Neoplastic spindle cells are an independent prognostic factor in pancreatic cancer

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Abstract

Objective: Several reports showed that Neoplastic spindle cells (NSCs) may be strongly involved in the invasion, metastasis and poor prognosis and epithelial-mesenchymal transition (EMT). It has not yet been investigated that NSCs relate to the recurrence and prognosis in various cancers, Furthermore, NSCs are participate in EMT in pancreatic cancer (PC) too. We clinicopathologically investigated the association between NSCs and the recurrence, prognosis, and EMT in PC.

Methods: We studied 68 PC patients. Cancer cells with a spindle or oval shape that do not exhibit luminal structures were defined as NSCs. We graded NSCs regarding to an area of NSCs at hematoxylin and eosin stain (NSC grade) and examined the participation in NSCs and EMT by immunohistostaining of Snail antibody and E-cadherin antibody.

Results: In multivariate analysis, NSC grade was an independent risk factor for disease-free survival and overall survival. This was independent of TNM stage and histological grade. NSCs were related to EMT pattern in immunohistostaining, significantly.

Conclusion: NSC grade significantly related to the recurrence and prognosis of PC. NSC grade assessment can be not only performed inexpensively and conveniently, but also used to guide future individualized therapeutic approaches. Furthermore, NSCs was found to relate to EMT, profoundly.

Key words: Neoplastic spindle cells (NSC), Epithelial-Mesenchymal Transition (EMT), Pancreatic cancer, Overall survival (OS), Snail, Prognostic factor

1. Introduction

Pancreatic cancer is a type of gastrointestinal cancer that is highly malignant,[1] and its mortality is increasing every year. In addition to surgical treatment, chemotherapy and radiation therapy are currently given in combination such that treatment outcomes have gradually improved; nonetheless, satisfactory outcomes have yet to be obtained.[2]

Some of the histopathological factors that determine the prognosis of pancreatic cancer include degree of differentiation, staging, Lymphatic invasion(Ly), Venous invasion(v), Intrapancreatic neural invasion(ne), Invasion to the extrapancreatic nerve plexus(PL), and Invasion to anterior pancreatic capsule(S), Invasion to the retroperitoneal tissue(RP), Duodenal invasion(DU), Intrapancreatic bile duct invasion(CH), Residual tumor status(R), and these factors are widely used to histologically assess the prognosis in Japan.[3] These factors are similar to the factors that determine prognosis of other types of gastrointestinal cancer.

It was discovered that neoplastic spindle cells (NSCs) with a spindle shape may be detected in parts of human cancerous tumours.[4] Recently, when a cancer cell loses its polarity due to some type of effect, it is known to exhibit a spindle shape, and this change may be strongly involved in the migration, invasion and metastasis of cancer.[5,6] In addition, in several cancers, NSCs have been reported to associate with poor prognosis in several cancers.[5,7] Clarifying the biological characteristics and mechanisms of NSCs may greatly impact the treatment of pancreatic cancer, a disease in which metastasis and recurrence can easily occur, even in a relatively early stage. The emergence of NSCs in various cancers has been associated with epithelial-mesenchymal transition (EMT).[8,9]

The characteristics of NSCs in pancreatic cancer have been gradually clarified, but there are still very few reports that evaluate its mechanism or clinicopathological significance. In the present study, we clinicopathologically investigated the association between the emergence of NSCs and the metastasis and recurrence of pancreatic cancer utilizing our experience. Furthermore, we assessed how the emergence of NSCs in pancreatic cancer is involved in the characteristic transformation.

2. Materials and methods

2.1. Patient characteristics

We studied all 68 patients with invasive pancreatic ductal carcinoma who underwent curative resection at Kurume University School of Medicine, Japan between January 2006 and December 2012. The present study was planned according to the REMARK guidelines.[10] Clinical staging was categorized by using the TNM Stage.[11] Clinical data were obtained from medical records. The clinicopathological information collected included: age, sex, tumour size, TNM stage, peritoneal dissemination, venous invasion(v), lymphatic invasion(ly), neural invasion(ne), duodenal invasion(DU), bile duct invasion(CH), invasion to anterior pancreatic capsule(S), invasion to the retroperitoneal tissue(RP), peripancreatic neural invasion (PL), and histological grade. Computed tomography scans from the thoracoabdominal area to the pelvic area were performed in all patients to verify distant metastasis prior to operation. This study was performed in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and was approved by local Institutional Research Ethics Committees (research number :

13248). A written informed consent was obtained for the use of the samples in this study.

2.2. Assay methods

2.2.1. Immunohistochemistry

Paraffin-embedded tissue samples were cut at 4-μm thickness and examined on a coated slide glass. The largest tumour section was selected per patient to be assessed. Immunostaining with SNAIL (×500, Abcam, Cambridge, MA, USA) was performed by the manual method. The specimens were boiled in a microwave for 30 min in 1 mmol/L EDTA, pH 9.0, target retrieval solution (DakoCytomation) to recover antigens. After washing in Tris-buffered saline (TBS; DakoCytomation) for 10 min, the SNAIL antibody was applied to the cells. The histological specimens were incubated 4°C overnight, washed in TBS for 15 min, and incubated with labelled polymer-HRP secondary antibody (ChemMate ENVISION Kit; DakoCytomation) for 30 min at room temperature. After washing in TBS for 10 min, the slides were visualized by using DAB. Immunostaining with E-cadherin (×100, DakoCytomation, Denmark) was performed on the same fully automated Bond-Max system by using onboard heat-induced antigen retrieval with ER2 for 20 min and a Refine polymer detection system (Leica Microsystems, Newcastle, UK). DAB was used as the chromogen in all immunostainings.

2.2.2 Assessment of NSCs, snail and E-cadherin

Cancer cells with a spindle or oval shape that do not exhibit luminal structures were defined as NSCs (Figure 1). The largest tumour section (one section) was selected per patient to be assessed. Whole high-power fields (HPFs) of selected sections were observed at 100-times magnification, and the amount of NSCs was evaluated on a 3-level scale depending on the area they occupied in the field of view.

