Tumour stem cell markers CD133 and CD44 are useful prognostic factors after surgical resection of pancreatic neuroendocrine tumours

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Abstract. The aim of the present study was to investigate the expression profiles and prognostic values of CD133 and CD44 in a cohort of patients with pancreatic neuroendocrine tumours (PNETs). PNET data from patients who underwent radical resection at the Guangdong Provincial People's Hospital were retrospectively analysed. Immunohistochemistry was performed on PNET samples, and CD133 and CD44 expression was examined. Survival analysis was performed using the Kaplan-Meier method and the log-rank test. A total of 71 cases were included in the study. The mean age of the patients was 45.2 years, and the mean tumour size was 3.3 cm. CD44 expression was positively associated with poor tumour differentiation (P=0.007), high Ki-67 index (P=0.001), added mitotic count (P=0.003), high histological grade (P=0.001) and advanced stage (P=0.025). Similarly, CD133 expression was positively associated with high Ki-67 index (P=0.014) and added mitotic count (P=0.012). However, CD133 expression was not associated with tumour differentiation (P=0.118), histological grade (P=0.126) and stage (P=0.203). Survival analysis revealed that both CD44 and CD133 were prognostic factors for overall survival (OS) and/or disease-free survival (DFS), and that increased co-expression of CD44 and CD133 indicated poor OS and DFS rates in patients with PNET. In patients with no expression or low expression of either CD44 or CD133, a DFS rate of 100% was observed, indicating a low recurrence risk. The present findings suggested that high CD44 and CD133 expression was associated with a poor prognosis in patients with PNET. CD44 and CD133 may be used as prognostic indicators of OS and/or DFS in patients with PNETs.

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Abbreviations: PNETs, pancreatic neuroendocrine tumours; CSC, cancer stem cell; ALDH, aldehyde dehydrogenase; OS, overall survival; DFS, disease-free survival

Key words: PNETs, CD44, CD133, CSC, prognosis

Introduction

Pancreatic neuroendocrine tumours (PNETs) are rare neuroendocrine neoplasms that originate from diffuse neuroendocrine cells (1). The incidence of PNETs has been increasing rapidly in the last 50 years. The age-adjusted incidence rate increased 6.4-fold from 1973 (1.09/100 000) to 2012 (6.98/100 000), partly as a result of increased detection using endoscopic and imaging techniques (2,3). Surgery remains the mainstay of therapy for patients diagnosed with both functional and non-functional PNETs (4). Regarding biological behaviour, PNETs have traditionally been considered to be less aggressive than pancreatic adenocarcinomas; however, the pathological potential of PNETs is increasingly being recognized as highly variable (5). Outcomes after surgical resection vary widely, with recurrence rates ranging between 17 and 76% (6-8). The prominent heterogeneity of PNETs creates an urgent need for prognostic factors. Various studies have specifically investigated factors that are associated with PNET progression (1,4,8). However, the pathophysiology involved in the progression and prognosis of PNETs remains incompletely characterized.

CD44 belongs to the adhesion molecule family (9), which serves important roles in cell proliferation, apoptotic resistance, motility, metastasis and chemotherapy resistance (10-12). Studies have reported that CD44 overexpression is associated with metastasis and a poor prognosis in various types of cancer, including gastric cancer, breast cancer and hepatocellular carcinoma (13-20). Additionally, CD44 has been used as a specific marker of cancer stem cells (CSCs) in a number of human tumours (10,21,22). Furthermore, CD44 serves an

important role in invasion and metastasis in a variety of human cancer types, including pancreatic adenocarcinoma (23,24).

CD133, a member of the pentaspan transmembrane glycoprotein family, is another marker of CSCs (25). CD133 was first described as a hematopoietic stem cell marker and later reported as a marker of CSCs in solid tumours (26). Previous studies have focused on CD44 and CD133 co-expression; high CD133 and CD44 expression is associated with invasion, metastasis, recurrence and decreased survival time in colon cancer, gastric cancer, oesophageal cancer, medullary thyroid carcinoma and hepatoblastoma (14,19,27-32).

