Japanese Journal of Clinical Oncology Advance Access published October 23, 2014

JJCO Japanese Journal of Clinical Oncology

Japanese Journal of Clinical Oncology, 2014, 1–6
doi: 10.1093/jjco/hyu159
Original Article

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Prognostic value of neutrophil–lymphocyte ratio and level of C-reactive protein in a large cohort of pancreatic cancer patients: a retrospective study in a single institute in Japan

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Received 7 July 2014; Accepted 21 September 2014

Abstract

Objective: Recent studies suggest that systemic inflammatory response is closely associated with cancer patient prognosis. Although several inflammatory prognostic markers have been proposed, the data to support their validity are lacking in large Japanese cohorts.

Methods: This is a retrospective study to examine the prognostic value of inflammatory markers, such as C-reactive protein, neutrophil–lymphocyte ratio, platelet–lymphocyte ratio and modified Glasgow prognostic scale, in pancreatic cancer. Selection criteria were admittance to hospital between January 2008 and December 2012, histologically confirmed adenocarcinoma, diagnosis of invasive ductal pancreatic cancer compatible by computed tomography imaging, and followed-up until death or for 180 days or longer. The primary end point was overall survival, which was measured from the day of histological diagnosis.

Results: There were 440 patients who met the selection criteria. Of the 440 cases, 200 (45.5%) received curative resection (166 Stage I/II and 34 Stage III patients), 237 (53.9%) received chemotherapy (4 Stage I/II, 92 Stage III and 141 Stage IV patients), and the remaining 3 received palliative care. Univariate and multivariate regression analyses revealed that advanced computed tomography stage, high level of C-reactive protein (0.45 mg/dl or greater), neutrophil–lymphocyte ratio (2.0 or greater) and CA19-9 level (1000 U/ml or greater) were significantly associated with worse prognosis.

Conclusions: We verified the results of previous studies, and showed that neutrophil–lymphocyte ratio and C-reactive protein also had prognostic value in a large Japanese PC cohort.

Key words: NLR, CRP, mGPS, PLR, survival

Introduction

Pancreatic cancer (PC) has become the fifth most common cause of cancer-related mortality in Japan; it has been estimated that PC was responsible for 29 916 deaths in 2012 (1), representing ~8% of all cancer deaths. Despite recent improvements in diagnostic techniques, only a small proportion of patients are eligible for surgery, even though resection represents the only curative treatment available thus far. Accordingly, the prognosis of PC patients is extremely poor, with a 5-year survival rate after diagnosis of <5% (2).

Recent studies suggest that the systemic inflammatory response is closely associated with cancer patient prognosis (3,4). Several parameters of the systemic inflammatory response, including level of C-reactive protein (CRP), neutrophil–lymphocyte ratio (NLR), derived NLR (dNLR), platelet–lymphocyte ratio (PLR) and modified Glasgow prognostic score (mGPS), have been demonstrated in numerous reports as good prognostic indicators in lung cancer (5), hepatocellular carcinoma (6), melanoma (7), renal cell carcinoma (8), gastric cancer (9) and colorectal cancer (10). Moreover, some studies have shown that these parameters can predicted clinical outcome in regardless of the primary site (11,12).

Further, initial reports have already indicated that the inflammatory response is predictive of prognosis in patients with PC, but most of these studies included only relatively small number of cases (13–17). An Austrian group has reported the prognostic value of NLR, dNLR and CRP as useful inflammatory markers in their large cohort of PC patients (18–20). In the present study, we aimed to validate the prognostic significance of inflammatory markers in a large cohort of Japanese PC patients with reference to the Austrian studies.

Patients and Methods

This retrospective study included data from 493 consecutive patients who were diagnosed with PC at the Gastroenterology Center, Cancer Institute Hospital of Japanese Foundation for Cancer Research between January 2008 and December 2012. Among these 493 patients, we selected those for the current study if all of the following criteria were met: (i) histologically or cytologically confirmed adenocarcinoma, (ii) invasive ductal PC compatible by computed tomography (CT) imaging and (iii) followed-up until death or for 180 days or longer.

Clinical variables collected in this study were: age, gender, height, weight and performance status (PS) according to the Eastern Cooperative Oncology Group grading system; white blood cell (WBC) count; fraction of neutrophil and lymphocyte in WBC differentiation (%); levels of albumin, bilirubin, CRP and carbohydrate antigen 19-9 (CA19-9); location of the primary pancreatic tumor; clinical CT stage according to the seventh edition of TNM classification; type of therapy (i.e. tumor resection, chemotherapy or symptomatic treatment); date of surgical intervention or biopsy and date of the final follow-up or death. The baseline data were obtained within 30 days prior to surgical intervention or biopsy.

The relationship between each baseline variable and long-term survival was investigated by univariate and multivariate analyses, with special focus on the prognostic impact of systemic inflammation markers. On the basis of previous studies, CRP level of 0.45 mg/dl, NLR of 2.0, dNLR (absolute count of neutrophils divided by the absolute WBC count minus the absolute count of neutrophils) of 2.3 and PLR of 150 were selected as cutoff values for validation. The mGPS was applied by combining CRP and albumin levels: 0 was defined as normal values of CRP and albumin; 1 was defined as increased CRP (1.0 mg/dl or greater) and normal albumin; and 2 was defined as increased CRP and decreased albumin (<3.5 g/ml). Other than the five inflammatory markers, variables included in the prognostic analysis were: age (65 years or younger versus older than 65); gender; PS (0 versus 1); body mass index (>25 versus 25 or greater); location of the primary tumor (head versus body–tail); clinical CT Stage (I/II, III or IV); and CA 19-9 (>1000 U/ml versus 1000 U/ml or greater).

The primary end point of this study was overall survival (OS), defined as the time from the date of histological confirmation (the date of surgery or biopsy) to death due to any cause or to the last known date alive. All patients were assessed in December 2013. Kaplan–Meier survival plots were generated, and differences in survival among subgroups classified by each factor were evaluated by log-rank tests. Cox regression was used to determine univariate hazard ratios for OS. Age, PS and all variables with significant prognostic value in the univariate analysis were selected for further evaluation in the final multivariate Cox proportional hazard model. Multivariate Cox proportion analysis by backward elimination method was performed to determine the influence of the different variables on OS. Hazard ratios estimated by the Cox analysis were reported as relative risks with corresponding 95% confidence intervals. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using the PASW Statistics 18 program (SPSS Inc., Chicago, IL, USA).

The Institutional Review Board of the Cancer Institute Hospital of the Japanese Foundation for Cancer Research approved this study, and waived the need for written informed consent from the participants because this was a retrospective non-intervention study.

Results

Of the 493 patients, 440 met the selection criteria. Of the remaining 53, 28 had other tumor histologies including neuroendocrine tumor, and 25 were transferred to a community hospital to receive palliative care within 6 months after diagnosis. Patient characteristics are summarized in Table 1. Of the 170 patients diagnosed with Stage I/II potentially resectable disease, 4 received chemotherapy because micro-metastases were found by laparotomy. Of the 127 patients diagnosed with Stage III disease, 34 underwent resection of the pancreas, 92 received chemotherapy and the remaining 1 received symptomatic treatment. Of the 143 patients diagnosed with Stage IV disease, 141 received chemotherapy and the remaining 2 received symptomatic treatment. Consequently, 200 (45.5%) patients received curative resection (166 Stage I/II and 34 Stage III cases), 237 (53.9%) received chemotherapy (4 Stage I/II, 92 Stage III and 141 Stage IV patients) and the remaining 3 received palliative care. Of the 440 selected patients, 313 (71.1%) died and the remaining 127 were still alive at the time of analysis. The median follow-up time of the 127 survivors was 18.7 months, ranging from 6.1 to 68.2 months. The median survival time of patients from the whole cohort was 11.6 months (interquartile range: 7.1–20.1 months).

Univariate Cox regression revealed that advanced CT stage, pancreatic body–tail cancer, high level of CRP, NLR, dNLR and CA19-9 level were significantly associated with worse prognosis (Table 2). We continued to analyze NLR but not dNLR in the multivariate analysis because the hazard ratio of NLR was higher than that of dNLR (1.894 versus 1.576, respectively). PLR and mGPS did not show any evident prognostic impact on survival in our cohort. In the multivariate analysis, CT stage, level of CRP, NLR and CA19-9 level were identified as independent prognostic factors in our cohort (Table 3).