(NSC 1+: -1/16, NSC 2+: 1/16-1/4, NSC 3+: 1/4-)

Each microscopic field of view was divided both vertically and horizontally in two such that the whole field of view was divided into four areas, and if NSCs covered more than 1/4 of the whole area, the amount of NSC was considered to be NSC 3+. Similarly, each microscopic field of view was divided both vertically and horizontally in four such that the whole field of view was divided into sixteen areas, and if NSCs covered less than 1/16 of the whole area, the amount of NSC was considered to be NSC 1+. All other occupied areas were considered to be NSC 2+.

Snail: Tumour cells that were stained in the nucleus were counted as Snail antibody positive.[12] Whole HPFs of selected sections were observed at 100-times magnification. Cancer cells were divided into two groups: NSCs and cancer cells that show glandular lumen. The number of cancer cells that 1) maintained the number of NSCs and glandular lumen, 2) were stained with Snail antibody in the nucleus in NSCs, and 3) were stained with Snail antibody in the nucleus and exhibited glandular lumen were each counted. Subsequently, (rate of NSCs positive with nuclear staining) = (number of NSCs positive with nuclear staining) / (number of NSCs) and (number of cancer cells with positive nuclear staining that maintained glandular lumen) / (number of cancer

cells that maintained glandular lumen) were calculated, and the Snail positive rate of each group of cells was determined after rounding off the numbers. Snail positive rate was evaluated on an 11-level scale from 0%-100% (0, 10, 20...100). Sixteen patients who did not exhibit NSCs in the selected sections and one patient who did not exhibit cancer cells with glandular lumen were excluded.

E-cadherin: Whole HPFs of selected sections were observed at 100-times magnification, and the staining intensity of the cell membranes of cancer cells was evaluated on a 3-level scale. The maximum staining intensity was considered as the appropriate grade.

All histopathologies were diagnosed by two pathologists who were blinded to clinical end-points. The study design is illustrated in Figure 2.

2.3. Observation

Serum tumour marker assessments and diagnostic imaging (simple chest X-ray, ultrasound, and computed tomography) were conducted every 3-6 months during the observation period to evaluate recurrence. The recurrence of peritoneal dissemination was diagnosed by pathological methods or by diagnostic imaging. For most patients, the last visit date was considered as the last survival verification date. For some patients, survival was confirmed by telephone or in writing, and this date was considered as the last survival verification date. Date of death was confirmed from the clinical data. For some patients, the date of death was confirmed patient family members by telephone or in writing.

2.4. Statistical analysis

Other covariate distributions between the 3 NSC grades were compared by using the χ^2 test or Fisher's exact test. Subsequently, the effects of each covariate on overall survival (OS) and disease-free survival (DFS) were assessed by using a univariate Cox proportional hazards model. OS and DFS Kaplan-Meier curves by the 3 NSC grades were depicted to assess the effects on OS and DFS by using the log-rank test. Next, by using a multivariate Cox proportional hazards model adjusted for factors that were associated with NSC ($P<0.2$) and with OS and DFS ($P<0.2$), the effects of NSCs on OS and DFS were assessed. The median and interquartile ranges of the Snail staining were calculated, and the Snail staining rate between NSCs and cancer cells with glandular lumen were compared by using the Wilcoxon signed-rank test. Spearman's rank correlation coefficient was used to assess the association between NSCs and E-cadherin grades. $P<0.05$ was considered statistically significant. SAS version 9.3 (SAS Institute Inc., Cary, NC, USA) was used for analysis.

3. Results

3.1. Data

Clinical staging was categorized in accordance with the TNM classification.[11]

Of the 69 patients with invasive pancreatic ductal carcinoma who underwent curative resection at our hospital between January 2006 and December 2012, one patient was excluded due to a change in the postoperative final pathological diagnosis from invasive pancreatic ductal carcinoma to intraductal papillary-mucinous carcinoma.

The study population consisted of 45 men and 23 women with an average age of 65.35 years (range: 37-81 years). For staging, there were 7 patients who were classified as Stage IA, 5 as Stage IB, 20 as Stage IIA, 33 as Stage IIB, and 3 as Stage III. For surgical procedures, there were 45 patients who underwent pancreaticoduodenectomy, 18 who underwent distal pancreatectomy, and 5 who underwent total pancreatectomy. The histological grades were: well-differentiated adenocarcinoma (n=43), moderately differentiated adenocarcinoma (n=18), poorly differentiated adenocarcinoma (n=5), and anaplastic carcinoma (n=2). The extent of pancreatic localized tumour progression was: 10 patients with T1, 6 with T2, 49 with T3, and 3 with T4. There were 35 patients who were positive for local lymph node metastasis, 26 patients who were positive for pancreatic bile duct invasion, and 24 patients who were positive for duodenal invasion. Eleven patients underwent preoperative chemoradiotherapy, and 55 patients underwent postoperative adjuvant chemotherapy. The observation period spanned an average of 813.3 days (range: 41-2228 days). There were 42 patients (61.8%) who experienced recurrence during the observation period. There were 31 patients (45.6%) who died due to primary disease, and 3 patients (4.4%) who died due to other diseases. (Table 1)

The 1-year survival was 83.8%, 3-year survival was 49.4%, and 5-year survival was 36.2%.

3.2. Frequency and association of NSC with clinicopathological parameters

The following are the number of patients with each NSC grade: NSC 1+, 25 patients (36.8%); NSC 2+, 20 patients (29.4%); and NSC 3+, 23 patients (33.8%). Of the 43 patients with well-differentiated adenocarcinoma, 23 patients (53.5%) were determined

to be either NSC 2+ or NSC 3+, indicating that a large number of NSCs appeared in at least half of the patients with well-differentiated adenocarcinoma.

Clinicopathological factors associated with NSC grade (P<0.2) in the covariate distribution by NSC grade were: age (≥65 years old vs. <65 years old) (p=0.03), tumour size (p=0.01), PL (p=0.14), TNM stage (p=0.002), N (p<0.001), histological grade (p<0.001), DU (p=0.15), RP (p=0.006), ly (p=0.053), and v (p=0.06) (Table 2)

3.3. Prognostic effect of NSC grade in univariate statistical analysis and multivariate statistical analysis

NSC grade significantly affected DFS (p<0.0001) and OS (p<0.0001).