CSC subpopulations are critical in cancer progression and serve as a promising therapeutic target (33). Numerous investigations have sought to identify CSC populations based on their surface markers (33-35). CSCs are also present in NETs (35), where several CSC markers have been investigated, including aldehyde dehydrogenase (ALDH), CD73 and CD24 (35-37). NET cells with high ALDHA expression exhibit CSC-like properties (35). High CD73 expression in PNET tissues is strongly associated with invasion into adjacent organs (37). CD24 expression is frequently noted in primary and metastatic midgut NETs, but is rare in PNETs (36). However, to the best of our knowledge, studies on CD44 and CD133 expression in PNETs and their prognostic value have not been performed. Therefore, the present study aimed to analyse CD44 and CD133 expression in a cohort of patients with PNETs, as well as the association between protein expression and clinicopathological characteristics, while further investigating the prognostic values of CD44 and/or CD133 in this group.

Materials and methods

Patients and samples. Patients who underwent radical surgery for a PNET between January 2,000 and December 2016 at the Department of General Surgery, Guangdong Provincial People's Hospital (Guangzhou, China) were included. Formalin-fixed paraffin-embedded primary specimens were obtained from all patients, with protocols approved by the Medical Ethics Review Committee of Guangdong General Hospital, and written informed consent was provided by all patients. The entire study was performed in accordance with the Declaration of Helsinki.

The histological types and grades of all samples were determined by experienced pathologists. The clinical stage of patients with PNETs was evaluated based on the TNM classification system (American Joint Committee on Cancer, TNM Staging System for Pancreatic Neuroendocrine Tumours, 7th edition, 2010) (38). Histological grades of the tumours were assessed according to the World Health Organization (WHO) 2010 classification (39). Routine pathology staining was used for Ki-67 and to calculate percentage as Ki-67 index, the detail is the same as percentage of CD44/CD133 in the immunohistochemistry method. Mitotic count and Ki-67 index were assessed independently by two pathologists who evaluated ≥10 high-power fields for each section. The results of Ki-67 index and mitotic count were further verified by a senior chief pathologist.

The inclusion criteria for patients were as follows: i) Initial treatment, including radical resection; ii) pathological confirmation of PNET by postoperative histopathological diagnosis; iii) no adjuvant therapy prior to surgery; iv) tumour lacking involvement of the celiac axis or the superior mesenteric artery, or without exhibiting distal metastasis; and v) no history of other malignancies. In total, 5 patients were excluded based on these criteria. Additionally, a single patient succumbed to a massive abdominal haemorrhage during the perioperative period and was excluded. Finally, a total of 71 eligible patients were identified.

Information regarding clinicopathological characteristics was collected for each patient. Follow-up information on prognosis was collected through clinic visits in outpatient departments, telephone calls and questionnaires. Disease-free survival (DFS) was calculated from the date of diagnosis to local recurrence or distal metastasis. Overall survival (OS) was measured from the date of diagnosis to death due to any cause, in addition to perioperative death caused by surgical complications.

Immunohistochemistry. Slides (4-μm thick, two serial sections for each sample) of formalin-fixed (37-40% for 24 h) at room temperature, paraffin-embedded specimens with the highest tumour content were used for immunohistochemical staining. Briefly, immunochemistry for CD44 (rabbit monoclonal antibody; 1:100; cat. no. ab51037; Abcam) and CD133 (rabbit polyclonal antibody; 1:200; cat. no. orb99113; Biorbyt, Ltd.) and Ki-67 (rat polyclonal antibody; 1:2,000; MIB-1; Gene Tech Co., Ltd.) was performed using commercially available antibodies. Sections were heated at 60˚C for 1 h and de-paraffinized in xylene and rehydrated in a graded ethanol series. Subsequently, antigen retrieval was performed using a microwave at 110˚C for 3 min. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide. Non-specific binding was blocked using 3% bovine serum albumin (cat. no. G5001; Servicebio, http://www.servicebio.cn/search-result?search=G5001) in PBS at room temperature for 30 min. The aforementioned primary antibodies were added overnight at 4˚C. After sufficient PBS washes at room temperature for 5 min (three times), sections were stained at room temperature for 1 h with horseradish peroxidase-labelled goat anti-rabbit antibodies (1:200; cat. no. K5007; Dako; Agilent Technologies, Inc.). The sections were subsequently stained with 3,3'-diaminobenzidine. Slides were observed under a light microscope (XSP-C204; CIC, magnification, x100).