Figure 1 demonstrates OS curves stratified by NLR in each CT stage, respectively. The number of patients with NLR >2.0 and those with NLR ≥2.0 were 71 (41.8%) and 99 (58.2%) in Stage I/II, 48 (37.8%) and 79 (62.2%) in Stage III and 21 (14.7%) and 122 (85.3%) in Stage IV. The prognostic value of NLR was clear especially in CT Stage I/II disease ($P = 0.014$, log-rank test). But there was no significant difference between Stages III and IV ($P = 0.079$ and $P = 0.125$).

Figure 2 demonstrates OS curves stratified by CRP in each CT stage, respectively. The number of patients with CRP <0.45 and

Table 1. Patient characteristics

Age (years)		
Median (range)	67	32–88
65 or younger	179	40.7%
Older than 65	261	59.3%
Gender		
Male	249	56.6%
Female	191	43.4%
Performance status		
0	378	83.3%
1	62	13.7%
Body mass index		
Median (range)	21.6	13.0–33.8
<25	375	85.2%
25 or greater	65	14.8%
Location of the primary tumor		
Head	220	50.0%
Body–tail	220	50.0%
Clinical CT stage		
I/II	170	38.6%
III	127	28.9%
IV	143	32.5%
C-reactive protein (mg/dl)		
Median (range)	0.12	0.01–21.9
<0.45	321	73.0%
0.45 or greater	119	27.0%
Neutrophil–lymphocyte ratio		
Median (range)	2.47	0.7–27.7
<2	140	31.8%
2 or greater	300	68.2%
Derived neutrophil–lymphocyte ratio		
Median (range)	1.77	0.5–13.3
<2.3	324	73.6%
2.3 or greater	116	26.4%
Platelet–lymphocyte ratio		
Median (range)	140.0	40.4–930.8
<150	239	54.3%
150 or greater	201	45.7%
Modified Glasgow prognostic score		
0	367	83.4%
1	49	11.1%
2	24	5.5%
Albumin (g/dl)		
Median (range)	4.0	2.4–5.0
<3.5	48	10.9%
3.5 or greater	392	89.1%
CA19-9 (U/ml)		
Median (range)	436.2	2.0–50 000
<1000	275	62.5%
1000 or greater	165	37.5%

those with CRP ≥0.45 were 147 (86.5%) and 23 (13.5%) in Stage I/II, 102 (80.3%) and 25 (19.7%) in Stage III and 72 (50.3%) and 71 (49.7%) in Stage IV, respectively. The prognostic value of CRP was evident in CT Stage III and IV disease ($P = 0.015$ and $P < 0.001$).

Figure 3 shows box plots of CRP and NLR in each CT stage. The dotted line means the cutoff level. The fraction of patients with NLR under the cutoff level was small especially in Stage IV, whereas most patients in Stage I/II had lower CRP level than the cutoff level.

Figure 4 demonstrates plots of the cumulative distribution function of NLR and CRP. The degree of asymmetric distribution of CRP was larger than that of NLR, with skewness coefficients of 5.568 and 4.803, respectively.

Table 2. Univariate cox regression

	HR	95% CI	*P* value
Age			
65 or younger	1		
Older than 65	0.806	0.644–1.008	0.059
Gender			
Male	0.985	0.788–1.232	0.897
Female	1		
Performance status			
0	1		
1	1.261	0.924–1.720	0.143
Body mass index			
<25	1		
25 or greater	1.192	0.883–1.609	0.252
Location of the primary tumor			
Head	1		
Body–tail	1.499	1.199–1.873	<0.001
Clinical CT stage			
I/II	1		
III	2.225	1.666–2.972	<0.001
IV	5.351	3.996–7.166	<0.001
C-reactive protein (mg/dl)			
1			
0.45 or greater	2.323	1.820–2.966	<0.001
Neutrophil–lymphocyte ratio			
<2.0	1		
2.0 or greater	1.894	1.474–2.435	<0.001
Derived neutrophil–lymphocyte ratio			
<2.3	1		
2.3 or greater	1.576	1.234–2.012	<0.001
Platelet–lymphocyte ratio			
<150	1		
150 or greater	1.048	0.838–1.309	0.683
Modified Glasgow prognostic score			
0	1		
1	2.61	1.89–3.605	<0.001
2	1.465	0.906–2.369	0.119
Albumin (g/dl)			
<3.5	1		
3.5 or greater	1.161	0.801–1.683	0.431
CA19-9 (U/ml)			
<1000	1		
1000 or greater	2.002	1.591–2.519	<0.001

HR, hazard ratio; CI, confidence interval.

Discussion

Previous studies suggest that disease progression in cancer patients is not only driven by the intrinsic properties of tumor cells, but also by systemic host reactions. Some systemic factors, in the shape of cytokines and other chemical messengers, may play an important role in cellular proliferation and metastatic ability (3,4). Although the detailed mechanisms have not been fully elucidated yet, several markers that reflect systemic inflammation have been reported to be closely associated with patient prognosis in different types of cancer (5–12). Among these inflammatory factors, we tested level of CRP, NLR, dNLR, PLR and mGPS in a large Japanese PC cohort in the current study. An Austrian group had already reported that NLR (18), dNLR (19) and CRP (20) predicted clinical outcome, and our study aimed to validate their findings. As a result, we confirmed that NLR and CRP have prognostic value in a large Japanese cohort similar to the Austrian studies. On the other hand, PLR and mGPS did not

Table 3. Multivariate cox regression

	HR	95% CI	*P* value
Age			
65 or younger	1		
Older than 65	0.834	0.665–1.045	0.115
Performance status			
0	1		
1	1.284	0.923–1.788	0.138
Location of the primary tumor			
Head	1		
Body–tail	1.07	0.842–1.359	0.582
Clinical CT stage			
I/II	1		
III	2.191	1.638–2.931	<0.001
IV	4.141	3.035–5.648	<0.001
C-reactive protein (mg/dl)			
<0.45	1		
0.45 or greater	1.695	1.308–2.197	<0.001
Neutrophil–lymphocyte ratio			
<2.0	1		
2.0 or greater	1.404	1.078–1.830	0.012
CA19-9 (U/ml)			
<1000	1		
1000 or greater	1.435	1.127–1.826	0.003

demonstrate any prognostic value in our cohort, possibly due to ethnic difference and/or specificity of cancer type.

As compared with the Austrian cohort, there were more patients with earlier stage disease in our cohort. The fraction of Stage IV patients was 70% in the Austrian studies and 33% in this report. The mean values of NLR and CRP were 4.75 and 2.32 mg/dl, respectively, in the Austrian reports, and 3.06 and 0.80 mg/dl, respectively, in the current one. The median survival time and interquartile range were 7 and 3–17 months, respectively, in the Austrian cohort, and 11.6 and 7.1–20.1 months, respectively, in ours. Due to a high surgeon volume in our institute, we fortunately had an advantage in recruiting many PC patients with earlier stage. In any case, the important fact was that the prognostic impacts of NLR and CRP were confirmed in resectable and unresectable PC patients, respectively, in both European and Asian cohorts.

Although we verified the prognostic value of NLR and CRP in PC patients, there were differences between the characters of NLR and CRP as prognostic markers. One important point is that NLR is a relative value. Because a neutrophil count of zero is not a realistic situation, thus, NLR cannot approach zero (Fig. 4). Figure 3 shows the distribution of NLR and CRP in each clinical stage. The level of NLR tended to become higher as the clinical stage progressed. Accordingly, the cutoff level of 2.0 was appropriate for resectable disease but