At 1-year OS rate was 91.7% (95% CI: 70.6-97.8%) for patients with NSC 1+ and 93.8%(95% CI: 63.2-99.1%) with NSC 2+ and 63.4% (95% CI: 39.8-79.8%) with NSC 3+. At 3- year OS rate was 81.1% (95% CI: 56.5-92.6%) for patients with NSC 1+ and 47.4%(95% CI: 21.8-69.4%) with NSC 2+ and 23.4% (95% CI: 8.1-43.3%) with NSC 3+. At 5- year OS rate was 68.0% (95% CI: 41.1-84.6%) for patients with NSC 1+ and 31.6%(95% CI: 7.0-60.6%) with NSC 2+ and none (95% CI: NA) with NSC 3+. (Figures 3A and 3B)

The prognostic effects of NSC results and each clinicopathological factor were assessed in all patients with univariate Cox proportional hazards regression analysis. Clinicopathological factors that were associated with DFS (P<0.2) were: tumour size (p=0.0036), TNM stage (p=0.0001), histological grade (p=0.0808), CH (p=0.1049), S (p=0.0159), RP (p=0.0205), ly (p=0.071), v (p=0.0239), and ne (p=0.1712). From these findings, we selected tumour size, UICC stage, histological grade, ly, v, and RP as

adjustment factors of DFS.

Additionally, clinicopathological factors that were associated with OS (P<0.2) were: sex (p=0.137), tumour size (p=0.0031), TNM stage (p=0.0018), v (p=0.0675), S (p=0.0351), NSC 2+ (p=0.086), and NSC 3+ (p<0.0001). From these findings, we selected tumour size, UICC stage, and v as adjustment factors of OS.

Based on these results, we conducted multivariate Cox proportional-hazards regression analysis and found that both DFS and OS significantly decreased in NSC 3+ compared to NSC 1+. Specifically, the risk of recurrence or death increased 3.38 times, and the risk of death increased 4.99 times in NSC 3+ as compared to NSC1+.

In the present study, NSC grade was an independent risk factor for recurrence and death (Tables 3A and 3B).

3.4. Relation to NSC and snail

The Snail-positive rates were assessed in NSCs and in cancer cells showing glandular lumen from the same section of the same patient. The median (interquartile range) of Snail-positive rates were 80% (60%-90%) for the NSC group and 30% (20%-60%) for cancer cells showing glandular lumen. Comparisons within the same section demonstrated that Snail expression was significantly greater in NSCs (Figure 3C). Additionally, the distribution of the difference = (snail positive rate of NSC)-(snail positive rate of cancer cells showing glandular lumen) ranged from 0%-90%, and the Snail-positive rate was greater in NSCs than in cancer cells that showed glandular lumen in all patients (Figure 3D).

3.5. Relation to NSC and E-cadherin

There were 23 patients with NSC 3+, and 19 of these patients (82.6%) were E-cadherin 1+ (and 4 of these patients (17.4%) were E-cadherin 2+ and none of these patient was E-cadherin 3+.). Of the 20 patients with NSC 2+, 11 (55%) were E-cadherin 2+ (and 8 of these patients (40%) were E-cadherin 1+ and 1 of these patient (5.0%) was E-cadherin 1+). Of the 25 patients with NSC 1+, 17 (68%) were E-cadherin 3+ (and 1 of these patients (4.0%) were E-cadherin 1+ and 7 of these patients (28%) were E-cadherin 2+). Spearman's rank correlation coefficient between NSC grade and E-cadherin grade was ρ=-0.76 (P<0.0001), demonstrating a strong negative correlation. (Table 4)

4. Discussion

Pancreatic cancer is a gastrointestinal cancer with high malignancy. While multidisciplinary therapy primarily consisting of chemotherapy and radiation therapy is performed in addition to surgical treatment, satisfactory outcomes are not usually obtained. Treatment strategies should be further investigated in the future, and useful predictors of pancreatic cancer prognosis and reoccurrence will be indispensable at that time.

Many pancreatic cancers are well-differentiated adenocarcinomas, and in our experience, approximately 60% (43 patients) were diagnosed as well-differentiated adenocarcinoma. We found that more than half of these cases that diagnosed as well-differentiated contained NSCs. Thus, we conducted the present study focusing on NSCs that appear in varying percentages regardless of the differences in the degree of histological grade. In

this study, we discovered that NSC grade is an independent factor for poor prognosis in invasive pancreatic ductal carcinoma.

When patients who underwent resection were assessed histologically, the percentage of NSCs within cancerous tumours varied greatly. As a result of evaluating and assessing the emergence of NSCs on a 3-level scale, we found that both DFS and OS were shorter as the percentage of NSCs increased.

Clinicopathological factors with $P<0.2$ in covariate distribution by NSC grade were: age (≥65 years old), tumour size, peripancreatic neural invasion(PL), TNM stage, lymph node metastasis, histological grade, duodenal invasion(DU), invasion to anterior pancreatic capsule(S), lymphatic invasion(ly), and venous invasion(v). It has been previously reported that NSC may be strongly involved in the migration, invasion, and metastasis of cancer, and our current results of frequently observing lymph node metastasis, lymphatic invasion(ly), venous invasion(v), and peripancreatic neural invasion(PL) depending on the emergence of NSCs corroborate these previous findings.

Furthermore, after multivariate analysis by NSC grade of each factor that showed significance with univariate analysis, we found that NSC grade is an independent risk factor for DFS and OS.

From the current findings, we considered that presence of NSCs in invasive pancreatic ductal carcinoma is a useful predictor of prognosis and recurrence.

Trietsch et al.[5] studied 108 vulvar cancer patients, and reported that those who exhibited NSCs had a poor prognosis, which is consistent with the results of the present study.

Next, we further investigated the association between NSCs and EMT in invasive pancreatic ductal carcinoma. EMT is profoundly involved in the metastasis and

recurrence of cancer. EMT is a reversible process in which epithelial cells morphologically change into mesenchymal cells that have high migratory abilities. EMT in tumours causes loss of cell polarity and enhancement of migratory properties by cancer cells gaining mesenchymal characteristics and is thought to be profoundly associated with the invasion and metastasis of cancer cells.

