Prognostic significance of stem cell/epithelial-mesenchymal transition markers in periampullary/pancreatic cancers: FGFR1 is a promising prognostic marker

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Abstract

Background: Periampullary cancers (PAC) including pancreatic, ampulla of Vater (AOV), and common bile duct (CBD) cancers are highly aggressive with a lack of useful prognostic markers beyond T stage. However, T staging can be biased due to the anatomic complexity of this region. Recently, several markers related to cancer stem cells and epithelial-mesenchymal transition (EMT) such as octamer transcription factor-4 (Oct4) and fibroblast growth factor receptor 1 (FGFR1) respectively, have been proposed as new promising markers in other solid cancers. The aim of this study was to assess the expression and prognostic significance of stem cell/EMT markers in PACs.

Methods: Formalin-fixed, paraffin-embedded tissues of surgically excised PACs from the laboratory archives from 1998 to 2014 were evaluated by immunohistochemical staining for stem cell/EMT markers using tissue microarray. The clinicopathologic parameters were documented and statistically analyzed with the immunohistochemical findings. Survival and recurrence data were collected and analyzed.

Results: A total of 126 PAC cases were evaluated. The average age was 63 years, with 76 male and 50 female patient samples. Age less than 74 years, AOV cancers, lower T & N stage, lower tumor size, no lymphatic, vascular, perineural invasion and histologic well differentiation, intestinal type, no fibrosis, severe inflammation were significantly associated with the better overall survival. High expression levels of FGFR1 as well as CK20, CDX2, and VEGF were significantly related to better overall survival, while other stem cell markers were not related. Similar findings were observed for tumor recurrence using disease-free survival.

Conclusions: In addition to other clinicopathologic parameters, severe fibrosis was related to frequent tumor recurrence, and high FGFR1 expression was associated with better overall survival. Histologic changes such as extensive fibrosis need to be investigated further in relation to EMT of PACs.

Keywords: Pancreatic ductal carcinoma, Duodenal neoplasms, Common bile duct neoplasm, SOX transcription factor, Duodenal neoplasms, Fibroblast growth factor receptor, Octamer transcription factor-4
Background

Periampullary cancers (PAC) including pancreatic, ampulla of Vater (AOV), and common bile duct (CBD) cancers are highly aggressive tumors, and their 5-year survival rate is less than 4, and 90% of the patients die from the disease within a year after diagnosis [1]. PACs are expected to rank as the second leading cause of cancer death in 10 years after lung cancers [2]. This is presumed to be because of the invasive growth of the tumor, delayed diagnosis due to the absence of specific symptoms, and limited treatment options. To date, the T stage, which represents the operability of curative resection is the most convincing prognostic marker, according to the American Joint Cancer Committee/Union International Contre le Cancer staging system (AJCC/UICC) [1]. However, T stage evaluation completely depends on the pathologist’s decision based only on gross and pathologic examination, which can be subjective by individuals due to the anatomical complexity of this region [2].

Moreover, as the tumor grows and invades more than two adjacent tissues, such as duodenum and AOV, or distal CBD and pancreatic head, determining the epicenter of the tumor is not straightforward, and might not produce reproducible results between examiners. In a recent review by Adsay et al., the T stage in 39% of the examined cases differed from the original reports [2]. Moreover, the T staging system is different for each cancer, which depends on the primary location (Supplementary Table 1) [3]. For example, a tumor that lies on the distal CBD and AOV with duodenal involvement is T3 for CBD cancer while it is T2 for AOV cancer according to the AJCC cancer staging 7th edition; There’s a higher chance to get the same results by 8th edition that the CBD tumors with duodenal involvement would likely invade the bile duct wall with a depth greater than 12 mm. According to the official guidelines of AJCC, the survival of patients with T1 and T2 stages was reversed during the first year after surgery [3]. Therefore, there is an immediate need for a feasible substitute for proper prognostic anticipation, but thus far, no effective markers have been developed.

Recent studies have found that many solid tumors, such as breast and colon cancers, are composed of heterogeneous tumor clusters showing different molecular genetic characteristics. Cancer stem cells are believed to play a major role in tumor development, progression, metastasis, and resistance. More recently, in pancreatic cancer cell lines, stem cell markers, such as Oct4, NANOG, and SOX2, were found to be aberrantly overexpressed compared to normal pancreatic cell lines [4–6]. This aberrant overexpression resulted in uncontrolled proliferation and dedifferentiation of tumor cells by causing changes in the expression of genes that control the G1/S phase of the cell cycle, and epithelial-mesenchymal transition (EMT) [4, 7–9]. Likewise, several EMT markers such as FGFRI, IGF-1, VEGF, and recently ZEB1/2, SNAIL/SLUG, are being considered as promising prognostic markers [10, 11]. Moreover, these markers are thought to be very important as potential targets for tailored therapy. However, the expression of these markers and their prognostic significance in clinical cases of PACs have not been fully studied.

The purpose of this study was to assess the expression and prognostic significance of the stem cell markers and the markers related to EMT in PACs, and finally to identify a panel of prognostic markers that can classify PACs into more relevant groups.

Methods

Patients and samples

This study was approved by the Institutional Review Board of the Catholic University of Korea (SC14SISI0052). We investigated formalin-fixed, paraffin-embedded tissues of surgically excised PACs from our laboratory archives (Yeouido St. Mary’s Hospital, Seoul, Korea) from 1998 to 2014 (15 years). These included pancreatic head cancers, distal CBD cancers, AOV cancers, and duodenal cancers with periampullary involvement, excised by Whipple surgery or pylorus-preserving pancreaticoduodenectomy. None of the cases had undergone any type of preoperative chemotherapy. We sequentially retrieved a total of 126 cases after excluding intraductal papillary mucinous neoplasms, which are considered as benign or premalignant lesions, metastatic cancers from other organs, and duodenal cancers without ampullary or pancreatic involvement. The mean age of the enrolled patient cases was 63 years (ranging from 36 to 82 years), and out of 126 cases, 76 were male, and 50 were female. The retrieved cases were blinded by sequential numbering. The hematoxylin and eosin-stained slides were independently reviewed by two pathologists (Y. Chong and EJ. Lee) to confirm the original diagnosis. The cases with not enough tissue available for tissue microarray analysis were excluded. The epicenter of the tumors was reevaluated and compared with the evaluation from the original diagnosis.