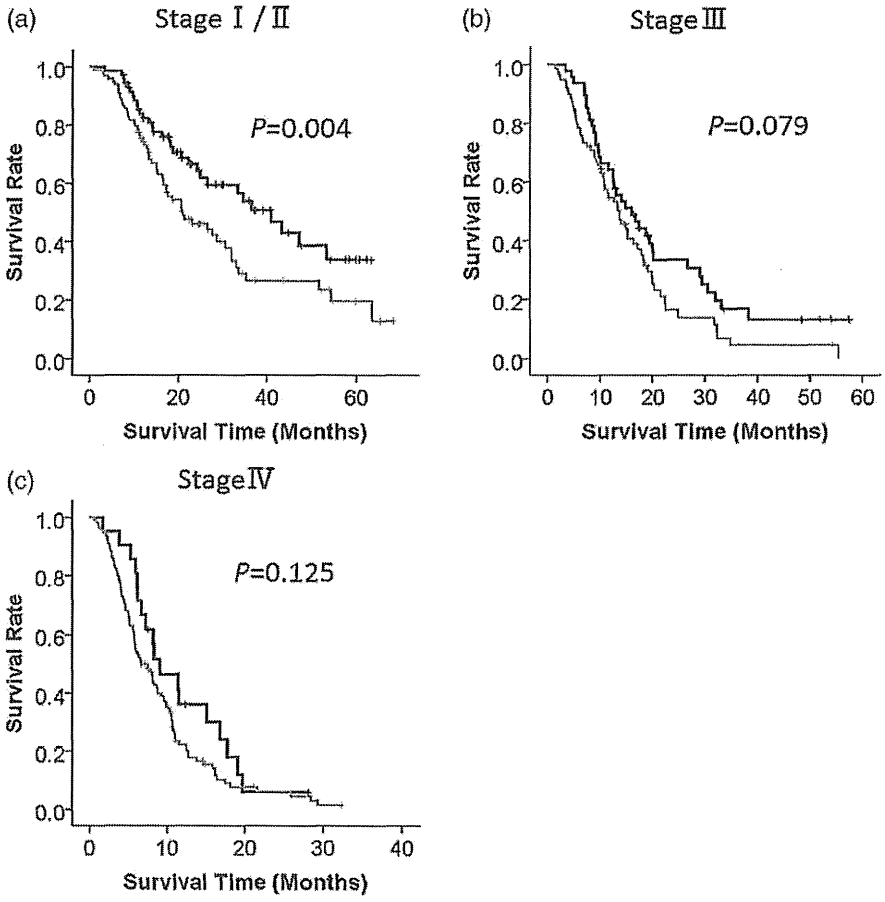

Figure 1. Overall survival curves stratified by neutrophil–lymphocyte ratio (NLR) for Stage I/II (a), Stage III (b) and Stage IV (c). Vertical lines represent censoring of data. Black and gray lines indicate subgroup of patients with NLR <2.0 and those with NLR ≥2.0, respectively. Prognosis of patients with increased NLR was significantly poorer in Stage I/II ($P = 0.004$, log-rank test).

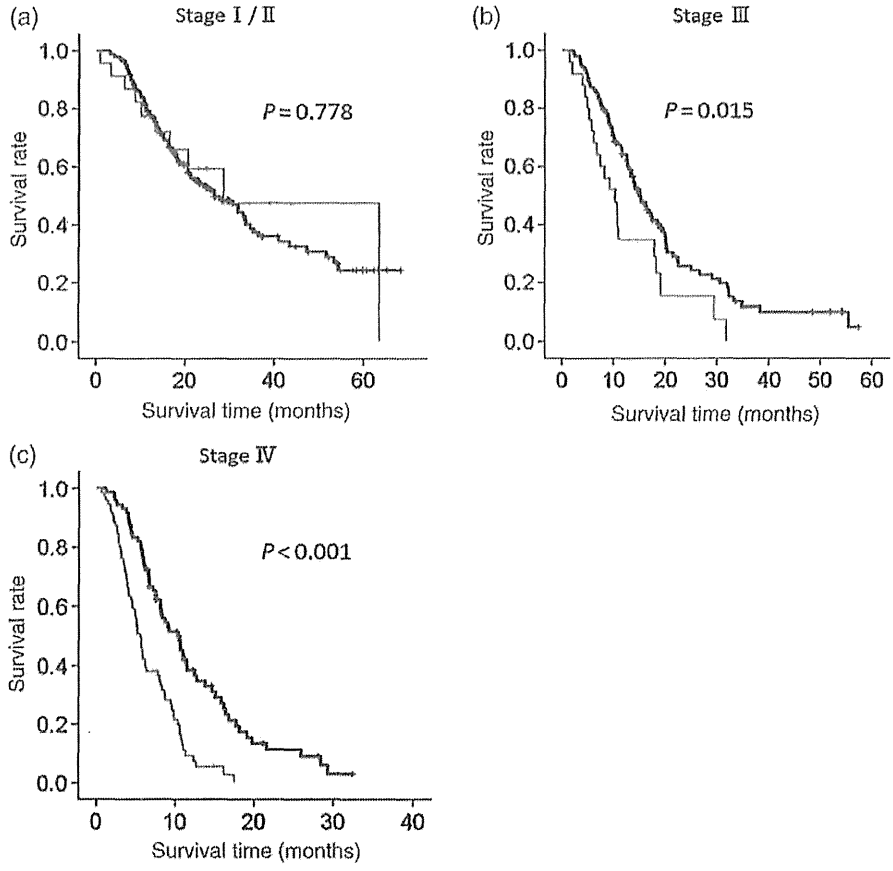

Figure 2. Overall survival curves stratified by C-reactive protein (CRP) for Stage I/II (a), Stage III (b) and Stage IV (c). Vertical lines represent censoring of data. Black and gray lines indicate subgroup of patients with CRP <0.45 and those with CRP ≥0.45, respectively. Prognosis of patients with increased CRP was significantly poorer in Stage III ($P=0.015$) and Stage IV ($P<0.001$, log-rank test).

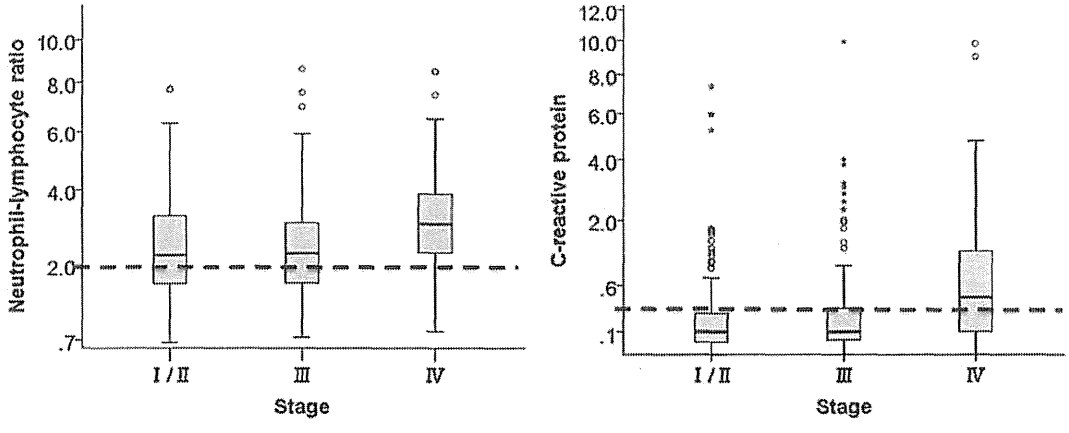

Figure 3. Box plots of CRP and NLR stratified by clinical stage. The dotted line denotes the cutoff level. The fraction of patients with NLR under the cutoff level was small especially in Stage IV, whereas most patients in Stage I/II had lower CRP level than the cutoff level.

it was too low to show the statistical significance in unresectable disease. If the cutoff level of NLR was set separately in each clinical stage, the prognostic value of NLR would be evident in both resectable and unresectable diseases. In practice, when we applied the cutoff level of 5.0 for NLR, the result was opposite from the result mentioned above, namely, the prognostic value of NLR was evident in unresectable disease, but not evident in resectable disease. On the other hand, CRP level is an absolute value, and small values close to zero represent a normal condition in general. To determine the cutoff level of CRP for patients especially in early stage was difficult because almost all

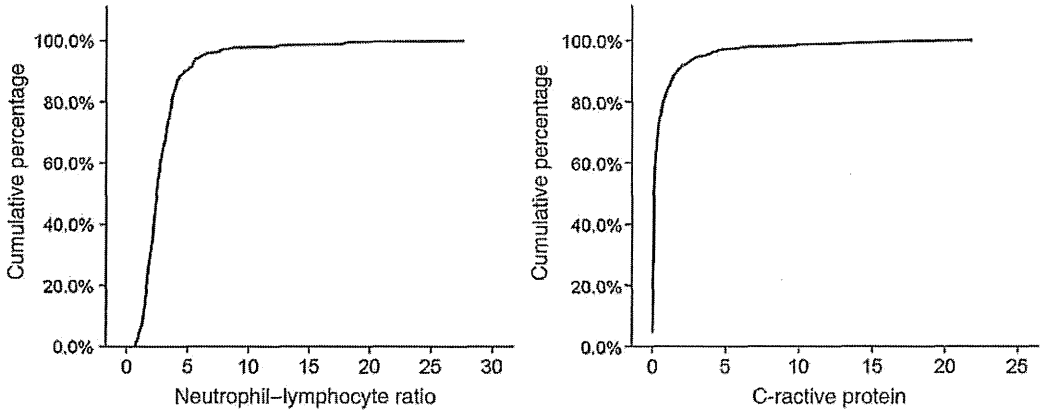

Figure 4. Cumulative distribution function plots of NLR and CRP. NLR cannot approach zero (95% of the NLR in our cohort were distributed between 1.1 and 6.2). On the contrary, small CRP values close to zero represent a normal condition. In the present study, 74% of the CRP levels were <0.5 mg/dl.

of the patients had a normal CRP level. For that reason, the prognostic value of CRP was relatively clear for advanced disease.