When EMT is induced in cancer cells, Snail, one of the EMT-induced transcription factors, increases in the nucleus, and E-cadherin, a cell adhesion factor, decreases.[13-15] Increased Snail and decreased E-cadherin are involved in the progression of cancer and the shortening of survival.[12, 16]

In the present study, we assessed the Snail-positive rate of NSCs and cancer cells that show glandular lumen in each section, and we found that Snail-positive rate was significantly greater in NSCs than in cancer cells that show glandular lumen. In addition, the Snail-positive rate in NSCs was greater than that in cancer cells that show glandular lumen in all patients. (In all patients, (Snail-positive rate in NSCs) $\geqq$ (Snail-positive rate in cancer cells that show glandular lumen))

In addition, a strong negative correlation was observed between NSC grade and E-cadherin staining, and there was a marked decrease in E-cadherin on the cell membranes of cancer cells in patients with a greater number of NSCs significantly.

From these findings, we considered that the presence of NSCs is profoundly associated with EMT. Our results are similar to those from a report on nasopharyngeal cancer by Wei et al.[17] and a report on malignant mesothelioma resection by Kobayashi et al.[12]

NSC grade was an independent risk factor for DFS and OS. This was also

independent of TNM stage and histological grade, which are widely used as clinical diagnosis of pancreatic cancer. NSC grade may be useful as an indicator of cancer activity or factor for poor prognosis. In addition, NSC was considered to be profoundly involved in the invasion, metastasis, and de-differentiation of cancer, or in EMT.

In addition, in this study, NSCs were not necessarily localized to the area of the invasive front of cancer alone but were spread throughout the tumour. Thus, we evaluated the entire tumour.

NSC grading in pancreatic cancer may become a new criterion for diagnosis or treatment of pancreatic cancer. For example, even in relatively early stages of cancer, in patients who exhibit many NSCs, prognosis could be improved by proactively performing postoperative adjuvant chemotherapy. Moreover, a new type of drug that selectively suppresses or blocks Snail expression in pancreatic cancer may contribute to an improved prognosis.

This study is the first to report the strong relationship between NSCs in resected invasive pancreatic ductal carcinoma and its recurrence and prognosis. NSC grade assessment in invasive pancreatic ductal carcinoma not only can be performed inexpensively and conveniently, but also is useful in predicting recurrence and prognosis.

In the future, NSC grade may be included in the routine assessment to diagnose invasive pancreatic ductal carcinoma. The present study investigated a small number of patients from one facility; however, we believe it is of significance from the perspective that complete clinicopathological data with information on chemotherapy and follow-up were used and that the association between NSCs and EMT was investigated. In the

future, further development in this field, including a multicenter, prospective investigation, is anticipated.

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Figure legends

Figure 1: Pathology

Example of NSC(arrowhead) and cancer cells showing glandular lumen(arrow) in pancreatic cancer.

Figure 2: Study design

This is a well-characterised cohort study. 83 pancreatic cancer patients were initially considered and 14 patients were excluded based on non-curative operation, and 1 patient was excluded based on the diagnosis changing to IPMC. Sixty-eight patients with full clinicopathological information constituted the analysis cohort. Univariate and multivariate analysis was performed on NSC grading of 68 patients, and the Snail-positive rate and graded E-cadherin staining was determined in selected sections from 68 patients.

Figure 3: Statistical analysis

Kaplan-Meier curve showing that high-NSC grade had a major adverse effect on the disease free survival(DFS) ($p<0.0001$) (A) and the overall survival(OS) ($p<0.0001$) (B) of pancreatic carcinoma of patients (NSC 1+ < NSC 2+ < NSC 3+).

Box plot of snail positive rate in NSC and cancer cells showing glandular lumen of pancreatic carcinoma (C) and difference = (snail positive rate of NSC) - (snail positive rate of cancer cells showing glandular lumen) (D).

Table 1. Pathological Characteristics of the Patients (n = 68)

Characteristics	n	rate (%)
Gender		
Male	45	66.2
Female	23	33.8
median age (range) =	65.35 (37-81)	
Tumor status		
pT1	10	14.7
pT2	6	8.8
pT3	49	72.1
pT4	3	4.4
Lymph node status		
pN0	33	48.5
pN1	35	51.5
pStage		
p IA	7	10.3
p IB	5	7.4
p IIA	20	29.4
p IIB	33	48.5
P III	3	4.4
Pl		
Pl(-)	64	94.1
Pl(+)	4	5.9
S		
S(-)	53	77.9
S(+)	15	22.1
RP		
RP(-)	38	46.3
RP(+)	44	53.7
R-status		
R0	68	100.0
R1 , R2, RX	0	0

Histologic grade		
Well differentiated tubular adenocarcinoma	**43**	**63.2**
Mod differentiated tubular adenocarcinoma	**18**	**26.5**
Poor differentiated adenocarcinoma	**5**	**7.4**
Anaplastic carcinoma	**2**	**2.9**
Neoadjuvant chemoradiotherapy		
yes	**11**	**16.2**
No	**57**	**83.8**
Adjuvant chemotherapy		
yes	**55**	**80.9**
No	**13**	**19.1**
Disease-free interval (n=68)		
median(range) = 411 (46 - 2228)		
Overall survival (n=68)		
median(range) = 616 (41 - 2228)		
Surgical procedure		
DP	**18**	**26.5**
PD	**45**	**66.2**
TP	**5**	**7.4**
Prognosis		
Survivor	**34**	**50.0**
Death of complication	**31**	**45.6**
Death of other diseases	**3**	**4.4**
Style of reccurence (includes multiple responses) (n = 42)		
Peritoneal metastasis	**9**	**13.2**
Liver metastasis	**12**	**17.6**
Periarterial plexus of Superior mesentric artery(SMA) or No.16 lymph node	**10**	**14.7**
Lung metastasis	**3**	**4.4**
Local reccurence	**8**	**11.8**
Other Lymph node Peritoneal metastasis	**1**	**1.5**

Table 2. Clinicopathological characteristics in patients and univariate analysis (n = 68)