CD44 and CD133 immunostaining were blindly scored by two independent pathologists using a semi-quantitative method that included staining intensity (scored from 0 to 3) and the percentage of positively stained tumour cells (scored from 0 to 100). Briefly, staining intensities were scored as follows: 0, no staining; 1, weak staining; 2, moderate staining; or 3, intense and strong staining. The percentage of positively stained tumour cells was determined by counting the number of positive staining cells and the number of all tumour cells in ≥10 random-selected high-power fields (HPFs), and calculated by the formula: Percentage (range, 0-100)=Number of stained cells/Total number of cells x100. A total score was calculated for each sample using the following formula: Total score (range, 0-300)=Staining intensity scores (range, 0-3)xPercentage of positively stained cells (range, 0-100).

Statistical analysis. Statistical analysis was performed using SPSS software v24.0 (IBM Corp.). The presentation of data

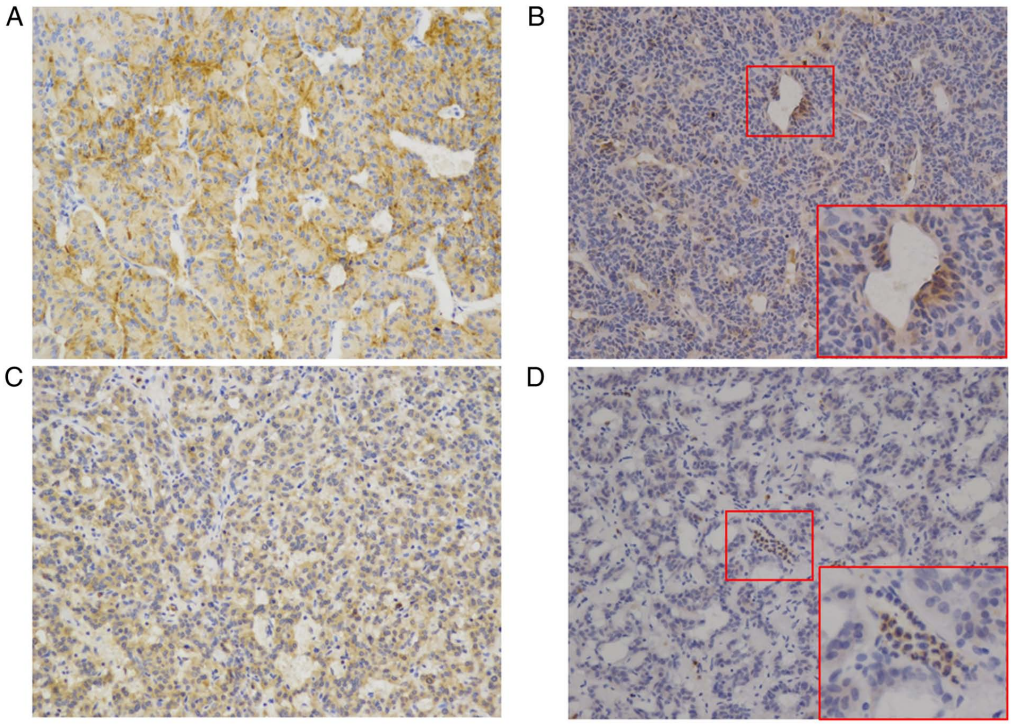

Figure 1. CD44 and CD133 expression in PNETs. (A) CD44 is primarily expressed in the cytoplasm and cytomembrane. (B) CD44 is expressed in a scattered pattern. The red box depicts a magnified image of a local stain. (C) Image depicting the diffuse staining of CD133. (D) Image depicting scattered CD133-positive PNET cells. The red box depicts a magnified image of a local stain. Magnification, x100. PNET, pancreatic neuroendocrine tumour.

adopt mean ± SD. Frequency distributions and categorical variables were compared using the χ^2 test or ordinal regression, and continuous variables were compared using one-way ANOVA, differences among groups were compared using one-way ANOVA followed by LSD post hoc test. The Kaplan-Meier survival method with the log-rank test was used to assess survival time. P<0.05 (two-tailed) was considered to indicate a statistically significant difference.

Results

Patient characteristics. The present study included a total of 71 patients, of whom 42 were men (59.2%). The mean age was 45.2 years (range, 10-78 years). A total of 31 (43.7%) patients had functional PNETs, while 40 (56.3%) patients had non-functional PNETs. All patients underwent an intended curative resection. A total of 40 (56.3%) of these patients underwent a pancreaticoduodenectomy or distal pancreatectomy, 7 patients (9.9%) had a segmental pancreatectomy, 16 patients (22.5%) had an enucleation, 7 patients (9.9%) had a local resection and 1 patient (1.4%) had a duodenum-preserving resection of the pancreatic head,. Only 1 patient exhibited an R1 surgical margin, where the tumour was adjacent to the adrenal gland. A total of 31 (43.6%) patients were categorized as G1 grade and 30 (42.3%) patients were categorized as G2 grade and 10 (14.1%) patients were categorized as G3 grade, according to the 2010 WHO classification of tumours of the digestive system. A total of 15 patients (21.1%) experienced recurrence, with a median time to recurrence of 2.5 years (range, 0.5-8.0 years; data not shown).