The clinicopathologic data were documented: location of the mass, gross type (fungating/polypoid, sessile, ulceroinfiltrative), presence of ulceration, tumor size (largest diameter), radial resection margin involvement, TNM stage, lymphatic, vascular, perineural invasion, histologic differentiation (pancreatobiliary vs. intestinal subtype, and well, moderately, poorly for each type), degree of fibrosis and inflammatory cell infiltration (mild, moderate, severe), date of surgery, follow-up duration, date of recurrence, and date of death. The degree of fibrosis was described by the fourth -tier system based on H&E finding (none (< 10%), mild (10–33%), moderate(34–66%), and severe (67–100%)). The epicenter of the tumor was
reevaluated for each case and compared with the original
diagnosis, based on the staging system of AJCC/UICC 7th
dition because most of the cases were originally diag-
osed based on the 7th edition. T stage was rescored ac-
cording to the revised tumor epicenter and compared
with the original T stage. Pancreatobiliary and intestinal
subtype was determined based on the results of immuno-
istochemical staining for CK7, CK20, CDX-2, and MUC-
2 (Mucin 2). Information regarding the cause and date of
death was collected based on National Death Certificate
data and medical records from our institute. Since Na-
tional Death Certificate records have a year of delay for
data collection, the death data of recent cases of 2013 and
2014 were documented according to our institutional
medical records. Tumor recurrence was defined as a
newly detected tumor or metastasis upon radiological
examination such as computed tomography (CT) or po-
position emission tomography (PET-CT) with or without an
increase of serum CA19–9 level in the surgically resect-
able cases. Incompletely resected cases with progressive
disease were excluded from assessment for tumor recur-
rence and, death resulting from early complications such
as bleeding, bile leakage, infection, and pulmonary embol-
ism were not considered as cancer-related death. Cases
with the unknown or unspecified cause of death were ex-
cluded from survival analysis.

Tissue microarray
Nine TMA recipient blocks were made using Quick-
Ray® Tissue Microarray recipient block (UB06–2, Uni-
TMA Co., LTD., Seoul, Korea). Three 2 mm sized tumor
spots representing each case were taken from donor
blocks to avoid tissue loss and edge artifact. Each recipi-
ent block consisted of 45 cores of tumor tissue (15
cases), and 4 cores of positive controls for stem cell
markers, and 1 core of negative control. To reduce tissue
loss, the recipient blocks were incubated at 30 °C for 25
min before core insertion. The positive control cores
were normal lung alveoli and squamous cell carcinoma
of the lung for CD24 and SOX2, testicular seminoma for
Oct4, benign urothelial epithelium of the bladder for
CD44v6, normal umbilical cord for FGFR1, normal
blood vessel for VEGF, and normal liver tissue for IGF-1.

Immunohistochemistry
The TMA blocks were cut into 4 μm thick sections,
mounted on silanized glass slides, and dried in an oven
at 70 °C for 60 min. The slides were automatically proc-
cessed and stained by BenchMark XT (Ventana, Roche
Diagnostics, USA) according to the manufacturer’s in-
suctions. The procedure included antigen retrieval by
heating at 70 °C for 1 h, followed by pretreatment with
cell conditioner 2 (pH 6) for 60 min, and subsequent in-
cubation with each antibody at optimal temperature for
32 min, and then counterstained by hematoxylin for 4
min and bluing agent for 4 min, followed by chromo-
genic detection using UltraView Universal DAB Detection
Kit for mins, and final washing step for mins. The
stained slides were covered with balsamic acid. Immuno-
histochemical staining was performed for CK7, CK20,
CDX-2, and MUC-2 and the tumors were classified into
pancreatobiliary and intestinal subtype. In addition,
immunohistochemical staining was performed for stem
cell markers including CD24, SOX2, Oct4, and CD44v6
and epithelial-mesenchymal transition markers, includ-
ing VEGF, IGF-1, and FGFR1. The dilution and incuba-
tion conditions for each antibody are as follows: CK7
(Prediluted, Ventana, Roche Diagnostics, USA), CK20
(Prediluted, Ventana, Roche Diagnostics, USA), CDX-2
(Prediluted, Ventana, Roche Diagnostics, USA), MUC-2
(Prediluted, Ventana, Roche Diagnostics, USA), CD24 (1:
50, Thermo Scientific, UK), Oct4 (ChIP Grade ab19857,
1:100, Abcam, CB4, UK), SOX2 (SP76, 1:100, Cell
Marque, CA, USA), VEGF (1:50, Quartett Immunodiag-
nostika, Berlin, Germany), IGF-1 (1:100, Abcam, CB4,
UK), FGFR1 (ChIP Grade ab10646, 1:100, Abcam, CB4,
UK) and CD44v6 (1:200, Invitrogen, CA, USA). The im-
munoreactivity was scored for each tissue microarray
core as a three-tier system (0, 1+, 2+, 3+) according to
the intensity. If the intensity is heterogeneously high or
low, the most commonly found intensity was scored.