In conclusion, we verified the results of the Austrian studies, and revealed the prognostic value of NLR and CRP in a large PC cohort. We also found that the cutoff value of 2.0 for NLR clearly demonstrated prognostic value in potentially resectable disease, whereas CRP was a useful prognostic factor in patients who are not good candidates for curative resection. Further investigations to clarify the optimal NLR and CRP cutoff levels are warranted.

Conflict of interest statement

None declared.

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DOI: 10.3748/wjg.v20.i46.17456

World J Gastroenterol 2014 December 14; 20(46): 17456-17462
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE CONTROL STUDY

Case-control study of diabetes-related genetic variants and pancreatic cancer risk in Japan

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Supported by Grants-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare, Japan
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Received: March 4, 2014 Revised: April 18, 2014
Accepted: July 24, 2014
Published online: December 14, 2014

Abstract

AIM: To examine whether diabetes-related genetic variants are associated with pancreatic cancer risk.

METHODS: We genotyped 7 single-nucleotide polymorphisms (SNPs) in *PPARG2* (rs1801282), *ADIPOQ* (rs1501299), *ADRB3* (rs4994), *KCNQ1* (rs2237895), *KCNJ11* (rs5219), *TCF7L2* (rs7903146), and *CDKAL1* (rs2206734), and examined their associations with pancreatic cancer risk in a multi-institute case-control study including 360 cases and 400 controls in Japan. A self-administered questionnaire was used to collect detailed information on lifestyle factors. Genotyping was performed using Fluidigm SNPtype assays. Unconditional logistic regression methods were used to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for the association between these diabetes-associated variants and pancreatic cancer risk.

RESULTS: With the exception of rs1501299 in the

ADIPOQ gene (P = 0.09), no apparent differences in genotype frequencies were observed between cases and controls. Rs1501299 in the *ADPIOQ* gene was positively associated with pancreatic cancer risk; compared with individuals with the AA genotype, the age- and sex-adjusted OR was 1.79 (95%CI: 0.98-3.25) among those with the AC genotype and 1.86 (95%CI: 1.03-3.38) among those with the CC genotype. The ORs remained similar after additional adjustment for body mass index and cigarette smoking. In contrast, rs2237895 in the *KCNQ1* gene was inversely related to pancreatic cancer risk, with a multivariable-adjusted OR of 0.62 (0.37-1.04) among individuals with the CC genotype compared with the AA genotype. No significant associations were noted for other 5 SNPs.

CONCLUSION: Our case-control study indicates that rs1501299 in the ADIPOQ gene may be associated with pancreatic cancer risk. These findings should be replicated in additional studies.

Key words: Single-nucleotide polymorphisms; Pancreatic cancer; Risk; Case-control study; Odds ratio

Core tip: Although it is likely that a common genetic background predisposes individuals to developing both diabetes and pancreatic cancer, very few molecular epidemiologic studies have addressed this issue. We therefore genotyped 7 diabetes-related genetic variants and found that rs1501299 in the ADIPOQ gene may be associated with pancreatic cancer risk. The role of adiponectin variants needs further study.

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INTRODUCTION

The etiology of sporadic pancreatic cancer remains largely unknown. Epidemiologic studies have consistently shown that pancreatic cancer is positively associated with cigarette smoking and long-standing diabetes[1,2]. A 2005 meta-analysis reported that the risk for pancreatic cancer is 82% higher among diabetics compared with those without diabetes[3], though it is unclear which factors underlying diabetes are associated with pancreatic cancer. Most epidemiological studies have been limited by self-reporting of diabetes and by the lack of objective biomarkers, such as fasting plasma glucose or insulin levels, to address the temporal relationship between diabetes and pancreatic cancer. There is increasing evidence from clinical studies that pancreatic cancer induces new-onset diabetes[4,5]. The evidence available thus far strongly suggests that the relationship between diabetes and pancreatic cancer is bi-directional.

Given the well-recognized, positive association between type 2 diabetes and pancreatic cancer risk in epidemiological studies, it may be interesting to examine whether diabetes-related genetic variants may also be associated with pancreatic cancer risk. Genome-wide association studies (GWAS) have reported that at least 30 loci are associated with susceptibility to diabetes in various populations, with the majority originating from individuals of European descent[6]. Because of the potential differences in fat distribution and genetic background between Asian and Western populations[7,8], we focused on diabetes-related genetic variants reported in studies of Japanese populations, and variants that were first reported in GWAS of other populations and then replicated in Japanese populations. Among the 7 diabetes susceptibility genes we chose for the present study, *PPARG2*, *ADIPOQ*, and *ADRB3* have been shown to be closely associated with diabetes risk in Japanese subjects[9]; *KCNQ1* was reported as a diabetes susceptibility gene simultaneously by 2 independent Japanese research groups in 2008[10,11]; *KCN11*, *TCF7L2*, and *CDKAL1* were also reported to be associated with diabetes susceptibility in GWAS of Japanese subjects[12,13].

Although it is likely that a common genetic background predisposes individuals to developing both diabetes and pancreatic cancer, very few molecular epidemiologic studies have addressed this issue. We hypothesized that diabetes susceptibility genetic variants may be associated with an increased risk of pancreatic cancer in Japanese subjects. We therefore genotyped 7 single-nucleotide polymorphisms (SNPs) in *PPARG2* (rs1801282), *ADIPOQ* (rs1501299), *ADRB3* (rs4994), *KCNQ1* (rs2237895), *KCNJ11* (rs5219), *TCF7L2* (rs7903146), and *CDKAL1* (rs2206734) and examined their associations with pancreatic cancer risk in a multi-institute, case-control study in Japan.

MATERIALS AND METHODS

Study subjects

The purpose of our case-control study was to evaluate the role of genetic polymorphisms and gene-environment interactions in the development of pancreatic cancer in Japanese subjects. The details of the study design have been described elsewhere[14]. Briefly, cases were defined as patients who were newly diagnosed with pancreatic ductal adenocarcinoma at five participating hospitals from April 1, 2010, through May 15, 2012. A diagnosis was made according to imaging modalities and further confirmed by pathology reports. Pathologically confirmed cases represented approximately 90% of all cases in this study. During the same time period, we recruited the majority of control subjects from in-

Table 2 Selected characteristics of cases and controls *n* (%)

	Cases *n* = 360	Controls *n* = 400
Age, mean ± SD	67.8 ± 8.8	64.8 ± 9.5
Sex		
Male	215 (59.7)	226 (56.5)
Female	145 (40.3)	174 (43.5)
BMI, mean ± SD	22.9 ± 3.3	22.8 ± 3.2
History of diabetes		
Yes	87 (24.1)	35 (8.7)
No	269 (74.7)	362 (90.5)
Cigarette smoking		
Ever	215 (59.7)	198 (49.5)
Never	145 (40.2)	202 (50.5)
Age upon starting smoking (mean ± SD)	21.8 ± 4.8	20.5 ± 4.5
Number of cigarettes smoked per day (mean ± SD)	20.3 ± 9.0	16.2 ± 9.2

BMI: Body mass index.

Table 2 Single-nucleotide polymorphisms profile

Rs number	Gene	Chromosome location	Risk allele[1]	Alternative allele
rs1801282	*PPARG2*	3p25	C	G
rs1501299	*ADIPOQ*	3q27	C	A
rs4994	*ADRB3*	8p12	C	T
rs2237895	*KCNQ1*	11p15	C	A
rs5219	*KCNJ11*	11q23	T	C
rs7903146	*TCF7L2*	10q25	T	C
rs2206734	*CDKAL1*	6p22	A	G

[1]Based on the odds ratios reported for the association between T2D risk allele and T2D risk in previous studies.

patients and outpatients as well as from individuals who underwent medical checkups at one of the participating hospitals. None of the control subjects had a history of cancer. The diagnoses for hospital control subjects included a variety of diseases, such as anemia, gastric ulcers, and irritable bowel syndrome. The response rate was 85% (441/516) for cases and 98% (525/534) for control subjects as of July 1, 2012. The control subjects were frequency matched to the case patients on sex and age (within 10-year categories). As a result, 360 case patients and 400 control subjects were included in the present analysis.

