs9939609 TA/AA genotype and a history of diabetes significantly increased the pancreatic cancer risk, with an OR of 3.70 (95%CI: 1.59–8.63).

We found that obesity, defined as a BMI of 30 or more, was associated with 1.2-fold increased risk of pancreatic cancer, but this association was not statistically significant. In contrast to evidence of a positive association between obesity and pancreatic cancer in Western countries, available data on the role of obesity in pancreatic cancer in Japanese are inconclusive. There have been no prospective studies that have observed a clear, dose–response relation between baseline BMI and pancreatic cancer risk in the Japanese population [15,16]. Given that less than 5% of the subjects were obese in this study, it might be difficult to observe significant associations. The small percentage of obese people may be the main reason for the inconclusive results on BMI and pancreatic cancer in Asians, including Japanese [15-18]. In addition, differences in body fat distribution, in genetic predisposition to obesity and in lifestyle factors between Caucasians and Asians may contribute to the inconsistent results on BMI and pancreatic cancer risk in Asian populations [33,34].

Table 3 Joint associations of the FTO rs9939609 and BMI with respect to pancreatic cancer risk

Genotype	BMI	Cases/control subjects	Age- and sex-adjusted OR	Multivariable-adjusted OR
TT	<25	166/220	1.00	1.00
TA/AA	<25	112/92	1.69 (1.20-2.40)	1.68 (1.18-2.41)
TT	≥25	45/51	1.29 (0.81-2.04)	1.20 (0.75-1.94)
TA/AA	≥25	35/36	1.35 (0.81-2.25)	1.21 (0.71-2.07)
				P for interaction=0.29

Multivariable OR: adjusted for age, sex, cigarette smoking and history of diabetes.

Table 4 Joint associations of the FTO rs 9939609 and history of diabetes with respect to pancreatic cancer risk

Genotype	History of diabetes	Cases/ control subjects	Age- and sex-adjusted OR	Multivariable-adjusted OR
TT	No	163/243	1.00	1.00
TA/AA	No	106/119	1.38 (0.99-1.93)	1.41 (1.00-1.98)
TT	Yes	34/26	1.76 (1.01-3.07)	1.70 (0.96-3.00)
TA/AA	Yes	24/8	4.03 (1.75-9.24)	3.70 (1.59-8.63)
				P for interaction=0.28

Cases were excluded if the onset of diabetes was within 2 years prior to the diagnosis of pancreatic cancer.
Multivariable OR: adjusted for age, sex, body mass index, and cigarette smoking.

Because of the positive association between obesity and pancreatic cancer in Caucasians and the plausible mechanisms, several research groups have hypothesized that variants in obesity-related genes might be associated with pancreatic cancer risk. The association between rs9939609 in the FTO gene was reported in one previous hospital–based case–control study conducted at the MD Anderson Cancer Center, Texas, USA [30]. Of the 15 obesity- and diabetes-associated genotypes in the FTO gene, rs9939609 was found to be positively associated with pancreatic cancer risk in persons who were overweight, whereas no increased risk was observed in persons who had a BMI of less than 25 kg/m^2 [30]. In contrast, our study showed a significant, positive association between rs9939609 TA/AA genotype and pancreatic cancer risk in individuals with a BMI of less than 25 kg/m^2. We consider that the difference in minor allele frequency (MAF) may be the main reason, given the fact that the MAF was 18% in our study, much lower than the 38% in the MD Anderson Cancer Center case–control study. The possible differences in selection of cases and controls, patterns of linkage disequilibrium and effects of gene-gene interactions may also account for the inconsistent findings. In addition to rs9939609, rs8050136 in the FTO gene was found to be associated with pancreatic cancer risk in individuals of European ancestry [31]; however, no association was noted in another case–control study [32].

In our study, FTO rs9939609 genotypes were associated with pancreatic cancer risk. However, the mean BMI did not differ among rs9939609 genotypes for control subjects, and no significant interaction was observed between rs9939609 TA/AA genotypes and BMI with

respect to pancreatic cancer risk. It is possible that the positive association observed between rs9939609 genotypes and pancreatic cancer risk may be driven by a mechanism other than adiposity. Diabetes, a well-established risk factor for pancreatic cancer, is a possible candidate. There is evidence suggesting that Asian people are more susceptible to insulin resistance at a lesser degree of obesity than Caucasians [33,34]. Besides its close association with adiposity, FTO has been shown to be associated with susceptibility to type II diabetes [21,22]. We found that individuals with a TA/AA genotype and a history of diabetes were at a 3.7-fold increased risk of pancreatic cancer. However, a test for the interaction was not statistically significant. Another possibility is that FTO is just a proxy of as yet unidentified causal variants, and it is those variants that exert their effects on rs9939609 and influence pancreatic cancer risk. Given that the function of the FTO gene is largely unknown, further studies are needed to comprehensively evaluate multiple SNPs in the FTO gene and elucidate the mechanisms by which FTO rs9939609 influences pancreatic cancer risk.