CD44 and CD133 expression in PNETs. Both CD44 and CD133 expression were observed in PNET tissues. CD44 and CD133 were primarily detected in the cytoplasm and cytomembrane of cells. CD44 exhibited two staining patterns: Diffuse staining and scattered staining (Fig. 1A and B). The same staining patterns were also noted for CD133 (Fig. 1C and D).

The expression levels of CD44 and CD133 were evaluated in serial sections. The obtained staining scores ranged from 0 (no staining) to 264 for CD44 staining and from 0 to 243 for CD133 staining. For further analysis, CD44/133 expression was divided into 4 levels: Level 0, no staining; level 1, score 1-100; level 2, score 101-200; and level 3, score 201-300. Representative images of staining levels are presented in Fig. 2. The number of cases in each level is presented in Table I. Overall, CD44 staining was stronger than CD133 staining (Table I; Fig. 3), and a significant association was observed between CD44 and CD133 expression (P<0.001; Table I).

CD44/CD133 expression and clinicopathological parameters in PNETs. Patients were stratified according to the total score of IHC staining, and the association between CD44/CD133 expression, and clinical characteristics of the enrolled patients were compared. The associations between CD44 or CD133 expression and the clinicopathological characteristics of patients with PNET are presented in Tables II and III, respectively. Increased CD44 expression was associated with poor tumour differentiation (P=0.007), high Ki-67 index (P=0.001), added mitotic count (P=0.003), high histological grade (P=0.001) and advanced stage (P=0.025) (Table II). Increased CD133 expression was also associated with high Ki-67 index (P=0.014), age (P=0.028) and added mitotic count (P=0.012), but not with tumour differentiation (P=0.118), tumour histologic grade (P=0.126) and stage (P=0.203) (Table III). No significant associations were observed between CD44/133

Table I. Association between CD44 and CD133 expression levels.

	CD44 levels				
CD133 levels	0 (n=10)	1 (n=19)	2 (n=32)	3 (n=10)	P-value[a]
0 (n=16)	6	8	2	0	<0.001
1 (n=27)	4	8	12	3	
2 (n=21)	0	3	15	3	
3 (n=7)	0	0	3	4	

[a]P-value was calculated using ordinal regression.