All the study subjects provided written informed consent. Our study was approved by the Ethics Board of Aichi Medical University and by all the participating hospitals.

Data collection

Using a self-administered questionnaire, we collected detailed information on demographic characteristics, medical history, and lifestyle factors. In addition to the questionnaire survey, we obtained a 7-mL venous blood sample from all consenting participants. Genomic DNA was extracted from peripheral lymphocytes and subsequently stored at -30 ℃ until analysis.

Genotyping assays

Genotyping was performed using Fluidigm 192.24 Dynamic Array with BioMark HD Systems and EP1 (Fluidigm Corp., CA). We applied SNPtype assay (Fluidigm Corp., CA) which employs allele-specifically designed fluorescences (FAM or VIC) primers and a common reverse primer. We analyzed the data by the BioMark SNP Genotyping Analysis software to obtain genotype calls. The software defined genotype of each sample based on the relative intensities of fluorescences. The laboratory staff members 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

A χ^2 test was used to test genotype frequencies in control subjects for Hardy-Weinberg equilibrium (HWE) by comparing the observed genotype frequencies with those expected under HWE. The differences in genotype frequencies between cases and controls were also tested using a χ^2 test. Because the biological function of most SNPs has not been clearly defined, a co-dominant genomic model was assumed for SNP effects. We used unconditional logistic regression methods to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for the association between diabetes-associated variants and pancreatic cancer risk. All analyses were adjusted for age (continuous), sex (male or female), BMI (< 20, 20-22.4, 22.5-24.9, or ≥ 25.0), and cigarette smoking (current, former, or never smokers). ORs were also estimated for the risk allele on the basis of a log-additive model.

All *P* values were two-sided, with $P < 0.05$ indicating statistical significance. All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University), which is a graphical user interface for the R program (The R Foundation for Statistical Computing). More precisely, EZR is a modified version of R commander designed to add statistical functions frequently used in biostatistics.

RESULTS

The distribution of genotypes for all SNPs among control subjects did not deviate from HWE. Table 1 shows the selected characteristics of cases and controls. The mean BMI was similar between cases and controls. The number of individuals who had a history of diabetes was 87 (24.1%) in cases and 35 (8.7%) in controls. The OR was 2.95 (95%CI: 1.90-4.57) for those who had a history of diabetes. Individuals who had a BMI of 30 or greater had a 1.21-fold increased risk; however, the association was not statistically significant. The number of ever smokers (including current and former smokers) was 215 (59.7%) in cases and 198 (49.5%) in controls. The SNP profile is summarized in Table 2.

Table 3 shows the associations of pancreatic cancer with individual SNPs in the following genes: *PPARG2*

Table 3 Associations between diabetes-associated single-nucleotide polymorphisms and pancreatic cancer risk

Gene	SNP	Genotype	Case, *n*	Control, *n*	Age- and sex-adjusted OR (95%CI)	[1]Multivariable-adjusted OR (95%CI)
PPARG2	rs1801282	GG + CG	26	27	1.00	1.00
		CC	334	373	0.83 (0.47-1.46)	0.77 (0.43-1.38)
ADIPOQ	rs1501299	AA	19	38	1.00	1.00
		AC	155	167	1.79 (0.98-3.25)	1.71 (0.93-3.15)
		CC	186	195	1.86 (1.03-3.38)	1.85 (1.01-3.39)
CDKAL1	rs2206734	GG	114	138	1.00	1.00
		AG	184	195	1.15 (0.83-1.59)	1.18 (0.85-1.64)
		AA	62	67	1.16 (0.75-1.79)	1.21 (0.78-1.89)
ADRB3	rs4994	TT	228	255	1.00	1.00
		CT	114	131	0.92 (0.67-1.25)	0.88 (0.64-1.21)
		CC	18	14	1.37 (0.66-2.83)	1.36 (0.65-2.87)
KCNQ1	rs2237895	AA	153	156	1.00	1.00
		AC	175	193	0.95 (0.70-1.29)	0.92 (0.67-1.26)
		CC	32	51	0.62 (0.37-1.02)	0.62 (0.37-1.04)
KCNJ11	rs5219	CC	150	159	1.00	1.00
		CT	157	192	0.87(0.64-1.18)	0.90 (0.66-1.24)
		TT	53	49	1.14 (0.72-1.79)	1.19 (0.75-1.90)
TCF7L2	rs7903146	CC	354	394	1.00	1.00
		CT + TT	6	6	1.20 (0.38-3.83)	1.16 (0.36-3.72)

[1]Adjusted for age, sex, body mass index, and cigarette smoking.

(rs1801282), *ADIPOQ* (rs1501299), *ADRB3* (rs4994), *KCNQ1* (rs2237895), *KCNJ11* (rs5219), *TCF7L2* (rs7903146), and *CDKAL1* (rs2206734). With the exception of rs1501299 in the *ADIPOQ* gene ($P = 0.09$), no apparent differences in genotype frequencies were observed between cases and controls. Rs1501299 in the *ADPOQ* gene was positively associated with pancreatic cancer risk; the age- and sex-adjusted OR was 1.79 (95%CI: 0.98-3.25) among those with the AC genotype and 1.86 (95%CI: 1.03-3.38) among those with the CC genotype when compared with individuals with the AA genotype. The ORs remained similar after additional adjustment for cigarette smoking and BMI. Under the log-additive model, each additional copy of risk allele C was associated with a 1.2-fold increased risk of pancreatic cancer (OR = 1.22, 95%CI: 0.96-1.55). In contrast, rs2237895 in the *KCNQ1* gene was inversely related to pancreatic cancer risk, with a multivariable-adjusted OR of 0.62 (0.37-1.04) among individuals with the CC genotype compared with those with the AA genotype. No significant associations were noted for the other 5 SNPs.

DISCUSSION

In this case-control study, we genotyped 7 diabetes-associated genetic polymorphisms, and found that 2 variants in the *ADIPOQ* and *KCNQ1* genes were associated with pancreatic cancer risk in Japanese subjects. The risk variant in the *ADIPOQ* gene had a 1.9-fold increased risk, whereas the risk variant in the *KCNQ1* gene was inversely associated with risk.

Studies examining the association between diabetes-related genetic variants and pancreatic cancer risk were very limited, and the results were inconsistent. In a case-control study examining 15 SNPs in several obesity- and diabetes-related genes, two *FTO* gene variants (rs8050136 and rs9939609) and one *ADIPOQ* gene variant (rs17366743) were positively associated with pancreatic cancer risk; however, these associations were observed only in individuals who were overweight[15].

Of the 37 diabetes risk alleles examined by Pierce *et al*[16], only two SNPs (rs8050136 in *FTO* and rs1387153 in *MTNR1B*) showed significant positive associations with pancreatic cancer risk. However, *ADIPOQ* gene variants were not included in their analyses. We found that rs1501299 in the *ADIPOQ* gene had a positive association with pancreatic cancer risk, with the risk variant CC genotype conferring an approximate 1.9-fold increased risk compared with the AA genotype. The precise mechanism linking this SNP to pancreatic cancer risk is not clear. Adiponectin, which is secreted by adipose tissue, acts as an endogenous insulin-sensitizing hormone[17] and activates intracellular signaling pathways, including AMPK, PPARα, and NF-κB, by binding to two receptors, AdipoR1 and AdipoR2[17]. AdipoR1 has been reported to be upregulated in pancreatic cancer[18]. The adiponectin gene is located on chromosome 3q26, a region associated with susceptibility to the development of type 2 diabetes[19]. Rs1501299 in the *ADIPOQ* gene has been shown to be correlated with adiponectin levels, with the CC genotype exhibiting decreased levels of adiponectin compared with the AA genotype[9,20]. Low adiponectin concentrations contribute to insulin resistance, type 2 diabetes, and atherosclerosis[21] as well as obesity-related cancers, including breast and colorectal cancers[22,23]. A prospective study showed that low plasma adiponectin levels are associated with an increased risk of pancreatic cancer, independent of other markers of insulin resistance[24]. Given the essential role of adiponectin in insulin resistance and the strong evidence supporting the positive association of pancreatic cancer with obesity, insulin resistance, and hyperinsulinemia in both

epidemiological and mechanistic studies, it is likely that genetic variations in the adiponectin pathway may affect pancreatic cancer risk through their effects on circulating adiponectin.