Our study has several limitations. First, it is well-known that two significant issues, namely selection bias and recall bias, plague case–control studies. Our results might have been biased if hospital controls did not represent the same population from which the cases were derived. However, the allele frequencies observed among control subjects in our study were similar to those reported in the studies of Asian populations [22]. In particular, the MAF of FTO rs9939609 was 18% in our control subjects, which is very close to that reported from a sample of 100 Japanese included in the HapMap project. Moreover, the risk estimates for current smokers and individuals with a history of diabetes were comparable to those estimated from cohort or population-based case–control studies [2,3], providing indirect evidence that selection bias might not be a serious concern in our study. Second, as for recall bias, while the analysis of the association between pancreatic cancer and BMI based on self-reported weight and height might be affected by recall bias, the association with the obesity-related genotype was not. Third, although our study included a relatively large sample size compared with previous studies conducted in Japan, the sample size may not have been large enough to detect significant gene-environment interactions in subgroups. Finally, it is possible that the results could represent a chance association and therefore replication in other independent samples is required. Despite these limitations, there are several advantages of the hospital-based design adopted in our study, including rapid case ascertainment, a high response rate from both cases and controls, and high quality genotyping.

Conclusion

Our findings indicate that rs9939609 in the FTO gene is associated with pancreatic cancer risk in Japanese subjects, possibly through a mechanism that is independent of obesity. Because of the limited statistical power, our results need replication in other independent samples. The fast-increasing prevalence of overweight/obesity and type II diabetes in Asians provides a good opportunity to further address this association and its underlying mechanisms.

Competing interests

The authors declare no conflict of interest.

Authors' contribution

SK supervised the study, SK, YL, KY designed the study, YL drafted the manuscript and conducted the statistical analysis. JU and KM performed genotyping and SNP data analysis. HI, MU, NE, HN, MM participated in data collection. All authors read and approved the final manuscript.

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BRIEF ARTICLE

Ultrasound-guided *vs* endoscopic ultrasound-guided fine-needle aspiration for pancreatic cancer diagnosis

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Abstract

AIM: To clarify the effectiveness and safety of endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) for the diagnosis of pancreatic cancer (PC).

METHODS: Patients who were diagnosed with unresectable, locally advanced or metastatic PC between February 2006 and September 2011 were selected for this retrospective study. FNA biopsy for pancreatic tumors had been performed percutaneously under extracorporeal ultrasound guidance until October 2009; then, beginning in November 2009, EUS-FNA has been performed. We reviewed the complete medical records of all patients who met the selection criteria for the following data: sex, age, location and size of the targeted tumor, histological and/or cytological findings, details of puncture procedures, time from day of puncture until day of definitive diagnosis, and details of severe adverse events.

RESULTS: Of the 121 patients who met the selection criteria, 46 had a percutaneous biopsy (Group A) and 75 had an EUS-FNA biopsy (Group B). Adequate cytological specimens were obtained in 42 Group A patients (91.3%) and all 75 Group B patients ($P = 0.0192$), and histological specimens were obtained in 41 Group A patients (89.1%) and 65 Group B patients (86.7%). Diagnosis of malignancy by cytology was positive in 33 Group A patients (78.6%) and 72 Group B patients (94.6%) ($P = 0.0079$). Malignancy by both cytology and pathology was found in 43 Group A (93.5%) and 73 Group B (97.3%) patients. The mean period from the puncture until the cytological diagnosis in Group B was 1.7 d, which was significantly shorter than that in Group A (4.1 d) ($P < 0.0001$). Severe adverse events were experienced in two Group A patients (4.3%) and in one Group B patient (1.3%).

CONCLUSION: EUS-FNA, as well as percutaneous needle aspiration, is an effective modality to obtain cytopathological confirmation in patients with advanced PC.