Statistical analysis
All analyses were performed using R statistical software
(version 3.2.3 via http://web-r.org/) and IBM SPSS Sta-
tistics (ver. 1.0.0.1347 64-bit). Statistical analysis was
performed using the Mann-Whitney test for continuous
variables and Fisher’s exact test or chi-square tests for
categorical variables to compare the groups with and
without recurrence For multivariable regression analysis,
logistic regression analysis was used to evaluate the asso-
ciation between the clinicopathologic parameters and
the recurrence.
Kaplan-Meier analysis was used for survival analysis
to search the parameters affecting the overall survival
and disease-free survival and results with a p-value
less than 0.05 were considered as statistically signifi-
cant. To find the novel panel of prognostic markers,
the combined immunoreactivity score was made using
the immunoreactivity of certain 2, 3, 4, 5 IHC
markers and analyzed with overall survival. The group
was divided into the lower and higher expression
groups according to the expression level of the IHC
panel. Using the statistically significant parameters in
Kaplan-Meier analysis, Cox regression analysis was
used to confirm the relationship and to determine the
odds ratio of each parameter.
Results

Patient and tumor characteristics

The clinicopathologic data of the evaluated cases are summarized in Table 1. Mean age of the patients was 63 years (ranging from 36 to 82). There were 76 male patient cases and 50 female patient cases (M:F = 1.52:1). Originally, the epicenter of the tumor was diagnosed as peripapillary duodenum in 3 cases, AOV in 37 cases, pancreatic head in 37 cases, distal CBD in 47 cases, and proximal CBD in 2 cases. After pathological revision, the epicenter of the tumor was AOV in 34 cases, pancreatic head in 44 cases, and distal CBD in 48 cases (Table 1). Changes in the original and revised diagnoses have been described in the following section and summarized in Table 2.

Grossly, 20 cases (15.9%) were categorized as fungating type, 93 (73.8%) as infiltrative, 3 (2.4%) as ulcerofungating, 5 (4.0%) as sessile, and 2 (1.6%) as solid type. The mean tumor size was 3.2 cm (ranging from 0.6 to 8.0 cm). Since tumor size (> 4.5 cm) is one of the important prognostic markers for pancreatic cancer, the cases were divided into two groups based on tumor size (< or > than 4.5 cm) and compared. There were 96 cases with smaller tumor size (76.2%) and 30 cases with larger tumor size (23.8%). Tumor classification based on N stage according to the AJCC staging system, showed that 73 cases (57.9%) were N0, 53 were N1 (42.1%), and there were no N2 cases. M stage classification indicated that 8 cases (6.3%) were M1, while the rest was M0 (117 cases, 93.7%). Lymphatic invasion was found in 56 cases (44.4%), vascular invasion in 16 (12.7%), and perineural invasion in 72 cases (57.1%). Positive radial resection margin was found in 8 cases (6.3%). Tumor ulcer was found in 10 cases (7.9%).

Classification according to histologic grade showed that originally, 37 cases (29.4%) were diagnosed as well-differentiated tumors, 79 (62.7%) as moderately differentiated, and 10 as (7.9%) poorly differentiated. After review, 32 (25.4%) were well-differentiated, 90 (71.4%) were moderately differentiated, and 4 (3.2%) were poorly differentiated. In histologic subtypes, 34 cases (27.2%) were pancreaticobiliary subtype, 58 (46.4%) were more likely to be pancreaticobiliary subtype, 19 cases (15.2%) were more likely to be intestinal subtype, and 14 cases (11.1%) were of the intestinal subtype. Degree of accompanying fibrosis, known as desmoplastic reaction, was absent in 4 cases (3.2%) in the original pathologic reports, mild in 20 cases (15.9%), moderate in 72 cases (57.1%), and severe in 30 cases (23.8%). After revision, it was absent in 1 case (0.8%), mild in 35 cases (27.8%), moderate in 57 cases (45.2%), and severe in 33 cases (26.2%). Degree of peritumoral inflammation was mild in 53 cases (42.1%) in the original pathologic reports, moderate in 62 cases (49.2%) and severe in 11 cases (8.7%). After revision, it was mild in 42 cases (33.3%), moderate in 70 cases (55.6%), and severe in 14 cases (11.1%). Tumor recurrence was observed in 61 cases (48.8%) during an average follow-up of 969.7 days (ranged from 3 to 5234 days). Disease-free survival duration was an average of 731.2 days (ranging from 3 to 4173 days). Eighty-seven out of 126 patients (69.0%) were dead during the follow-up.

Comparison of tumor epicenter and T stages between original and revised diagnoses

Comparison of original and revised diagnoses showed that the epicenter of the tumor was altered in 22 out of 126 cases (17.4%) (Table 2). Among the 22 cases, 10 cases showed a discrepancy between distal CBD and pancreatic head cancers, 6 cases showed a discrepancy between distal CBD and AOV cancers, 5 cases showed a discrepancy between pancreatic head and AOV cancers, 2 cases showed a discrepancy between peripapillary duodenum and AOV, 2 cases showed a discrepancy between proximal and distal CBD, and 1 case showed a discrepancy between peripapillary duodenum and distal CBD. As the tumor locations have been changed after the review, the T stages were also altered (Table 2). As a result, 6 cases showed different T stage between the original and revised diagnoses with 3 over-staged and 3 understaged cases, respectively (6 out of 126 cases, 4.8%).

Immunohistochemical staining and immunoreactivity results

The immunohistochemical staining conditions are summarized in Table 3 and the representative images of the immunohistochemical stainings are shown in Supplementary Fig. 1. The immunoreactivity of SOX2 in the nucleus was negative in 13 cases (10.3%), 1+ in 66 cases (52.4%), 2+ in 45 cases (35.7%) and 3+ in 2 cases (1.6%). The SOX2 immunoreactivity in the cytoplasm was negative in 66 cases (52.4%), 1+ in 56 cases (44.4%), 2+ in 4 cases (3.2%), and no cases showed 3+. CD24 staining was negative in 6 cases (4.8%), 1+ in 79 cases (62.7%), 2+ in 37 cases (29.4%), and 3+ in 4 cases (3.2%).Oct4 immunoreactivity was 1+ in 26 cases (20.6%), 2+ in 73 cases (57.9%), and 3+ in 27 cases (21.4%). IGF-1 staining was negative in 13 cases (10.3%), 1+ in 74 cases (58.7%), 2+ in 33 cases (26.2%), and 3+ in 6 cases (4.8%). The FGFRI immunoreactivity was 1+ in 7 cases (5.6%), 2+ in 52 cases (41.3%), and 3+ in 67 cases (53.2%). The VEGF immunoreactivity was 1+ in 18 cases (14.6%), 2+ in 78 cases (63.4%), and 3+ in 27 cases (22.0%). CD44v6 staining was negative in 14 cases (11.1%), 1+ in 41 cases (32.5%), 2+ in 43 cases (34.1%), and 3+ in 28 cases (22.2%).
Table 1 Summary of clinicopathological data of the enrolled cases