Characteristics	Total N=68 n (%)	NSC 1+ n=25(36.8%)	NSC 2+ n=20(29.4%)	NSC 3+ n=23(33.8%)	p Value※1
Gender					
male	45(66.2)	15(60.0)	16(80.0)	14(60.9)	0.30
female	23(33.8)	10(40.0)	4(20.0)	9(39.1)	
Age	Median age (range) = 65.35 yr (37-81 yr)				
≧65 yr	37(54.4)	14(56.0)	15(75.0)	8(34.8)	0.03
<65 yr	31(45.6)	11(44.0)	5(25.0)	15(65.2)	
Tumor size	Median size (range) = 25 (8 - 90)				
＞20 mm	38(55.9)	9(36.0)	11(55.0)	18(78.3)	0.01
≦20 mm	30(44.1)	16(64.0)	9(45.0)	5(21.7)	
Tumor status					
pT3,pT4	52(76.5)	16(64.0)	17(85.0)	19(27.9)	0.29
pT1,pT2	16(23.5)	9(36.0)	3(15.0)	4(5.9)	
Lymph node status					
pN1	35(51.5)	8(32.0)	7(35.0)	20(87.0)	<0.001
pN0	33(48.5)	17(68.0)	13(65.0)	3(13.0)	
pStage					
p I A	7(10.3)	6(24.0)	1(5.0)	0	
p I B	5(7.4)	2(8.0)	2(10.0)	1(4.3)	0.002
p II A	20(29.4)	9(36.0)	9(45.0)	2(8.7)	
p II B	33(48.5)	8(32.0)	7(35.0)	18(78.3)	
p III	3(4.4)	0	1(5.0)	2(8.7)	
Ly					
ly1,2,3	56(82.4)	17(68.0)	18(90.0)	21(91.3)	0.053
ly0	12(17.6)	8(32.0)	2(10.0)	2(8.7)	
V					
v1,2,3	41(60.3)	11(44.0)	11(55.0)	19(82.6)	0.06
v0	27(39.7)	14(56.0)	9(45.0)	4(17.4)	
Ne					
ne1,2,3	60(88.2)	20(80.0)	19(95.0)	21(91.3)	0.23
ne0	8(11.8)	5(20.0)	1(5.0)	2(8.7)	
Du					0.15

du(+)	24(35.3)	6(24.0)	9(45.0)	9(45.0)	
du(-)	44(64.7)	19(76.0)	11(55.0)	14(55.0)	
S					
S(+)	14(20.6)	3(12.0)	5(25.0)	6(26.1)	0.42
S(-)	54(79.4)	22(88.0)	15(75.0)	17(73.91)	
RP					
RP(+)	33(48.5)	7(28.0)	9(45.0)	17(73.9)	0.006
RP(-)	35(51.5)	18(72.0)	11(55.0)	6(26.1)	
PL					
PL(+)	4(5.9)	0	1(5.0)	3(13.0)	0.14
PL(-)	64(94.1)	25(100.0)	19(95.0)	20(87.0)	
Histologic grade					
Well	43(63.2)	20(80.0)	16(80.0)	7(30.4)	
Mod	18(26.5)	5(20.0)	4(20.0)	9(39.1)	<0.001
Por	5(7.4)	0	0	5(21.7)	
Other	2(2.9)	0	0	2(8.7)	

※[1]Determained by Chisquare or Fisher's Exact tests

Table 3A. Univariate Cox regression analysis and multivariate Cox regression analysis of disease-free (DFS) in pancreatic cancer patients (n = 68)

	univariate Cox regression analysis ※1		multivariate Cox regression analysis ※1 ※2	
NSC grade	HR(95%CI)	P	HR(95%CI)	P
NSC 1+	1	Ref	1	ref
NSC 2+	1.83(0.79-4.24)	0.16	1.60(0.66-3.88)	0.30
NSC 3+	5.71(2.62-12.4)	<0.0001	3.38(1.30-8.80)	0.013

※1 Determained by Cox proportional hazard regression analysis .

※2 Adjustment factors : size, pStage, histological grade, ly, v, RP

Table 3B. Univariate Cox regression analysis and multivariate Cox regression analysis of overall survival (OS) in pancreatic cancer patients (n = 68)

	univariate Cox regression analysis ※1		multivariate Cox regression analysis ※1 ※2	
NSC grade	HR(95%CI)	P	HR(95%CI)	P
NSC 1+	1	ref	1	ref
NSC 2+	2.48(0.88-6.99)	0.09	2.71(0.92-7.93)	0.07
NSC 3+	7.31(2.81-19.0)	<0.0001	4.99(1.82-13.7)	0.002

※1 Determained by Cox proportional hazard regression analysis .

※2 Adjustment factors : size, pStage, v

Table 4 : The items of NSC grade and E-cadherin grade in pancreatic cancer patients

	E-cadherin 1+	E-cadherin 2+	E-cadherin 3+	total
NSC 1+	1(4.0)	7(28.0)	17(68.0)	25(100)
NSC 2+	8(40.0)	11(55.0)	1(5.0)	20(100)
NSC 3+	19(82.6)	4(17.4)	0	23(100)
total	28	22	18	68

number of cases (%)

Figure 1: Pathology

Example of NSC(arrowhead) and cancer cells showing glandular lumen(arrow) in pancreatic cancer.