KCNQ1 (potassium voltage-gated channel KQT-like subfamily, member 1) encodes the pore-forming subunit of a voltage-gated K+ channel (KvLQT1) and plays a key role in the repolarization of cardiac action potential as a water and salt transporter in epithelial tissues[25]. *KCNQ1* is also expressed in pancreatic islets[26], and a blockade of the channel with the *KCNQ1* inhibitor 293B stimulated insulin secretion[27]. To date, variants in the *KCNQ1* gene exert the greatest effects on the risk of type 2 diabetes in Asians[28]. Of the several SNPs in the *KCNQ1* gene that are associated with increased type 2 diabetes risk in Asians[10,11], we selected rs2237895 because this SNP was reported to be significantly associated with diabetes risk in both GWAS of Japanese people. Furthermore, in a previous study examining the effects of 4 SNPs in the *KCNQ1* gene (rs2237892, rs2283228, rs2237895, and rs2237897) on serum insulin levels following an oral glucose tolerance test in approximately 6000 Scandinavian individuals, only the C risk allele of rs2237895 was associated with reduced insulin release[29]. A 2012 meta-analysis confirmed that the C risk allele of rs2237895 in the *KCNQ1* gene increases the risk of diabetes by 32%[30]. However, we found that the C risk allele of rs2237895 was associated with a decreased risk of pancreatic cancer, which is unexpected and contrary to our hypothesis. This finding may be due to chance, but the mechanisms underlying this inverse association should be explored in further studies.

Diabetes is a complex disease, and susceptibility is determined by both genetic and environmental factors. Additionally, pancreatic cancer develops only in a subset of diabetics. Thus, these factors led us to postulate that certain diabetes-predisposing variants may be associated with a decreased risk of pancreatic cancer. A nested case-control study offered supporting evidence that circulating markers of peripheral insulin resistance, rather than pancreatic β-cell dysfunction, were independently associated with pancreatic cancer risk[31]. This finding, together with our observation of the positive association between rs1501299 in the *ADIPOQ* gene and pancreatic cancer risk, indicates that genetic variations influencing insulin resistance and their impact on circulating biomarkers are closely associated with pancreatic cancer risk.

No significant differences were observed in the genotype distributions between cases and controls in this study, with the exception of rs1501299 in the *ADIPOQ* gene. Other than SNPs in the *ADIPOQ* and *KCNQ1* genes, none of the 5 SNPs we genotyped were associated with pancreatic cancer risk. Among the genes examined in this study, *TCF7L2*, the most significant diabetes-related gene in Western populations, did not show any significant associations in this study. One possible reason for this result is the difference in the minor allele frequency. The very low frequency of *TCF7L2* risk genotypes in this study might make the detection of significant associations difficult. The null association for these SNPs suggests that other causal SNPs in these genes may be involved in pancreatic cancer susceptibility, and further studies are warranted to identify novel risk variants.

Our findings should be interpreted cautiously due to several limitations of this study. First, the results obtained may be due to chance because of the inadequate statistical power or bias inherent in case-control studies. Second, pathology reports were not available for all cases. However, we performed an analysis excluding those cases without pathology reports, and found that the positive association between rs1501299 in the *ADIPOQ* gene and pancreatic cancer remained unchanged. Third, we did not genotype SNPs that have been shown to be related to diabetes-related quantitative traits, including fasting plasma glucose, insulin, and homeostasis model assessment of β-cell function (HOMA-β). These biomarkers have been shown to be associated with pancreatic cancer risk in previous prospective studies[32,33]. Fourth, we did not examine serum levels of adiponectin in this study. Additional studies are necessary to clarify the effects of genetic polymorphisms on serum levels of adiponectin and evaluate their roles in the development of pancreatic cancer. Finally, we cannot exclude the possibility that the observed SNPs are in linkage disequilibrium with causal variants in the same gene or other genes. Further comprehensive analyses of SNPs in the two genes are required to identify the causal variants that confer susceptibility to diabetes or pancreatic cancer.

In summary, the results of our case-control study indicate that rs1501299 in the *ADIPOQ* gene may be associated with pancreatic cancer risk. These findings should be replicated in additional studies.

ACKNOWLEDGMENTS

We thank Kiyoko Yagyu for the contribution to the study design and data collection. We thank Mayuko Masuda, Kikuko Kaji, Kazue Ando, Etsuko Ohara, and Sumiyo Asakura for assisting us with data collection. We also thank Miki Watanabe, Tomoko Ito, Sanae Inui, and Sachiko Mano for technical assistance with genotyping.

COMMENTS

Background

Given the well-recognized, positive association between type 2 diabetes and pancreatic cancer risk in epidemiological studies, it may be interesting to examine whether diabetes-related genetic variants may also be associated with pancreatic cancer risk.

Research frontiers

Although it is likely that a common genetic background predisposes individuals to developing both diabetes and pancreatic cancer, very few molecular epidemiologic studies have addressed this issue.

Innovations and breakthroughs

This case-control study indicates that rs1501299 in the *ADIPOQ* gene may be associated with pancreatic cancer risk in Japanese subjects.

Applications

Genetic variations in the adiponectin pathway may affect pancreatic cancer risk

through their effects on circulating adiponectin. Further comprehensive analyses of SNPs in this gene are required to identify the causal variants that confer susceptibility to diabetes or pancreatic cancer.

Terminology

Single-nucleotide polymorphisms (SNP) are the most common type of genetic variation among individuals. Some SNPs have been linked to increased susceptibility to disease.

Peer review

Very few molecular epidemiologic studies have addressed the issue about the common genetic background which predisposes individuals to developing both diabetes and pancreatic cancer. This a good case-control study, try to examine whether diabetes-related genetic variants are associated with pancreatic cancer risk in Japan. Seven diabetes-related genetic variants were therefore genotyped and it was found that rs1501299 in the *ADIPOQ* gene may be associated with pancreatic cancer risk, although the role of adiponectin variants has not been clarified yet.

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P- Reviewer: Fu DL, Li CY
S- Editor: Ma N **L- Editor:** A **E- Editor:** Liu XM

RESEARCH ARTICLE

Lack of Associations between Genetic Polymorphisms in GSTM1, GSTT1 and GSTP1 and Pancreatic Cancer Risk: A Multi-Institutional Case-Control Study in Japan

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Abstract

Background: We aimed to evaluate the role of genetic polymorphisms in tobacco carcinogen-metabolizing genes and their interactions with smoking in a hospital-based case-control study of Japanese subjects. Materials and Methods: We examine the associations of pancreatic cancer risk with genetic polymorphisms in GSTM1, GSTT1 and GSTP1, phase II enzymes that catalyze the conjugation of toxic and carcinogenic electrophilic molecules. The study population consisted of 360 patients and 400 control subjects, who were recruited from several medical facilities in Japan. Unconditional logistic regression methods were used to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for the associations between genotypes and pancreatic cancer risk. Results: Among the control subjects, the prevalence of the GSTM1-null genotype and the GSTT1-null genotype was approximately 56% and 48%, respectively. Cases and controls were comparable in terms of GSTM1 and GSTT1 genotype distributions. Neither of the deleted polymorphisms in GSTM1 and GSTT1 was associated with the risk of pancreatic cancer, with an age- and sex-adjusted OR of 0.99 (95%CI: 0.74-1.32) for the GSTM1-null genotype, and 0.98 (95%CI: 0.73-1.31) for the GSTT1-null genotype. The OR was 0.97 (95%CI: 0.64-1.47) for individuals with the GSTM1 and GSTT1-null genotypes compared with those with the GSTM1 and GSTT1-present genotypes. No synergistic effects of smoking or GST genotypes were observed. Conclusions: Our results indicate no overall association between the GSTM1 and GSTT1 deletion polymorphisms and pancreatic cancer risk in the Japanese subjects in our study.

Keywords: GSTM1 - GSTT1 - GSTP1 - pancreatic cancer - risk

Asian Pac J Cancer Prev, **15** (1), 391-395

Introduction

The etiology of pancreatic cancer remains largely unknown. Epidemiologic studies have consistently shown positive associations between pancreatic cancer with cigarette smoking and long-standing diabetes (Duell, 2012; Ben et al., 2011). According to a 2008 meta-analysis, current smokers had approximately double the risk of pancreatic cancer relative to nonsmokers (Iodice et al., 2008). Although the exact mechanisms underlying the smoking-pancreatic cancer association remain to be clarified, similar to tobacco-induced cancers, a DNA adduct is thought to play a crucial role in pancreatic carcinogenesis. The accumulation of unrepaired genetic mutations due to tobacco-derived carcinogen-DNA adducts can cause disruption of cell cycle checkpoints

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and chromosomal instability (Hecht, 2008).