Key words: Endoscopic ultrasound-guided fine needle aspiration; Percutaneous needle aspiration; Pancreatic cancer

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INTRODUCTION

Pancreatic cancer (PC) is currently the fifth leading cause of cancer-related mortality in Japan. Although complete surgical removal of the tumor is the only chance of cure, almost all PC patients are initially diagnosed as having advanced unresectable disease despite recent improvements in diagnostic techniques. In recent decades, techniques were developed to obtain proof of cancer from the primary tumor in PC patients. Pancreatic juice cytology *via* endoscopic retrograde pancreatography was initially developed to meet this challenge; however, in practical settings the positive rate for cancer cells has remained low, indicating the presence of false-negative results[1,2]. Ultrasonography-guided fine-needle aspiration (US-FNA) biopsy or computed tomography (CT)-guided FNA biopsy appears to provide a more definitive diagnosis of PC[3,4]. US-FNA is convenient but its usefulness is limited for masses in the pancreatic tail. In contrast, CT-guided FNA is the biopsy procedure of choice to assess pancreatic lesions. However, this technique is time-consuming and is limited by a substantial false-negative rate of approximately 20%[5]. In addition, there have been concerns about percutaneous cancer seeding[6,7]. Recently, endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) has been developed as a more feasible method to obtain definitive specimens for cytological and/or histological examinations for diagnosis of PC[8-12]. Three years ago, we began to perform EUS-FNA although until that time US-FNA was the standard technique at our institute.

In the current study, we retrospectively examined the diagnostic ability of EUS-FNA for PC compared with US-FNA.

MATERIALS AND METHODS

Patients

The inclusion criteria were: (1) the patient underwent US-FNA between February 2006 and October 2009 or EUS-FNA between November 2009 and September 2011 at the Cancer Institute Hospital, Tokyo, Japan for suspected PC; and (2) the patient was subsequently diagnosed as having clinical stage Ⅲ or Ⅳ PC. Unresectable PC, which was indicated by International Union Against Cancer clinical stage Ⅲ (locally advanced disease: T4N0-1 and M0) or Ⅳ (metastatic disease: T1-4N0-1 and M1), was diagnosed by CT.

The exclusion criteria were: (1) a contraindication for EUS (esophageal stenosis, duodenal stenosis, ileus, or perforation of the digestive tract); and (2) a contraindication for EUS-FNA and US-FNA (severe cardiovascular disease or respiratory disease, poor performance status, difficulty in visualization of the target, bleeding tendency, or impossibility of ensuring the puncture route).

Patients who met the selection criteria were identified from the database in our division, which was updated daily.

US- and EUS-FNA procedures

A short admission, usually for one or two nights, was mandatory according to the protocol for FNA biopsy of a suspected pancreatic tumor in our division. FNA biopsy for pancreatic tumors had been performed percutaneously under extracorporeal ultrasound guidance (US-FNA) until October 2009; then, beginning in November 2009, FNA biopsies have been performed under EUS guidance (EUS-FNA). In general, FNA examinations were performed and managed by Ishii H until October 2009 and by Matsuyama M since November 2009. Written informed consent was obtained from each patient before the examination.

US-FNA was performed using SSA-550A (Toshiba, Tokyo, Japan) as the ultrasound device and SONOPSY C1 21G (Hakko, Osaka, Japan) as the ultrasound-guided biopsy needle. After systemic premedication and percutaneous local anesthesia, FNA was performed 1-3 times repeatedly until adequate material was obtained. Pathological examination of the obtained materials and cytological examination of the needle-washing water were done. There was no on-site cytotechnologist during the performance of US-FNA.

EUS-FNA was performed using EU-ME1 and UCT240-AL5 (Olympus, Tokyo, Japan) as the EUS system and the Echo-Tip ULTRA 22G (Wilson-Cook, Bloomington, IN, United States) as the ultrasound-guided biopsy needle. After systemic premedication and pharyngeal local anesthesia, FNA was performed endoscopically *via* the stomach or duodenum. Aspiration puncture was repeated until an on-site cytology screener confirmed that adequate materials had been obtained.

After the examination, patients stayed in the hospital overnight and were discharged the following morning if no problems were revealed by physical examination, complete blood count tests and biochemistry tests that included serum amylase level. Three to 7 d later, the patients came to the outpatient clinic for an explanation of the results of the biopsy and examination for late adverse events, and were then able to start chemotherapy.

The final diagnosis was based on pathology results or clinical follow-up of > 6 mo.