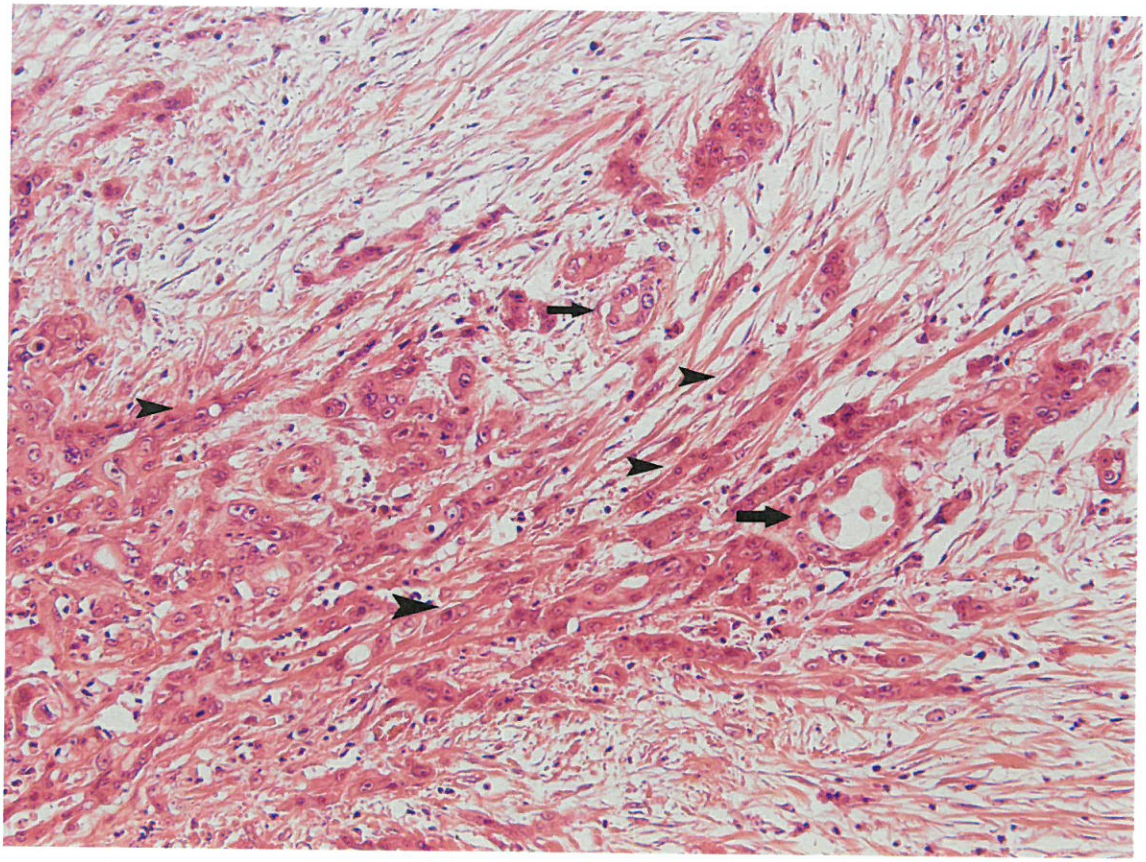


Figure 2: Study design

This is a well-characterised cohort study. 83 pancreatic cancer patients were initially considered and 14 patients were excluded based on non-curative operation, and 1 patient was excluded based on the diagnosis changing to IPMC. Sixty-eight patients with full clinicopathological information constituted the analysis cohort. Univariate and multivariate analysis was performed on NSC grading of 68 patients, and the Snail-positive rate and graded E-cadherin staining was determined in selected sections from 68 patients.

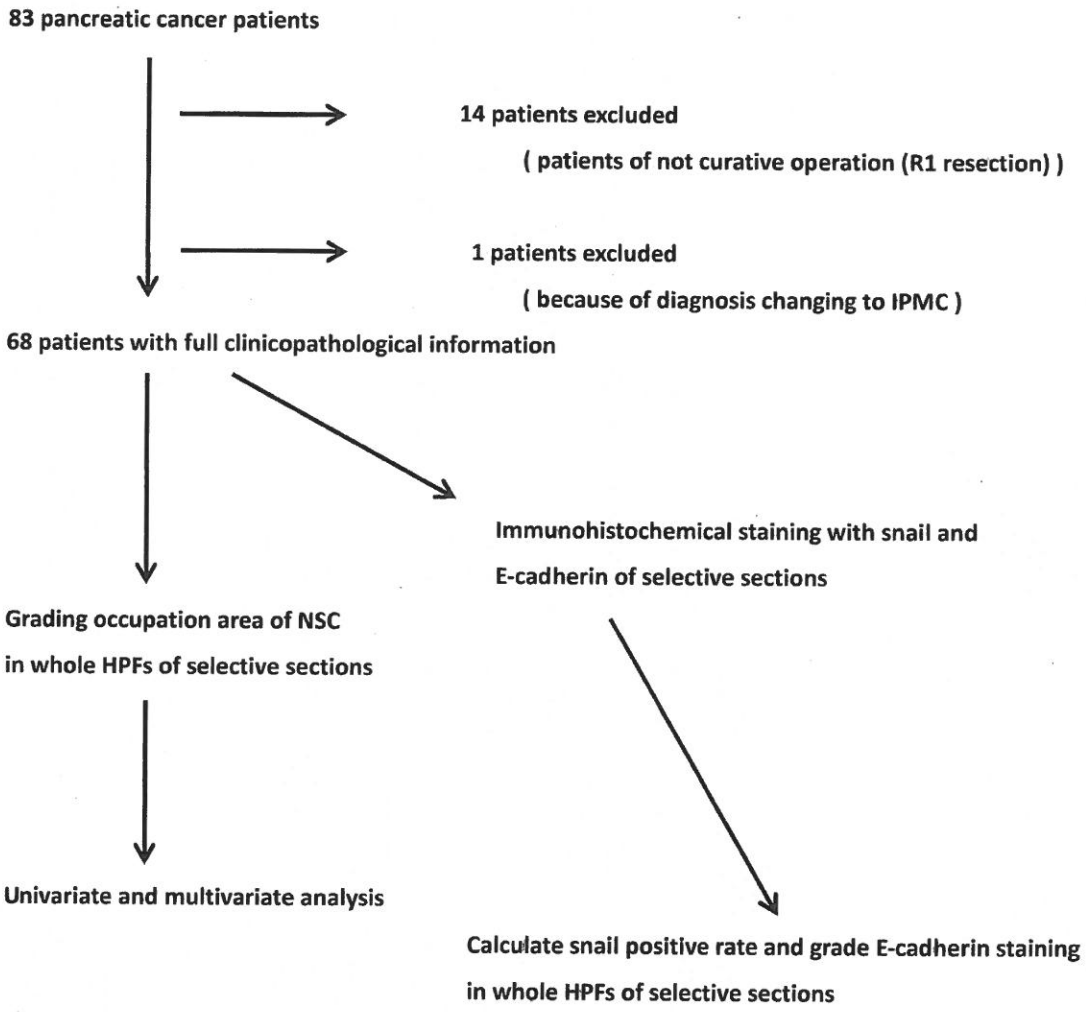


Figure 3: Statistical analysis

Kaplan-Meier curve showing that high-NSC grade had a major adverse effect on the disease free survival(DFS) ($p<0.0001$) (A) and the overall survival(OS) ($p<0.0001$) (B) of pancreatic carcinoma of patients (NSC 1+ < NSC 2+ < NSC 3+).