The process of carcinogen metabolism involves phase I metabolic activation and phase II detoxification [5], with a variety of enzymes involved in each phase. Cytochrome 450 (CYP1A1) is a phase I enzyme that initiates metabolic activation of carcinogens (Nebert et al., 2006). Glutathione S-transferases (GSTs) are the principal phase II enzymes that catalyze the conjugation of toxic and carcinogenic electrophilic molecules (Hayes et al., 2005). Three common polymorphisms in the GSTM1, GSTT1 and GSTP1 genes have been extensively studied in molecular epidemiologic studies due to their varied effects on enzyme activity (Moyer et al., 2007; Moyer et al., 2008). Variants in the GSTM and GSTT1 genes have attracted the most attention because inherited homozygous deletions of the GSTT1 and GSTM1 genes lead to an absence of enzyme activity, therefore increasing disease susceptibility. For GSTP1, a single nucleotide substitution (A→G) at position 313 of the GSTP1 gene (rs1695) substantially diminishes GSTP1 enzyme activity (Moyer et al., 2006). Loss-of-function (LoF) deletion polymorphisms in the GSTM1 and GSTT1 genes have been linked to an increased risk for many cancers, including head and neck, lung, liver, colon and pancreatic cancers (Geisler et al., 2001; Moore et al., 2005; White et al., 2008; Carlsten et al., 2008; Cote et al., 2009; Jang et al., 2012). Compared with GSTT1 and GSTM1 genetic polymorphisms (Bartsch et al.,1998; Liu et al., 2000; Duell et al., 2002; Jiao et al., 2007; Vrana et al., 2009; Jang et al., 2012), very few studies have studied GSTP1 genetic polymorphisms and their associations with pancreatic cancer risk. Only one previous study reported that GSTP1 polymorphisms were significantly associated with pancreatic cancer survival (Jiao et al., 2007)

Because the frequencies of GST genotypes vary across populations and ethnicities (Di Pietro et al., 2010), large inter-individual differences might exist in the metabolic response to carcinogen exposure. As a result, the risk of pancreatic cancer could be partly determined by these factors. In this study, we examined the associations of pancreatic cancer risk with genetic polymorphisms in GSTM1, GSTT1 and GSTP1 in Japanese subjects, using a hospital-based case-control study.

Materials and Methods

Study subjects

We aimed to clarify the roles of genetic polymorphisms and gene-environment interaction in the development of pancreatic cancer, using data obtained from an ongoing multi-institutional case-control study. The details of our case-control study have been described elsewhere (Lin et al., 2013). Briefly, the eligible cases were patients who were newly diagnosed with pancreatic cancer at five hospitals from April 1, 2010, through May 15, 2012. Imaging modalities and pathologic reports (if available) were used for pancreatic cancer diagnosis. During the same time period, we enrolled control subjects from the following three sources: 1) inpatients and outpatients from the same participating hospitals where the cases were enrolled; 2) relatives of inpatients from the same participating hospitals where the cases were enrolled; and 3) individuals who were undergoing medical checkups at one of the participating hospitals. All of the control subjects who were recruited from among inpatients and outpatients had no prior diagnoses of cancer at the time of enrollment. The diagnoses for control subjects included a variety of diseases, such as anemia, gastric ulcers and irritable bowel syndrome. We achieved a response rate of 85% (441/516) for cases and 98% (525/534) for control subjects as of July 1, 2012. The control subjects were frequency matched to the case patients by sex and age (within 10-year categories). As a result, the data from 360 case patients and 400 control subjects were included in the present analysis.

We obtained written, informed consent from all of the study subjects. The ethical board of Aichi Medical University and all of the participating hospitals approved this study.

Data collection

The study participants completed a self-administered questionnaire covering information on demographic characteristics, medical history and lifestyle factors, such as cigarette smoking, alcohol consumption and dietary intake. Information on cigarette smoking included smoking status (never, former or current smokers), average number of cigarettes smoked per day, age at starting and quitting smoking and duration of smoking. In addition to lifestyle information, a 7-mL venous blood sample was collected from all of the consenting participants.

Genomic DNA was extracted from peripheral lymphocytes in the blood at SRL Hachioji Laboratory and was stored at -30°C until genotyping.

Genotyping assays

All of the genotyping was conducted in the laboratory of Aichi Cancer Center Research Institute in Nagoya, Japan, with the laboratory staff blinded to case or control status. For GSTM1 and GSTT1, the genotyping was performed using the Taqman SNP Genotyping assay. Two quality control samples were included in each assay. The assays were undertaken independently using 30-50 ng of genomic DNA in a 10-μL reaction. The reactions were performed in a 96-well plate format. The GSTM1 and GSTT1 real-time assays were conducted using 4 μL of the 2×Genotyping Master Mix Universal (ABI). The thermocycling conditions were as follows: 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles 95°C for 15 seconds and 56°C for 1 minute and 30 seconds. Real-time fluorescence was monitored during PCR amplification, and the results were analyzed using Applied Biosystems 7500 Real-Time PCR systems. The GSTP1 rs1695 polymorphism was genotyped using Fluidigm SNPtype assays.

Statistical analysis

Deviation from Hardy-Weinberg equilibrium (HWE) in the control subjects was evaluated using the chi-squared test. Unconditional logistic regression methods were used to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for the associations between GST genotypes and pancreatic cancer risk. All of the analyses were adjusted

for age (continuous) and sex (male or female). The interaction of genotype and smoking with regard to pancreatic cancer risk was assessed using a likelihood ratio test.

The statistical tests were two-sided, and a P-value less than 0.05 was considered statistically significant. All of the statistical analyses were performed using SAS software, version 9.2 (SAS Institute, Inc., Cary, NC, USA).

Results

Table 1 shows the distributions of selected characteristics and risk factors for pancreatic cancer. The cases were more likely to be current smokers and to have a history of diabetes, compared to the controls. The OR was 2.86 (95%CI: 1.79-4.57) for current smokers after adjustment for age, sex, BMI and history of diabetes. Subjects who reported a history of diabetes had a 2.9-fold increased risk of pancreatic cancer (OR=2.94; 95%CI: 1.90-4.57).

As shown in Table 2, the prevalence of the GSTM1-null genotype and GSTT1-null genotype among the control subjects was approximately 56% and 48%, respectively. The cases and controls were comparable

Table 1. Characteristics of Case Patients and Control Subjects

Characteristics	Case patients (N=360)	Control subjects (N=400)	OR (95% CI)
Mean age±SD	67.8±8.8	64.8±9.5	
Male (%)	215 (59.7)	226 (56.5)	
Body mass index (kg/m²)			
<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, SD; standard deviation, OR was adjusted for sex, age, body mass index, history of diabetes, and cigarette smoking. The numbers shown in parentheses are percentages

Table 2. Association of Pancreatic Cancer with GSTM1 and GSTT1 Polymorphisms

	Case patients (n=360)	Control subjects (n=400)	OR (95% CI)
GSTM1			
Present	160 (44.4)	175 (43.8)	1.00
Null	200 (55.6)	225 (56.2)	0.99 (0.74-1.32)
GSTT1			
Present	193 (53.6)	209 (52.3)	1.00
Null	167 (46.4)	191 (47.7)	0.98 (0.73-1.31)
*GSTP1 (rs1695)			
AA	266 (73.9)	284 (71.0)	1.00
AG	88 (24.4)	113 (28.3)	0.83 (0.60-1.16)
GG	6 (1.7)	3 (0.7)	2.41 (0.58-9.98)
AG+GG	94 (26.1)	116 (29.0)	0.87 (0.63-1.20)

**OR: odds ratio; CI: confidence interval, OR was adjusted for age and sex

Table 3. Joint Efects of GSTT1, GSTM1 Genotypes on Pancreatic Cancer Risk

GSTT1	GSTM1	Case patients	Control subjects	OR (95%CI)
Present	Present	85	87	1.00
Present	Null	108	122	0.95 (0.64-1.42)
Null	Present	75	88	0.93 (0.60-1.45)
Null	Null	92	103	0.97 (0.64-1.47)
				P for interaction=0.62

*OR: odds ratio; CI: confidence interval, OR was adjusted for age and sex

Table 4. Joint Effects of Smoking and GSTT1, GSTM1 Genotypes on Pancreatic Cancer Risk