| Age (yrs) | No. (%) | Mean 63.0 ± 9.4 |
|-----------|---------|-----------------|
| Ranged 36 ~ 82 |         |                 |

| Sex | Male 76, Female 50 | M:F = 1.52:1 |
|-----|--------------------|--------------|

| Location (tumor epicenter) | Original diagnosis | Revised diagnosis |
|---------------------------|--------------------|-------------------|
| Periampullary duodenum    | 3 (2.4%)           | 0 (0%)            |
| AOV                       | 37 (29.4%)         | 34 (27.0%)        |
| Pancreatic head           | 37 (29.4%)         | 44 (34.9%)        |
| Distal CBD                | 47 (37.3%)         | 48 (38.1%)        |
| Proximal CBD              | 2 (1.6%)           | 0 (0%)            |

| T stage | Original diagnosis | Revised diagnosis |
|---------|--------------------|-------------------|
| Tis     | 1 (0.8%)           | 1 (0.8%)          |
| T1      | 21 (16.7%)         | 21 (16.7%)        |
| T2      | 29 (23.0%)         | 29 (23.0%)        |
| T3      | 67 (53.2%)         | 67 (53.2%)        |
| T4      | 8 (6.3%)           | 8 (6.3%)          |

| Gross type | Original diagnosis | Revised diagnosis |
|------------|--------------------|-------------------|
| Fungating  | 20 (15.9%)         |                   |
| Infiltrative | 93 (73.8%)       |                   |
| Ulcerofungating | 3 (2.4%)     |                   |
| Sessile    | 5 (4.0%)           |                   |
| Solid      | 2 (1.6%)           |                   |

| Tumor size | Original diagnosis | Revised diagnosis |
|------------|--------------------|-------------------|
| < 4.5 cm   | 96 (76.2%)         |                   |
| ≥ 4.5 cm   | 30 (23.8%)         |                   |

| N stage | Original diagnosis | Revised diagnosis |
|---------|--------------------|-------------------|
| N0      | 73 (57.9%)         |                   |
| N1      | 53 (42.1%)         |                   |
| N2      | 0 (0%)             |                   |

| M stage | Original diagnosis | Revised diagnosis |
|---------|--------------------|-------------------|
| M0      | 117 (93.7%)        |                   |
| M1      | 8 (6.3%)           |                   |

| Lymphatic invasion | Original diagnosis | Revised diagnosis |
|--------------------|--------------------|-------------------|
| 70 (55.6%)         | 56 (44.4%)         |

| Vascular invasion | Original diagnosis | Revised diagnosis |
|-------------------|--------------------|-------------------|
| 110 (87.3%)       | 16 (12.7%)         |

| Perineural invasion | Original diagnosis | Revised diagnosis |
|---------------------|--------------------|-------------------|
| 54 (42.9%)          | 72 (57.1%)         |

| Positive radial resected margin | Original diagnosis | Revised diagnosis |
|---------------------------------|--------------------|-------------------|
| 118 (93.7%)                    | 8 (6.3%)           |

| Tumor ulceration | Original diagnosis | Revised diagnosis |
|------------------|--------------------|-------------------|
| 116 (92.1%)      | 10 (7.9%)          |

| Histologic grade | Original diagnosis | Revised diagnosis |
|------------------|--------------------|-------------------|
| Well differentiated | 37 (29.4%)       | 32 (25.4%)        |
| Moderately differentiated | 79 (62.7%)   | 90 (71.4%)        |
| Poorly differentiated | 10 (7.9%)      | 4 (3.2%)          |

| Histologic subtype | Original diagnosis | Revised diagnosis |
|--------------------|--------------------|-------------------|
| Pancreaticobiliary subtype | 34 (27.2%)  |                   |
| Prone to pancreaticobiliary subtype | 58 (46.4%) |                   |
| Prone to intestinal subtype | 19 (15.2%)   |                   |
Clinicopathological parameters related to tumor recurrence

There was no significant difference between the recurrence and non-recurrence groups based on age, gender, original, revised and combined locations, gross type, ulcer, tumor size, presence or absence of radial resection margin, N stage, and M stage. Although there were no statistically significant relationships between T stage and recurrence, there was a tendency that the recurrence group had a higher T stage than the non-recurrence group. Moreover, there was no statistically significant difference observed between the pathological parameters such as lymphatic invasion, vascular invasion, perineural invasion, histological differentiation, degree of fibrosis, degree of inflammation, histological subtype and the markers detected by IHC (CK7, CK20, CDX-2, MUC-2, ...

Table 1 Summary of clinicopathological data of the enrolled cases (Continued)

| Age (yrs) | No. (%) | Mean 63.0 ± 9.4 |
|-----------|---------|-----------------|
| Ranged 36 ~ 82 | 64 (51.2%) | 61 (48.8%) |
| Intestinal subtype | 14 | (11.2%) |
| Degree of fibrosis | | |
| Absent | 4 (3.2%) | 1 (0.8%) |
| Mild | 20 (15.9%) | 35 (27.8%) |
| Moderate | 72 (57.1%) | 57 (45.2%) |
| Severe | 30 (23.8%) | 33 (26.2%) |
| Degree of inflammation | | |
| Mild | 53 (42.1%) | 42 (33.3%) |
| Moderate | 62 (49.2%) | 70 (55.6%) |
| Severe | 11 (8.7%) | 14 (11.1%) |
| Tumor recurrence | | |
| Absent | 64 (51.2%) | 61 (48.8%) |
| Present | | |
| Death | 39 (31.0%) | 87 (69.0%) |
| Follow-up duration (days) | Ranged 3 ~ 5234 | 969.7 ± 1135 |
| Disease free survival (days) | Ranged 3 ~ 4173 | 731.2 ± 954.5 |