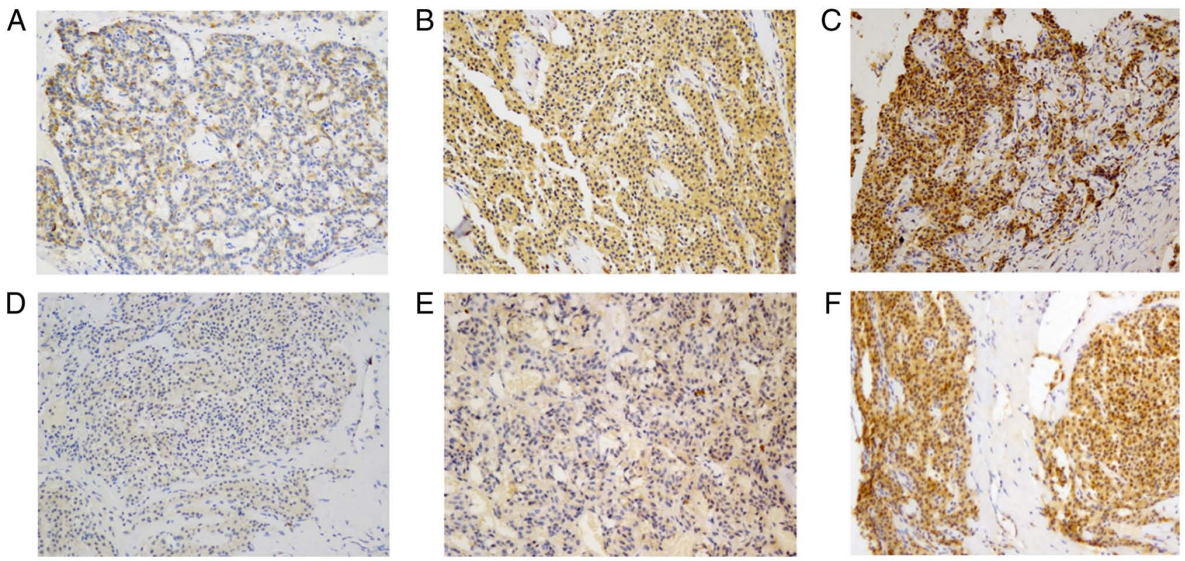

Figure 2. Representative images of immunohistochemical staining of CD44/CD133 in pancreatic neuroendocrine tumour tissues. CD44 staining levels (A) 1, (B) 2 and (C) 3. CD133 staining levels (D) 1, (E) 2 and (F) 3. Magnification, x100.

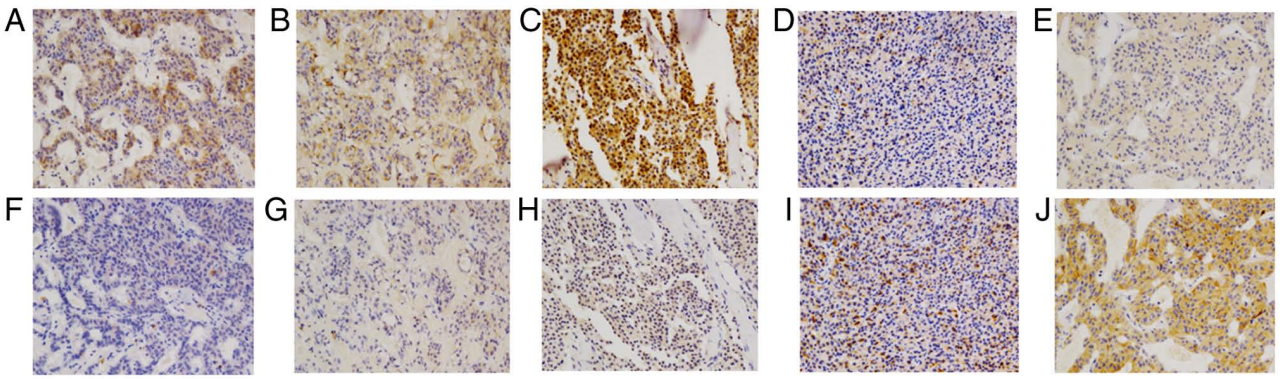

Figure 3. Comparison of CD44 and CD133 expression in serial sections. (A-E) Images of CD44 staining in PNET tissues. (F-J) Images of CD133 staining in PNET tissues in corresponding serial sections. CD44 staining in most samples was stronger than CD133 staining (A-C vs. F-H). Some samples displayed stronger CD133 expression compared with CD44 expression (D and E vs. I and J). Magnification, x100. PNET, pancreatic neuroendocrine tumour.

expression and other clinical parameters, such as sex, tumour location, tumour size, TNM stage and functionality (Tables II and III).

Survival analysis. The median follow-up time for this cohort was 57 months (range, 12-182 months). The single patient with an R1 surgical margin was excluded from the survival analysis to maintain sample homogeneity. Kaplan-Meier survival curves for DFS and OS stratified by Ki-67 index or histological grade are presented in Fig. 4. Consistent with the aforementioned immunohistochemistry observations, increased Ki-67 proliferative index and high histological

Table II. Association between CD44 expression and clinicopathological characteristics in patients with pancreatic neuroendocrine tumours (n=71).

Variables	Values	CD44 expression levels				P-value[a]
		0 (n=10)	1 (n=19)	2 (n=32)	3 (n=10)	
Mean age ± SD, years	45.2±17.5	40.3±15.1	47.1±21.6	45.0±16.4	47.2±16.2	0.856
Sex, n						0.738
Female	29	5	8	11	5	
Male	42	5	11	21	5	
Mean tumour size ± SD, cm	3.3±2.1	2.6±1.6	3.4±1.9	3.1±2.4	4.1±2.0	0.408
Function, n						0.395
Functional	31	5	8	16	2	
Non-functional	40	5	11	16	8	
Location, n						0.369
Head/uncinate	34	7	9	12	6	
Body and/or tail	37	3	10	20	4	
Margin status, n						0.601
R0	70	10	19	31	10	
R1	1	0	0	1	0	
Differentiation, n						0.007
Well/moderate	64	10	18	30	6	
Poor	7	0	1	2	4	
Ki-67 index, n						0.001
≤2%	39	9	13	14	3	
3-20%	26	1	6	16	3	
>20%	6	0	0	2	4	
Mitotic count[b], n						0.003
<2	38	10	14	13	1	
2-20	25	0	4	15	6	
>20	8	0	1	4	3	
Histological grade[c], n						0.001
G1	31	9	11	10	1	
G2	30	1	7	18	4	
G3	10	0	1	4	5	
TNM stage[d], n						0.025
I	49	7	13	26	3	
II	22	3	6	6	7	