GSTT1	Smoking	Case patients	Control subjects	OR (95%CI)
Present	Non-smokers	71	103	1.00
Present	Current smokers	47	28	3.22 (1.72-6.04)
Null	Non-smokers	74	99	1.08 (0.70-1.67)
Null	Current smokers	49	30	3.27 (1.76-6.06)
				P for interaction=0.79
GSTM1				
Present	Non-smokers	67	98	1.00
Presnt	Current smokers	44	23	3.67 (1.93-6.98)
Null	Non-smokers	78	104	1.08 (0.69-1.68)
Null	Current smokers	52	35	2.92 (1.63-5.25)
				P for interaction=0.39

*OR: odds ratio; CI: confidence interval, OR was adjusted for age and sex

in terms of GSTM1 and GSTT1 genotype distributions. Neither of the deleted polymorphisms in GSTM1 and GSTT1 was significantly associated with the risk of pancreatic cancer, with an age- and sex-adjusted OR of almost 1.0. The results remained unchanged after further adjustment for BMI, history of diabetes and cigarette smoking (data not shown). The distribution of GSTP1 rs1695 genotypes among the control subjects deviated from HWE (p=0.02). No significant associations were observed between rs1695 genotypes in GSTP1 and the risk of pancreatic cancer. Compared with individuals with the AA genotype, the age- and sex-adjusted OR was 0.87 (95%CI: 0.63-1.20) among those with the AG and GG genotype.

Table 3 shows the combined effects of GSTM1 and GSTT1 polymorphisms on pancreatic cancer risk. The OR was 0.97 (0.64-1.47) for individuals with the GSTM1 and GSTT1-null genotypes compared with those with the GSTM1 and GSTT1-present genotypes. No statistically significant interactions were noted (P=0.62). No synergistic effects of smoking or GST genotypes were observed (Table 4). The risk estimates were similar for current smokers with the GSTT1 or GSTM1-null genotypes, compared to current smokers with the GSTT1 or GSTM1-present genotypes.

Discussion

We evaluated the associations between genetic polymorphisms in GSTM1 and GSTT1 and pancreatic cancer risk in Japanese subjects. We found that neither the GSTM1-null genotype nor the GSTT1-null genotype was associated with increased pancreatic cancer risk. Furthermore, although smoking was significantly

associated in our study with an increased risk of pancreatic cancer, the results of the gene-environment interactions did not indicate a synergistic effect of smoking and GST-null genotypes on the risk.

The frequencies of GSTM1 and GSTT1-null genotypes vary widely across ethnicities. It has been shown that Asians and Caucasians display higher frequencies of GSTM1-null genotypes than African populations (Di Pietro et al., 2010). The prevalence of the GSTT1-null genotype is low in Caucasians, and it is significantly greater in Asian populations. In our control group, the GSTT1-null genotype represented approximately 48% of the subjects, which was greater than the percentage reported in Caucasians (Di Pietro et al., 2010). The allele frequencies for GSTT1 and GSTM1 in our study were similar to those reported in other Asian populations (Di Pietro et al., 2010).

Previous studies have yielded mixed results regarding the associations between GSTT and GSTM polymorphisms and pancreatic cancer. To date, at least six case-control studies have addressed this association (Bartsch et al., 1998; Liu et al., 2000; Duell et al., 2002; Jiao et al., 2007; Vrana et al., 2009; Jang et al., 2012). All the studies were conducted in Western countries, with the exception of a population-based case-control study in the San Francisco Bay area, in which a small number of Asian participants were included (Duell et al., 2002). No main effects of the GSTT1 and GSTM1-null genotypes on pancreatic cancer risk were noted in any of the studies, with the exception of a population-based case-control study conducted in Canada (Jang et al., 2012).

The lack of association between LoF variants, such as GSTM1 and GSTT1, and pancreatic cancer risk indicates that a common gene-disrupting variant alone might not confer major susceptibility. Two possibilities exist. First, given the high prevalence of null genotypes, such as GSTT1 and GSTM1, it is unlikely that any major effects exist, because natural selection is expected to prevent the most severely deleterious alleles from reaching high population frequencies (MacArthur et al., 2012). Another possibility is that the pancreas is not directly exposed to tobacco-derived carcinogens, suggesting that the effect of carcinogen-metabolizing enzymatic activity might be weaker than in other organs that are directly exposed to tobacco carcinogens, such as the lungs. Even for lung cancer, a meta-analysis of the GSTM1-null genotype showed a weakly positive association, with a summary OR of 1.22 (95%CI: 1.14-1.30) (Carlsten et al., 2008).

On the basis of a multiplicative interaction model, we observed no synergistic effect of smoking and GST-null genotypes on pancreatic cancer risk. Although the notion that smokers with GSTT1 or GSTM1-null genotypes had the highest risk compared with non-smokers with GSTT1 or GSTM1-present genotypes is biologically plausible, the clarification of genotype-environment interactions remains a challenge. This is due to a limited sample size and difficulty of obtaining accurate exposure information. In the population-based case-control study carried out in six San Francisco Bay areas, the OR was 5.0 (95%CI 1.8-14.5) for heavy smokers who had a deletion polymorphism in GSTT1, suggesting that inherited deletion polymorphisms in GSTT1 increase the susceptibility to smoking-related pancreatic cancer (Duell et al., 2002). The results, however, might have been due to chance because they were based on a very limited sample size.

We recognize several limitations of our study. First, as with other case-control studies, selection bias was an inherent limitation and should be addressed when interpreting the study results. Selecting an appropriate control group remains a major challenge, especially in hospital-based case-control studies. Ideally, the cases and controls should come from the same source population. However, the hospital controls did not necessarily represent the same population from which the cases were derived. The frequency of GST genotypes observed among the control subjects in this study was comparable to that obtained from other Asian populations, suggesting that our results regarding GST polymorphisms and pancreatic cancer were robust. Second, we were limited to detecting significant gene-environment interactions in the subgroups. For example, the numbers of cases and controls were small, especially after stratification by smoking status. Third, the genotyping methods, based on PCR techniques, used in our study and in other studies could not distinguish GSTM1 and GSTT1 homozygous wild-type +/+ from heterozygous +/- individuals. Only one previous study found phenotypic differences between these two groups based on a newly developed assay (Moore et al., 2005). Fourth, although we showed that a combination of GSTM1 and GSTT1-null genotypes was not associated with the risk of pancreatic cancer, the pathways of carcinogen metabolism are complex and are mediated by a variety of factors. These factors include the balance between metabolic activation and the detoxification of tobacco carcinogen compounds, as well as the efficiency of DNA repair. For example, it is likely that a deficiency in one class of GST enzymes due to a genetic polymorphism can be compensated for by the presence of other classes of GST enzymes. We genotyped rs1695 in GSTP1 and found no significant association between rs1695 polymorphisms and the risk of pancreatic cancer. It should be noted that the distribution of genotypes among control subjects was not in HWE. The reason for this fact is unclear, but genotyping error and population stratification are possible explanations (Pompanon et al., 2005). Further studies are needed to integrate genetic variations into different pathways, to define the risk of pancreatic cancer better.

In conclusion, our case-control study indicated no overall association between the GSTM1 and GSTT1 variants and pancreatic cancer risk in Japanese subjects. As common low-risk variants in different genes might act collectively to confer susceptibility to pancreatic cancer, further studies will be required to uncover the full spectrum of these variants and their effects on pancreatic cancer.

Acknowledgements

The authors declare no conflict of interests. This work was supported by Grants-in-Aid for Cancer Research

from the Ministry of Health, Labor and Welfare of Japan.

We thank Mayuko Masuda, Kikuko Kaji, Kazue Ando, Etsuko Ohara and Sumiyo Asakura for assisting us with the data collection. We also thank Miki Watanabe, Tomoko Ito, Sanae Inui, and Sachiko Mano for their technical assistance with genotyping.

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Online Submissions: http://www.wjgnet.com/esps/
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doi:10.3748/wjg.v19.i15.2368

World J Gastroenterol 2013 April 21; 19(15): 2368-2373
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

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

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Received: December 9, 2012 Revised: January 23, 2013
Accepted: February 5, 2013
Published online: April 21, 2013

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

Matsuyama M, Ishii H, Kuraoka K, Yukisawa S, Kasuga A, Ozaka M, Suzuki S, Takano K, Sugiyama Y, Itoi T. Ultrasound-guided *vs* endoscopic ultrasound-guided fine-needle aspiration for pancreatic cancer diagnosis. *World J Gastroenterol* 2013; 19(15): 2368-2373 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i15/2368.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i15.2368

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.