Table 2 Comparison of tumor epicenter and T stages between original and revised diagnoses

(A)

| Original tumor epicenter | Periampullary duodenum | AOV | Pancreatic head | Distal CBD | Proximal CBD | Total |
|--------------------------|------------------------|-----|----------------|------------|-------------|-------|
| Periampullary duodenum   | 0                      | 2   | 0              | 1          | 0           | 3     |
| AOV                      | 0                      | 29  | 3              | 5          | 0           | 37    |
| Pancreatic head          | 0                      | 2   | 33             | 2          | 0           | 37    |
| Distal CBD               | 0                      | 1   | 8              | 38         | 0           | 47    |
| Proximal CBD             | 0                      | 0   | 0              | 2          | 0           | 2     |
| Total                    | 0                      | 34  | 44             | 48         | 0           | 126   |

(B)

| Original T stage | Tis | T1 | T2 | T3 | T4 | Total |
|------------------|-----|----|----|----|----|-------|
| Tis              | 1   | 0  | 0  | 0  | 0  | 1     |
| T1               | 0   | 21 | 0  | 0  | 0  | 21    |
| T2               | 0   | 0  | 27 | 2  | 0  | 29    |
| T3               | 0   | 0  | 2  | 64 | 1  | 67    |
| T4               | 0   | 0  | 0  | 1  | 7  | 8     |
| Total            | 1   | 21 | 29 | 67 | 8  | 126   |
SOX2 (nuclear), SOX2 (cytoplasmic), CD24, Oct4, IGF-1, FGFR1, VEGF, and CD44v6). However, the only significant difference was observed in the original degree of fibrosis \((p = 0.020)\) as indicated in Table 4. However, the multivariable regression analysis showed no statistical differences among all clinicopathological parameters according to tumor recurrence (data not shown).

### Disease-free survival analysis in recurrence patient

The clinical parameters such as age \(\leq 74\) \((p = 0.0221)\), location of AOV \((p = 0.014)\), lower T stage \((p = 0.02)\), size less than 1.5 cm \((p = 0.0426)\), lower N stage \((N0)\) \((p = 0.000391)\) were significantly associated with better disease-free survival (DFS) in recurrent patients, whereas, no significant correlation was observed in other parameters (Supplementary Fig. 2).

In addition, other pathological parameters including no lymphatic invasion \((p < 0.0001)\), histological well differentiation \((p = 0.00121)\), intestinal subtype \((p = 0.0417)\), and mild fibrosis \((p = 0.0259)\) showed a significant association with better DFS (Supplementary Fig. 3). In addition, IHC markers such as CDX-2 \((p = 0.0245)\) and FGFR1 \((p = 0.0181)\) were also significantly correlated with better DFS (Supplementary Fig. 4). Cox regression analysis showed no significant relation of any clinicopathologic parameters to DFS (data not shown).

### Clinicopathological parameters related to overall survival

Among clinical parameters, age \((p = 0.0527)\) and gender \((p = 0.908)\) were not associated with overall survival. On the other hand, location of AOV \((p < 0.0001)\), lower T stage \((p = 0.000228)\), sessile and solid gross type \((p = 0.00278)\), size less than 1.5 cm \((p = 0.00727)\), lower N stage \((N0)\) \((p < 0.0001)\), and lower M stage \((M0)\) \((p = 0.000139)\) were significantly related to better overall survival (Fig. 1). Among pathological parameters, better overall survival was related to no lymphatic invasion \((P < 0.0001)\), no vascular invasion \((p = 0.000325)\), no perineural invasion \((p = 0.00145)\), histological well differentiation \((p = 0.000793)\), intestinal subtype \((p = 0.000483)\), no fibrosis \((p = 0.00497)\), and severe inflammation \((p = 0.036)\) (Fig. 2). In addition, expression of four IHC markers, higher expression of intestinal-type markers, CK20 \((p = 0.0135)\) and CDX2 \((p = 0.000135)\), and higher expression of EMT markers, FGFR1 \((p = 0.0014)\) and VEGF \((p = 0.0333)\) were significantly related to better overall survival (Fig. 3). The combined panel expression score more than 8 of CK20, CDX2, FGFR1, VEGF, and IGF-1 was significantly related to better overall survival \((p = 0.000445)\) as well as the combined panel expression score more than 6 of CK20, CDX2, FGFR1, and VEGF \((p < 0.0001)\) (Fig. 3). Cox regression analysis also showed a significant relationship of N stage, lymphatic invasion, degree of inflammation, pancreaticobiliary/intestinal subtypes, expression of intestinal markers, CK20 and CDX2, and EMT markers, FGFR1 and VEGF (Supplementary Table 2, Supplementary Fig. 5).

### Discussion

The two major findings in this study are, first, FGFR1 could be a promising prognostic marker for periampullary cancers, and second, peritumoral fibrosis was associated with tumor recurrence in periampullary cancer patients. We also confirmed significant relationship between overall survival and previously known clinicopathological prognostic markers, such as age, location, T stage, gross type, size, N stage, M stage, lymphatic invasion, vascular invasion, perineural invasion, histological differentiation, inflammation and the staining pattern of the IHC markers (CK20, CDX2), as described in the literature [1, 12].