Statistical analysis

We reviewed the complete medical records of all patients who met the selection criteria for the following data: sex, age, location and size of the targeted tumor, histological and/or cytological findings of the obtained specimens, details of puncture procedures, time from day of puncture until the day of definitive diagnosis, and details of severe adverse events, if any. The tumor status (location and size) was determined by dynamic CT before puncture. Frequency analysis was performed with Fisher's exact test for 2 × 2 tables, χ^2 test for 3 × 2 tables, and Mann-Whitney test. All analysis were performed using the statistical software SPSS 11.0J for Windows. Statistical significance was defined as a two-sided P value ≤ 0.05.

Table 1 Characteristics of patients and comparison of results of percutaneous biopsy with those of endoscopic ultrasound-guided fine-needle aspiration

	Percutaneous biopsy Group A	EUS-FNA Group B	*P* value
Patients	46	75	
Site of puncture			
Pancreas	46	74	> 0.9999
Head/body/tail	12/32/2	34/31/9	0.0114
Sex (male/female)	25/21	39/36	> 0.8525
Age, yr			> 0.8466
≥ 65	28	48	
< 65	18	27	
Tumor diameter, mm (range)	44.8 (18-111)	25.5 (7-70)	
≥ 40	30	25	0.0007
< 40	16	50	
Passes (range)	2.26 (1-4)	2.85 (2-5)	< 0.0001
Adequate specimens obtained[1] *n* (%)			
Cytology	42 (91.3)	75 (100)	0.0192
Histology	41 (89.1)	65 (86.7)	0.7812
Positivity for cancer *n* (%)			
Cytology	33 (78.6)	72 (94.6)	0.0079
Histology	33 (80.5)	51 (78.4)	> 0.9999
Total *n* (%)	43 (93.5)	73 (97.3)	0.3672
Complications *n* (%)	2 (4.3) Fever[1] Bleeding[1]	1 (1.3) Peritonitis[1]	> 0.5567
Time from puncture to definitive diagnosis			
Cytology, d (range)	4.05 (0-8)	1.65 (0-5)	< 0.0001
Histology, d (range)	3.95 (2-7)	3.18 (2-10)	0.7066

[1]An on-site pathologist was available for endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) but not for ultrasonography-guided-FNA.

RESULTS

US-FNA was performed in 48 patients from February 2006 until October 2009. Two cases (renal cell carcinoma and malignant lymphoma) were excluded from the analysis of US-FNA because the patients did not have primary PC. EUS-FNA was attempted in 125 cases and was successfully performed in 123 cases from November 2009 until September 2011. Among these, 48 patients did not meet the selection criteria (lymph node metastasis, 34 cases; other pancreatic tumor, 10 cases; other abdominal tumor, three cases, and mediastinum tumor, one case). EUS-FNA could not be performed in two patients because of difficulty of visualization due to total gastrectomy in one case, and impossibility of ensuring the puncture route in the other. Thus, 46 patients who underwent US-FNA (Group A) and 75 who underwent EUS-FNA (Group B) were eligible for analysis.

Table 1 shows the characteristics of the study subjects. The distribution of the target tumor in the pancreas differed significantly between the two groups, with the tumor location more frequent in the pancreatic head/tail than in the pancreatic body in Group B. The maximum diameter of the target tumor ranged from 18 to 111 mm (median, 44.8 mm) in Group A and from 7 to 70 mm (median, 25.5 mm) in Group B. A significantly larger number of target tumors were < 40 mm in Group B than in Group A ($P = 0.0007$).

Table 1 shows a comparison of the results of percutaneous biopsy with those of EUS-FNA. Adequate cytological and histological specimens were obtained in 42 (91.3%) and 41 (89.1%) Group A patients ($n = 46$), respectively, and in 75 (100%) and 65 (86.7%) Group B patients ($n = 75$).

Results of cytology indicated the presence of cancer cells in 33 Group A patients (78.6%) and in 72 Group B patients (94.6%). Histological studies showed cancer tissue in 33 (80.5%) and 51 (78.4%) patients in Group A and Group B, respectively. In total, a cancer diagnosis was made in 43 Group A (93.5%) and 73 Group B (97.3%) patients by cytology and/or histology. These 116 patients were diagnosed with pancreatic adenocarcinoma by cytology/histology as well as by imaging and their subsequent clinical course. The final diagnosis of PC in the remaining five patients for whom there was no cytological or histological proof was confirmed by the clinical course until April 2012. The positive cytology/histology rate did not differ between the two groups.