Box plot of snail positive rate in NSC and cancer cells showing glandular lumen of pancreatic carcinoma (C) and difference = (snail positive rate of NSC) - (snail positive rate of cancer cells showing glandular lumen) (D).

Figure 3A

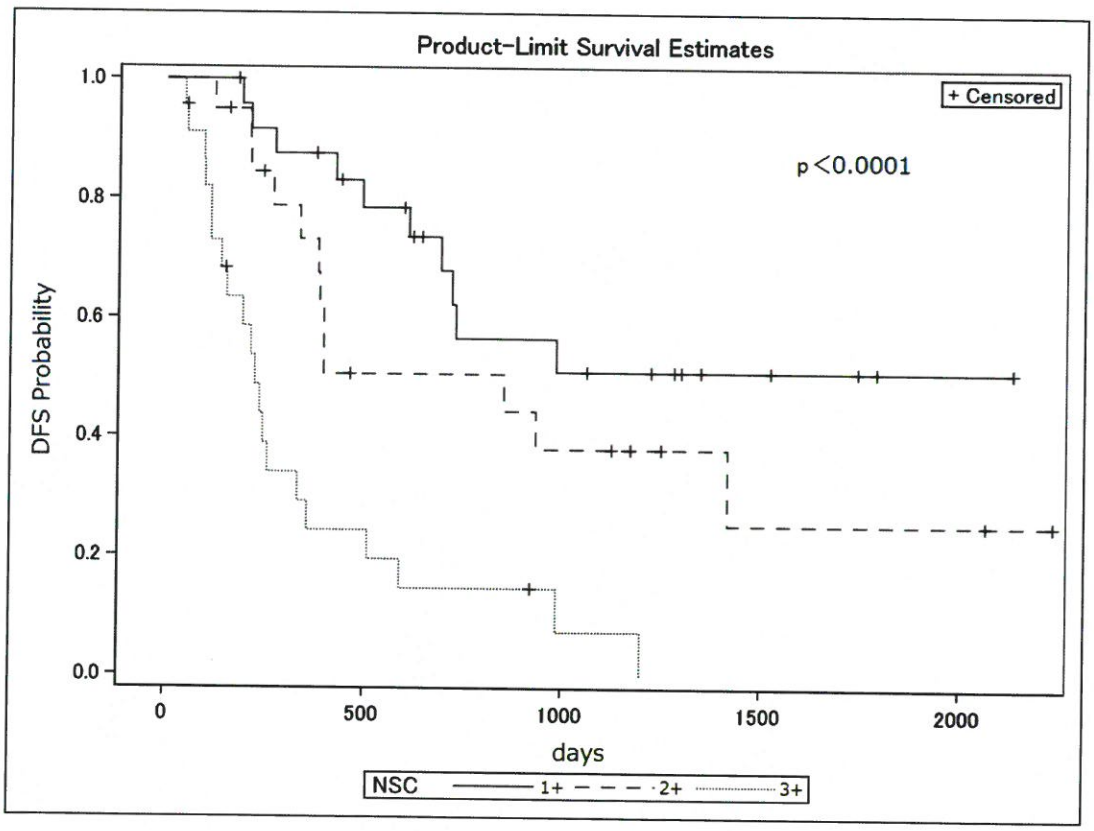