In the present study, FGFR1 is significantly associated with overall survival and disease-free survival in periampullary/pancreatic cancer patients. FGFR1 has been known to be related to the prognosis of several human cancers [9, 13]. It is a member of the tyrosine kinase family and shares similar structural morphology with VEGFRs and platelet-derived growth factor receptors.

| Clinicopathologic parameters          | No. (%)          | Non-recurrence (N = 58) | Recurrence (N = 61) | P value |
|--------------------------------------|------------------|-------------------------|---------------------|---------|
| Age (yrs)                            |                  |                        |                     |         |
|                                      |                  | 62.4 ± 9.9             | 64.4 ± 8.9          | 0.258   |
| Sex                                  |                  |                        |                     |         |
| Male                                 |                  | 37 (63.8%)             | 36 (59.0%)          | 0.729   |
| Female                               |                  | 21 (36.2%)             | 25 (41.0%)          |         |
| Original location (epicenter)        |                  |                        |                     | 0.347   |
| Periampullary duodenum               |                  | 2 (3.4%)               | 1 (1.6%)            |         |
| AOV                                  |                  | 20 (34.5%)             | 16 (26.2%)          |         |
| Pancreatic head                      |                  | 15 (25.9%)             | 16 (26.2%)          |         |
| Distal CBD                           |                  | 19 (32.8%)             | 28 (45.9%)          |         |
| Proximal CBD                         |                  | 2 (3.4%)               | 0 (0.0%)            |         |
| Revised location (epicenter)         |                  |                        |                     | NA      |
| AOV                                  |                  | 0 (0.0%)               | 0 (0.0%)            |         |
| Pancreatic head                      |                  | 20 (34.5%)             | 14 (23.0%)          |         |
| Distal CBD                           |                  | 38 (65.5%)             | 47 (77.0%)          |         |
| Combined location (epicenter)        |                  |                        |                     | NA      |
| AOV                                  |                  | 0 (0.0%)               | 0 (0.0%)            |         |
| Pancreatic head                      |                  | 17 (34.7%)             | 13 (26.5%)          |         |
| Distal CBD                           |                  | 32 (65.3%)             | 36 (73.5%)          |         |
| Original T stage                     |                  |                        |                     | 0.536   |
| Tis                                  |                  | 1 (1.7%)               | 0 (0.0%)            |         |
| T1                                   |                  | 13 (22.4%)             | 8 (13.1%)           |         |
| T2                                   |                  | 14 (24.1%)             | 15 (26.4%)          |         |
| T3                                   |                  | 28 (48.3%)             | 35 (57.4%)          |         |
| T4                                   |                  | 2 (3.4%)               | 3 (4.9%)            |         |
| Gross type                           |                  |                        |                     | NA      |
| Fungating                            |                  | 12 (21.4%)             | 8 (13.3%)           |         |
| Infiltrative                         |                  | 37 (66.1%)             | 50 (83.3%)          |         |
| Ulcerofungating                      |                  | 2 (3.6%)               | 1 (1.7%)            |         |
| Sessile                              |                  | 3 (5.4%)               | 1 (1.7%)            |         |
| Solid                                |                  | 2 (3.6%)               | 0 (0.0%)            |         |
| Ulcer                                |                  |                        |                     |         |
| Absent                               |                  | 54 (93.1%)             | 55 (90.2%)          | 0.805   |
| Present                              |                  | 4 (6.9%)               | 6 (9.8%)            |         |
| Average tumor size                   |                  | 3.0 ± 1.5              | 3.3 ± 1.7           | 0.199   |
| Tumor size < 4.5 cm                  |                  | 47 (81.0%)             | 44 (72.1%)          | 0.353   |
| ≥ 4.5 cm                             |                  | 11 (19.0%)             | 17 (27.9%)          |         |
| Radial resection margin Absent       |                  | 56 (96.6%)             | 57 (93.4%)          | 0.722   |
| Present                              |                  | 2 (3.4%)               | 4 (6.6%)            |         |
| No. of positive lymph nodes          |                  | 0.7 ± 1.2              | 1.2 ± 1.8           | 0.073   |
| No. of dissected lymph nodes         |                  | 11.6 ± 7.4             | 11.2 ± 7.0          | 0.730   |