Total puncture procedures per patient varied from one to five, with a median of 3. The frequency of multiple punctures, that is, > 2, was significantly higher in Group B than in Group A. Time from the day of puncture until the day of the final cytological diagnosis varied from 0 to 8 d (median, 4.1 d) in Group A and from 0 to 5 d (median, 1.7 d) in Group B. The period was significantly shorter in Group B than in Group A. The time from the day of puncture until the day of the final histological diagnosis varied from 2 to 7 d (median, 4.0 d) in Group A and 2 to 10 d (median, 3.2 d) in Group B, with no significant difference between the two groups.

Severe adverse events occurred in two Group A patients (4.3%) and in one Group B patient (1.3%). In Group A, one patient developed a high fever, which required hospitalization but resolved with only symptomatic treatment. The other Group A patient experienced upper gastrointestinal bleeding, which was confirmed by endoscopy to be related to the needle biopsy. This patient was treated by blood transfusion and antiulcer medication and was hospitalized for 1 wk without surgical intervention. The adverse event in Group B was an abdominal abscess that required surgical drainage. The patient experienced continuous abdominal pain one night after EUS-FNA, and dynamic CT demonstrated an abscess in front of the pancreatic body tumor, which was clearly related to the EUS-FNA puncture. Fortunately, she recovered after surgery and antibiotic therapy and could receive chemotherapy thereafter. There was no cancer seeding event up to 6 mo from the time of puncture in any patient in either group.

DISCUSSION

The aim of the current study was to investigate the results of two different approaches to obtain pancreatic biopsy specimens, which are a percutaneous approach and EUS-FNA, because this issue has seldom been ad-

dressed[12]. Our results confirmed the usefulness of EUS-FNA, especially with regard to cytology. The National Comprehensive Cancer Network Guidelines (2012) require that cytological or histological confirmation is needed for the diagnosis of unresectable pancreatic carcinoma[13]. In patients with stage IV PC, a biopsy of the metastatic lesion is preferred for proof of cancer. However, in those with stage III PC and some patients with stage IV PC in whom it is difficult to access metastatic sites for biopsy procedures, the primary tumor of the pancreas must be targeted to obtain proof of cancer. Pancreatic juice cytology was developed in the early 1980s and is still being performed; however, cancer cells cannot easily be observed by collection of pancreatic juice[1,2,14]. Percutaneous needle biopsy was developed with the expectation of a more definitive method to obtain proof of cancer from the primary pancreatic tumor[3,15,16]. Our institute then used percutaneous needle biopsy under extracorporeal US guidance as the standard for histological confirmation of the pancreatic primary tumor. Recently, EUS-FNA was introduced and was used mainly in high-volume cancer centers in Japan[17-22]. As a result of the risk of cancer seeding as well as other risks with percutaneous biopsy, we adopted EUS-FNA beginning in November 2009 in place of percutaneous biopsy. We expected that EUS-FNA would have advantages over a percutaneous procedure with regard to efficacy in confirmation of cancer and avoiding adverse reactions before administering chemotherapy to patients with PC.

Our results demonstrated that EUS-FNA is effective and feasible for obtaining proof of cancer in candidates for PC chemotherapy. In fact, EUS-FNA might have merits with regard to obtaining specimens from small tumors or tumors in the pancreatic tail, for which performance of percutaneous biopsy is difficult[2,23-27]. In this study, the location of the target tumor was most frequent at the body of the pancreas in Group A. In addition, the target tumors were larger in Group A than in Group B. These findings suggest that patients might have been excluded from Group A in which difficulty could be expected in making a puncture because the tumor was either small or difficult to delineate. In these cases, endoscopic retrograde cholangiopancreatography or liver biopsy might have been performed to obtain confirmation of malignancy, if possible.

Horwhat *et al*[12] have performed a randomized controlled trial of EUS-FNA and percutaneous biopsy of the pancreas (US- and CT-guided) in 2006. Although there was no statistically significant difference in accuracy between the two methods, the results showed that EUS-FNA had the advantage in the diagnosis of pancreatic malignancy. In our study, the diameters of the target tumors in the EUS-FNA group (Group B) were smaller than those in the US-FNA group (Group A) and the deviation of distribution around the puncture site was smaller in the EUS-FNA than the US-FNA group. Our results indicated high performance through the use of EUS-FNA and are not inconsistent with those of Horwhat *et al*[12]. In the present study, there was no analysis of accuracy in the two groups, because our institution is an oncology hospital and we rarely perform biopsies of benign cases.