Figure 3B

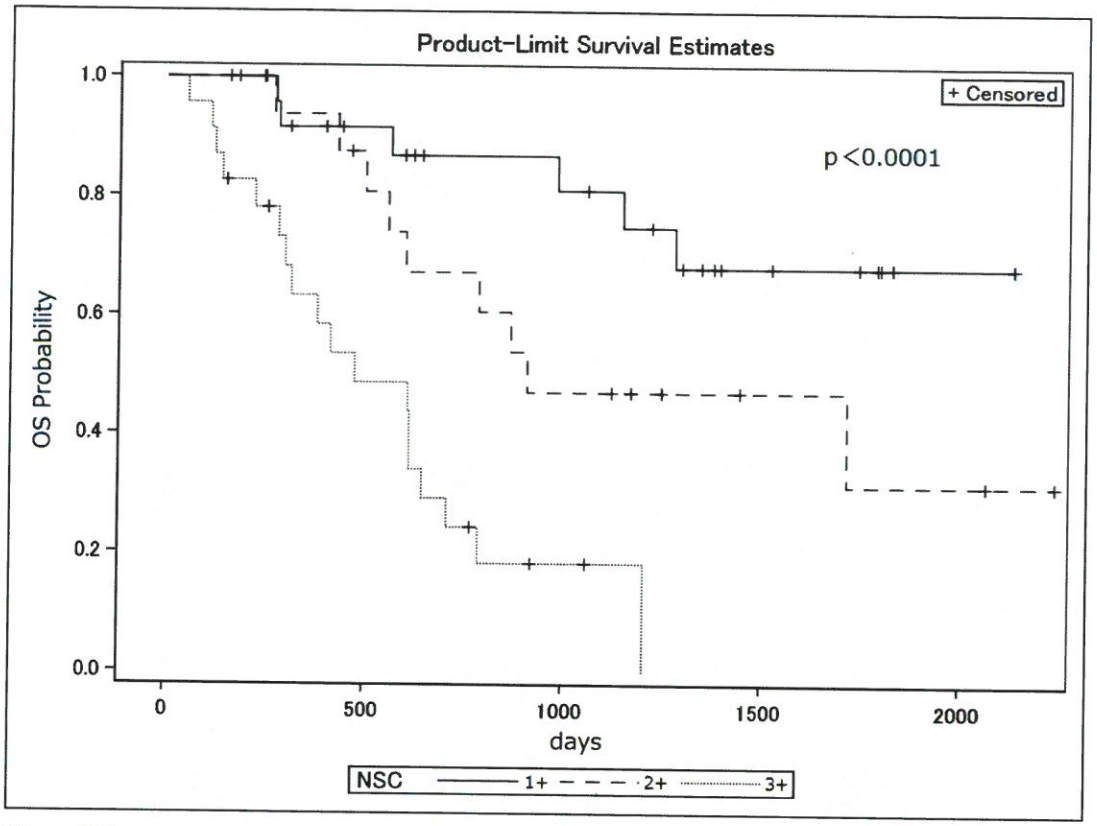


Figure 3C

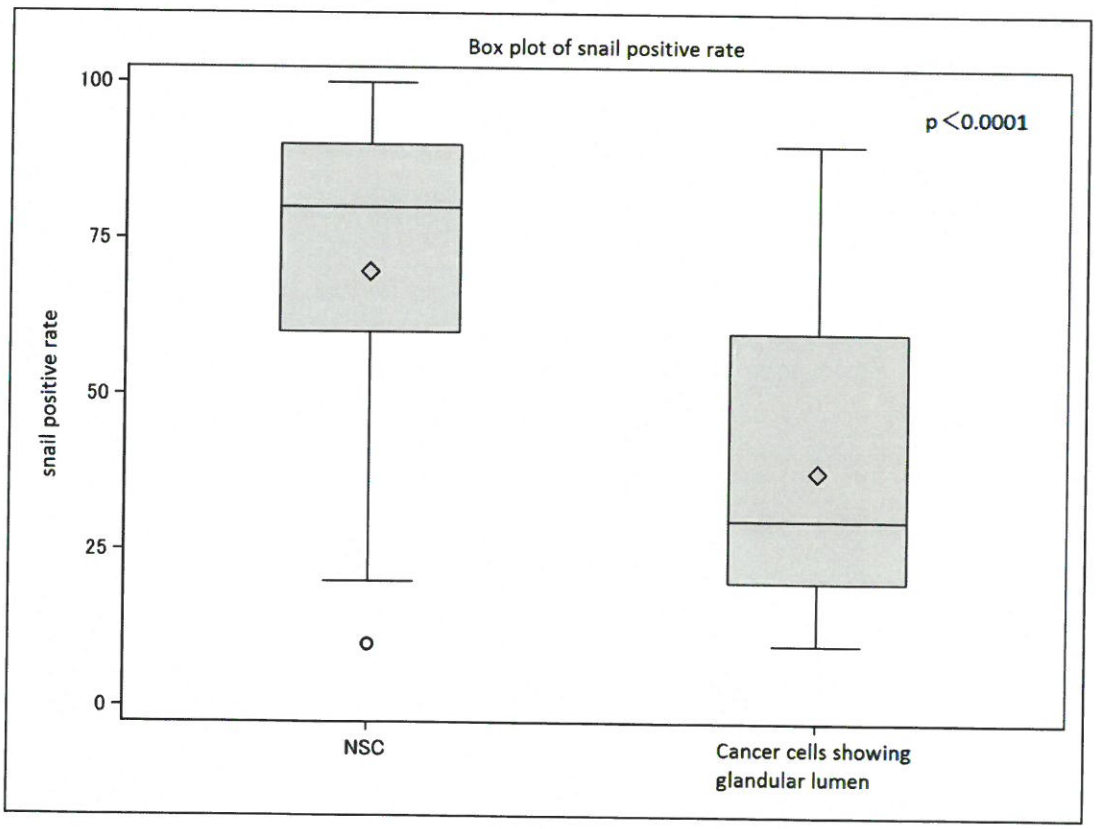
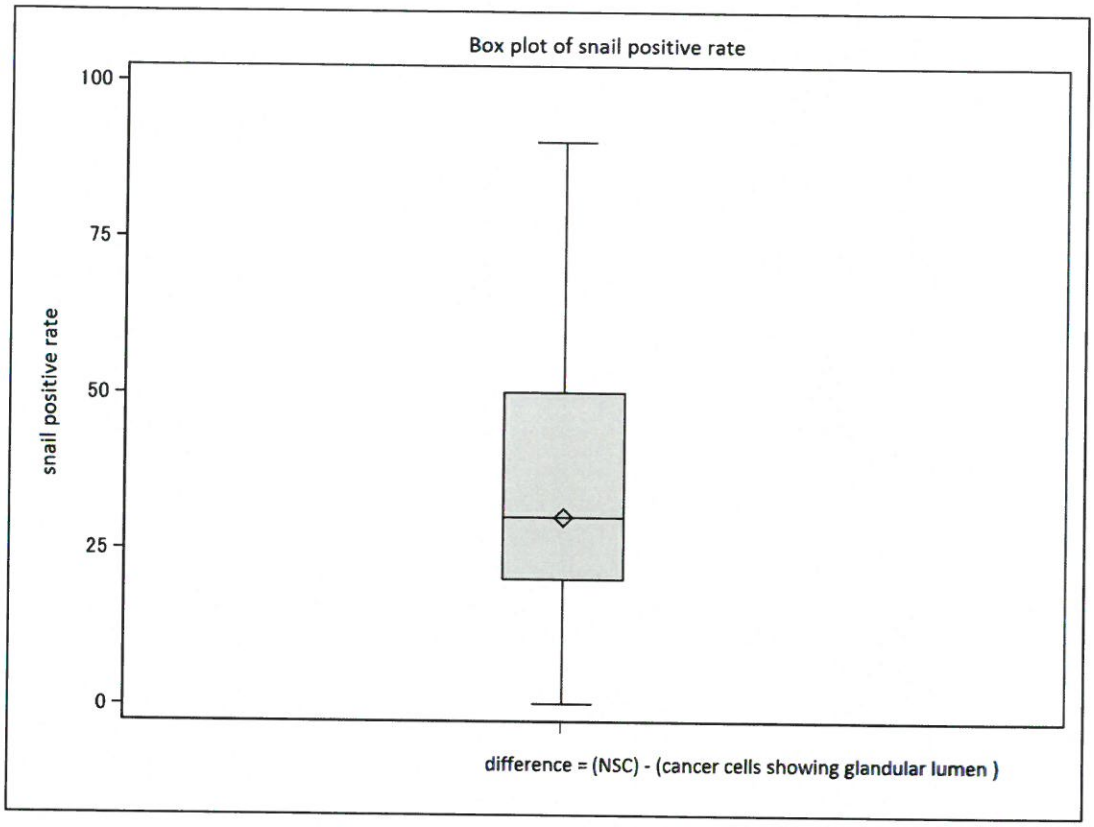


Figure 3D


Box plot of snail positive rate
100
75
50
25
0
snail positive rate
difference = (NSC) - (cancer cells showing glandular lumen)