| Clinicopathologic parameters          | No. (%)          | Non-recurrence (N = 58) | Recurrence (N = 61) | P value |
|--------------------------------------|------------------|-------------------------|---------------------|---------|
| N stage                              |                  |                        |                     |         |
| N0                                   |                  | 38 (65.5%)             | 34 (55.7%)          | 0.366   |
| N1                                   |                  | 20 (34.5%)             | 27 (44.3%)          |         |
| M stage                              |                  |                        |                     |         |
| M0                                   |                  | 55 (94.8%)             | 60 (98.4%)          | 0.575   |
| M1                                   |                  | 3 (5.2%)               | 1 (1.6%)            |         |
| Lymphatic invasion                   |                  |                        |                     | 0.106   |
| Absent                               |                  | 38 (65.5%)             | 30 (49.2%)          |         |
| Present                              |                  | 20 (34.5%)             | 31 (50.8%)          |         |
| Vascular invasion                    |                  |                        |                     |         |
| Absent                               |                  | 51 (87.9%)             | 53 (86.9%)          | 1.000   |
| Present                              |                  | 7 (12.1%)              | 8 (13.1%)           |         |
| Perineural invasion                  |                  |                        |                     | 0.805   |
| Absent                               |                  | 27 (46.6%)             | 26 (42.6%)          |         |
| Present                              |                  | 31 (53.4%)             | 35 (57.4%)          |         |
| Histologic grade                     |                  |                        |                     | 0.845   |
| Well differentiated                  |                  | 17 (29.3%)             | 17 (27.9%)          |         |
| Moderately differentiated            |                  | 37 (63.8%)             | 38 (62.3%)          |         |
| Poorly differentiated                |                  | 4 (6.9%)               | 6 (9.8%)            |         |
| Degree of fibrosis                   |                  |                        |                     | 0.020*  |
| None                                 |                  | 4 (6.9%)               | 0 (0.0%)            |         |
| Mild                                 |                  | 11 (19.0%)             | 9 (14.8%)           |         |
| Moderate                             |                  | 36 (62.1%)             | 33 (54.1%)          |         |
| Severe                               |                  | 7 (12.1%)              | 19 (31.1%)          |         |
| Degree of inflammation               |                  |                        |                     | 0.415   |
| Mild                                 |                  | 21 (36.2%)             | 28 (45.9%)          |         |
| Moderate                             |                  | 30 (51.7%)             | 29 (47.5%)          |         |
| Severe                               |                  | 7 (12.1%)              | 4 (6.6%)            |         |
| Histologic subtype                   |                  |                        |                     | 0.932   |
| Pancreaticobiliary subtype           |                  | 8 (14.0%)              | 21 (34.4%)          |         |
| Prone to pancreaticobiliary subtype  |                  | 29 (50.9%)             | 28 (45.9%)          |         |
| Prone to intestinal subtype          |                  | 10 (17.5%)             | 8 (13.1%)           |         |
| Intestinal subtype                   |                  | 10 (17.5%)             | 4 (6.6%)            |         |
| CK7                                  |                  |                        |                     |         |
| Negative                             |                  | 8 (13.8%)              | 2 (3.3%)            | 0.145   |
| 1+                                   |                  | 5 (8.6%)               | 5 (8.2%)            |         |
| 2+                                   |                  | 10 (17.2%)             | 17 (27.9%)          |         |
| 3+                                   |                  | 35 (60.3%)             | 37 (60.7%)          |         |
| CK20                                 |                  |                        |                     |         |
| Negative                             |                  | 28 (48.3%)             | 42 (68.9%)          | 0.110   |
| 1+                                   |                  | 15 (25.9%)             | 7 (11.5%)           |         |
| 2+                                   |                  | 8 (13.8%)              | 7 (11.5%)           |         |
| 3+                                   |                  | 7 (12.1%)              | 5 (8.2%)            |         |
(PDGFRs), which implies the potential role of FGFR1 in the carcinogenesis of many human cancers [9, 13]. Many reports including data from the cancer genome atlas study have provided evidence regarding the involvement of FGFRs in the carcinogenesis of several cancers such as primary lobular breast carcinomas (20%) [14], lung cancer (22%) [15], and pancreatic cancer (5%) [16]. However, the prognostic impact of FGFR1 expression in cancer shows conflicting results depending on cancer types. Higher expression of the FGFR1 gene predicts poor overall survival and shorter disease-free survival in esophageal squamous cell carcinoma [17] and similar results were observed in non-small-cell lung cancer, particularly squamous cell carcinoma [18].

On the other hand, a recent study performed in the Korean patients showed that FGFR1 positive pancreatic cancer had better overall survival as compared to FGFR1 negative pancreatic cancer [19], which is consistent with the findings of our study. Although the precise reason for this discrepancy in different cancer types is unclear, it could probably be due to the different pathogenic roles of FGFR1 in various cancers. High expression of FGFR1 might cause a severe desmoplastic reaction (increased fibrosis) and could be protective or antitumorigenic in pancreatic cancers.

On the other hand, in this study, we found that the degree of peritumoral fibrosis was related to tumor recurrence. Although this finding seems conflicting with the first finding, high FGFR1 expression is correlated with the degree of fibrosis but is not strictly linked together. In pancreatic cancer, pancreatic stellate cells (PSCs) in the stroma are considered as the sprouting seeds of cancer progression and metastasis [20]. These are essential components of the tumor-stromal organization and are usually present as quiescent or inactive cells in normal pancreatic tissue. These cells are believed to play a key role in extracellular matrix production and regulate or promote EMT [2, 20]. It is possible that after surgery, tumors with a higher degree of fibrosis might have increased remnant fibrotic tissue containing activated PSCs, and this could be the foci of recurrence. In a recent study in colorectal cancers, fibrosis in metastatic lymph nodes was strongly associated with poor overall survival and relapse-free survival [21], suggesting that the fibrosis in the metastatic lymph nodes can be a potential foci of tumor recurrence. In our study, recurrence was also related to lymph node metastasis.

EMT is very important for the progression and metastasis of many cancers. It is particularly crucial in pancreatic cancer because histologically, pancreatic cancer is characterized by increased mesenchymal as well as epithelial features compared to other adenocarcinomas in other organs. In EMT, the epithelial cells lose polarity and cell to cell contact, have decreased E-cadherin

| Table 4 Clinicopathological parameters related to tumor recurrence (Continued) |
|----------------------------------|----------------|----------------|----------------|----------------|----------------|
| Clinicopathologic parameters     | No. (%) | Non-recurrence (N = 58) | Recurrence (N = 61) | P value |
| CDX                              |         |                   |                   |         |
| Negative                         | 13 (22.4%) | 21 (34.4%) | 0.086 |
| 1+                               | 18 (31.0%) | 23 (37.7%) |
| 2+                               | 10 (17.2%) | 10 (16.4%) |
| 3+                               | 17 (29.3%) | 7 (11.5%) |
| MUC                              |         |                   |                   |         |
| Negative                         | 51 (87.9%) | 56 (91.8%) | 0.692 |
| Positive                         | 7 (12.1%) | 5 (8.2%) |
| SOX2 (nuclear) Negative          | 5 (8.6%) | 6 (9.8%) | 0.497 |
| 1+                               | 31 (53.4%) | 31 (50.8%) |
| 2+                               | 20 (34.5%) | 24 (39.3%) |
| 3+                               | 2 (3.4%) | 0 (0.0%) |
| SOX2 (cytoplasmic) Negative      | 30 (51.7%) | 32 (52.5%) | 0.107 |
| 1+                               | 24 (41.4%) | 29 (47.5%) |
| 2+                               | 4 (6.9%) | 0 (0.0%) |
| CD24                             |         |                   |                   |         |
| Negative                         | 4 (6.9%) | 2 (3.3%) | 0.728 |
| 1+                               | 37 (63.8%) | 37 (60.7%) |
| 2+                               | 15 (25.9%) | 20 (32.8%) |
| 3+                               | 2 (3.4%) | 2 (3.3%) |
| OCT4                             |         |                   |                   |         |
| 1+                               | 12 (20.7%) | 14 (23.0%) | 0.943 |
| 2+                               | 34 (58.6%) | 34 (55.7%) |
| 3+                               | 12 (20.7%) | 13 (21.3%) |
| IGF-1                             |         |                   |                   |         |
| Negative                         | 7 (12.1%) | 6 (9.8%) | 0.778 |
| 1+                               | 32 (55.2%) | 38 (62.3%) |
| 2+                               | 16 (27.6%) | 14 (23.0%) |
| 3+                               | 3 (5.2%) | 3 (4.9%) |
| FGFR                              |         |                   |                   |         |
| 1+                               | 3 (5.2%) | 4 (6.6%) | 0.697 |
| 2+                               | 22 (37.9%) | 27 (44.3%) |
| 3+                               | 33 (56.9%) | 30 (49.2%) |
| VEGF                              |         |                   |                   |         |
| 1+                               | 6 (10.3%) | 10 (17.2%) | 0.547 |
| 2+                               | 38 (65.5%) | 36 (62.1%) |
| 3+                               | 14 (24.1%) | 12 (20.7%) |
| CD44v6 Negative                   |         |                   |                   |         |
| 1+                               | 18 (31.0%) | 22 (36.1%) |
| 2+                               | 21 (36.2%) | 19 (31.1%) |
| 3+                               | 13 (22.4%) | 14 (23.0%) |
Fig. 1 Kaplan-Meier survival analysis on the relationship between overall survival and clinical parameters in periampullary/pancreatic cancers.