The benefits of EUS-FNA might be maximized to make a pathological diagnosis in patients with an abdominal tumor of an uncertain type. The definite merit of our EUS-FNA procedure was thought to be rapid cytological results, but perhaps success in this regard was mainly due to the contribution of an on-site cytotechnologist and not to the EUS-FNA procedure itself. Iglesias-Garcia *et al*[28] have claimed that on-site cytological evaluation improves the diagnostic yield of EUS-guided FNA for the cytological diagnosis of solid pancreatic masses. Savoy *et al*[29] have pointed out that even trained endosonographers have variable and, in some cases, inferior abilities in interpreting on-site cytology in comparison with cytotechnologists. In the present study, we had adequate specimens for all cases in the EUS-FNA group. This is natural because we continued the examination until we obtained a sufficient quantity of specimens that were checked by the on-site cytotechnologist. On the contrary, there was no difference in the rate of adequate specimens obtained for histological examination between the EUS-FNA and US-FNA groups, because the collected tissue was checked by the examiner's naked eye in both groups. The presence of an on-site cytotechnologist to accompany EUS-FNA is considered to be necessary, at least, in high-volume centers.

In the present study, the positivity rate for malignancy was higher for EUS-FNA cytology than for histology. Supporting the current results, another study has shown that the positivity rate for malignancy in EUS-FNA cytology of the pancreas was higher than that in histology[30].

As previously reported, EUS-needle core biopsy is useful for histological and cytological diagnosis in terms of sample volume[31]. In addition, the combined results of EUS-FNA cytology and EUS-needle core biopsy have been reported to improve diagnosis[32-34]. However, to confirm the malignancy, EUS-FNA cytology is more useful than EUS-needle core biopsy[35]. This result is similar to the results of our study, indicating that cytology might be more useful than histology for the diagnosis of malignancy.

In the current study, there was no cancer seeding in any patient in either group. As previously reported, there were rare cases of seeding among patients who underwent US-guided FNA[36]. With regard to the puncture route, we suggest that there is less possibility of seeding in patients who undergo EUS-FNA than in patients who undergo US-FNA, although some recent studies have shown the possibility of seeding in patients who undergo EUS-FNA[37-39]. We did inform patients who were scheduled to undergo EUS-FNA about the possibility of this complication.

The limitations of our study included its retrospective nature. Furthermore, there were no cases of benign pancreatic conditions to enable an evaluation of US and EUS-FNA for accurate differentiation between malignant

and benign diseases.

In conclusion, EUS-FNA, as well as percutaneous needle aspiration, is an effective modality to obtain cytopathological confirmation in patients with advanced PC. EUS-FNA cytology was able to detect malignancy at a high rate. We believe that EUS-FNA has advantages for smaller tumors located deeply and for tumors in which the diagnosis is uncertain by various other imaging modalities.

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COMMENTS

Background

Ultrasonography-guided fine-needle aspiration (US-FNA) biopsy or computed tomography (CT)-guided FNA biopsy was used for histological/cytological diagnosis of pancreatic cancer (PC). US-FNA is limited to masses in the pancreatic tail. CT-guided FNA is time-consuming and limited by a substantial false-negative rate. There have been concerns about percutaneous cancer seeding and difficulty in puncturing for small tumors. Endoscopic ultrasound (EUS)-guided FNA has been developed as a more feasible method of obtaining definitive specimens for the diagnosis of PC. Studies on the results of the two different approaches to obtain pancreatic biopsy specimens, which are the percutaneous approach and EUS-FNA, have rarely been conducted.

Research frontiers

The benefits of EUS-FNA might be maximized to be able to make a pathological diagnosis in patients with an abdominal tumor of an uncertain type.

Innovations and breakthroughs

EUS-FNA is effective and feasible for obtaining proof of cancer in PC chemotherapy candidates. In fact, EUS-FNA might have advantages with regard to obtaining specimens from small tumors or tumors in the pancreatic tail, for which performance of percutaneous biopsy is difficult.

Applications

The results suggest that EUS-FNA is the best method of obtaining cytological samples for diagnosis of unresectable PC. This method can be used for other types of cancer.

Terminology

On-site cytotechnologist: An on-site cytotechnologist should attend the puncture examination to confirm quickly the existence of atypical cells. The information of the cytotechnologist is more appropriate than that of the endoscopist.

Peer review

This is a good descriptive study in which EUS-FNA is a feasible and safe technique to acquire pancreatic specimens. The results are interesting in that the advantages of EUS-FNA over the percutaneous procedure are time between examination and diagnosis, the possibility of puncture of small tumors, and tumors in the tail of the pancreas.

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