There was no significant difference according to (a) age and (b) sex, while there was significant relationship according to (c) location, (d) T stage, (e) gross type, (f) size, (g) N stage, and (h) M stage.
Fig. 2 Kaplan-Meier survival analysis on the relationship between overall survival and the pathological parameters in the periampullary/pancreatic cancers. There was significant difference according to a lymphatic invasion, b vascular invasion, c perineural invasion, e histological differentiation, f pancreatobiliary/intestinal type, g fibrosis, and h inflammation, whereas there was no significant difference according to d radial margin involvement.
expression and increased vimentin expression during the mesenchymal transition [8]. However, the loss of E-cadherin is usually exclusively found in metastatic lesions and the vimentin is well known for non-specific, frequent coexpression in pancreatic cancers. Therefore, the feasibility as a prognostic marker seems to be limited although a few studies have suggested these as potential prognostic markers [22].

On the other hand, the EMT marker VEGF shares similar structural morphology with FGFRs, and both are very important for angiogenesis [9]. It has been reported that the expression of VEGF and FGFR1 were correlated with microvessels density [13]. Generally, VEGF is associated with poor overall survival in cancer. Yousuke et al. demonstrated that high VEGF expression showed significantly worse survival compared to low VEGF expression in pancreatic adenocarcinoma [23]. Surprisingly, in our study, we found a positive relationship between VEGF expression and overall survival, which is opposed to that of previous studies. In addition, the combined panel expression of CK20, CDX2, FGFR1, VEGF, and IGF-1 and CK20, CDX2, FGFR1, and VEGF were also useful prognostic markers for better survival.

Fig. 3 Kaplan-Meier overall survival analysis on the relationship between overall survival and expression level of the IHC markers in periampullary/pancreatic cancers. There was significant difference according to a CK20, b CDX2, c FGFR1, d VEGF, e combined CK20, CDX2, FGFR1, VEGF, and IGF-1 (combined score of 5 marker expression levels, 2−15), and f combined CK20, CDX2, FGFR1, and VEGF (combined score of 4 marker expression levels, 2−12)
In terms of stem cell markers, no stems cell markers including Oct4, SOX2, CD24, and CD44v6, showed significant relationship either with overall survival and disease-free survival. Higher expression of SOX2 and Oct4 has been reported to be associated with worse survival in some cancers, such as esophageal carcinoma [24]. As mentioned in the introduction, the aberrant expression of stem cell markers, such as Oct4, SOX2, CD24, CD44, PDX-1, SHH, and CD133, was suggested to be related to the prognosis in pancreatic cell lines, according to several studies [7, 25]. However, there are no studies so far conducted on the human hosts but only in the cell lines about the aforementioned markers. Our study investigated the clinical and prognostic significance of the stem cell marker expression in a large number of pancreatic cancer samples, and the result was not significant among all markers, which was opposed to the results of the studies using the cell lines. One thing to notice, Quint et al. have reported that the expression of stem cell markers, Ptc and PDX-1, is related to survival in certain grades of pancreatic cancers using 51 cases [26].

In this study, we reevaluated the T stage according to the tumor epicenter and, 22 out of 126 cases (17.4%) have been revised. Although it is not as high as reported by Adsay et al. (39%) [2], the number of cases with the revised T stage was considerably high and therefore, cannot be neglected. Due to the anatomical complexity, determining the tumor epicenter correctly when the tumor is detected in the advanced stage is a complicated issue. In this study, although we evaluated the difference in T staging based on the 7th AJCC cancer staging system, the results would be similar in the 8th edition as well. The determination of tumor epicenter is the most important factor to differentiate AOV, pancreatic, and CBD cancers and the T stage of 8th edition AJCC system is also based on the different size standards according to the location [27]. In our suggestion, pancreaticobiliary/intestinal subtypes, according to the expression status of CK20 and CDX2, can be a good supportive parameter for better TNM staging. The combined panel markers including CK20, CDX2, FGFR1, VEGF, and IGF-1 or CK20, CDX2, FGFR1, and VEGF, also can be good prognostic markers.

Conclusions
In this study, we identified fibrosis and FGFR1 expression as new prognostic markers to predict tumor recurrence and overall survival, respectively. Moreover, there was no significant correlation between stem cell markers and overall survival. Further basic studies on the VEGF and FGFR1 expression and the relationship between the markers need to be investigated in relation to EMT of PACs.

Supplementary information
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Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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