[a]P-values were calculated using one-way ANOVA for continuous variables of >2 groups and χ^2 test for categorical variables. [b]Per 10 high-power fields. [c]Histological grade was classified according to the World Health Organization 2010 classification system (42). [d]TNM stage was classified according to the 7th 2010 American Joint Committee on Cancer TNM classification (43).

grade were associated with a poor prognosis in patients with PNET. Additionally, Kaplan-Meier survival curves revealed that patients with PNET with low or no CD44 expression had significantly improved OS and DFS rates (Fig. 5A and B). Increased CD133 expression was associated with a poor OS rate (Fig. 5C); however, this association was not significant (P=0.0741). However, CD133 expression was a significant prognostic factor for DFS (P=0.0008; Fig. 5D).

To further evaluate the combined effect of CD44 and CD133 co-expression on the prognosis in patients with PNET, the CD44 expression levels were combined with the CD133 expression levels for each sample, obtaining combined scores ranging from 0 to 6. Kaplan-Meier analysis revealed that patients with high combined scores exhibited significantly decreased OS and DFS rates (Fig. 5E and F). Among the patients with a combined score ≤1, none of the patients developed recurrence during the follow-up period. Two G1 grade patients with a Ki-67 index ≤1% experienced recurrence during the follow-up period, suggesting that a total combined score ≤1 (indicating no CD44 and CD133 expression, or that one of them is not expressed and the other is expressed at a low level) may be a more effective predictor of a favourable prognosis in patients with PNETs than low histological grade or low Ki-67 index.

Discussion

Surgical resection remains the primary curative modality in the management of PNETs (4). However, heterogeneous

Table III. Association between CD133 expression and clinicopathological characteristics in patients with pancreatic neuroendocrine tumours (n=71).

Variables	Patients	CD133 expression levels				P-value[a]
		0 (n=16)	1 (n=27)	2 (n=21)	3 (n=7)	
Mean age ± SD, years	45.2±17.5	43.1±19.3	40.6±17.2	48.4±17.1	58.0±11.9	0.028
Sex, n						0.674
Female	29	8	13	5	3	
Male	42	8	14	16	4	
Mean tumour size ± SD, cm	3.3±2.1	3.0±1.7	2.6+±1.7	4.0±2.5	4.3±2.4	0.051
Function, n						0.061
Functional	31	6	15	10	0	
Non-functional	40	10	12	11	7	
Location, n						0.247
Head/uncinate	34	11	10	10	3	
Body and/or tail	37	5	17	11	4	
Margin status, n						0.491
R0	70	16	27	20	7	
R1	1	0	0	1	0	
Differentiation, n						0.118
Well/moderate	64	16	23	20	5	
Poor	7	0	4	1	2	
Ki-67 index,						0.014
≤2%	39	11	19	9	0	
3-20%	26	5	7	9	5	
>20%	6	0	1	3	2	
Mitotic count[b], n						0.012
<2	38	13	16	9	0	
2-20	25	3	7	9	6	
>20	8	0	4	3	1	
Histological grade[c], n						0.126
G1	31	10	13	8	0	
G2	30	6	10	9	5	
G3	10	0	4	4	2	
TNM stage[d], n						0.203
I	49	10	22	14	3	
II	22	6	5	7	4	

[a]P-values were calculated using one-way ANOVA for continuous variables of >2 groups and χ^2 test for categorical variables. [b]Per 10 high-power fields. [c]Histological grade was classified according to the World Health Organization 2010 classification system (42). [d]TNM stage was classified according to the 7th 2010 American Joint Committee on Cancer TNM classification (43).

behaviour and unpredictable pathology are a challenge to optimal treatment decision-making. The use of CD44 and CD133 as markers for CSCs, which may promote tumourigenesis and regeneration, has been actively investigated in various types of solid tumour, such as gastric cancer, breast cancer and colon cancer (14,40,41). Additionally, the presence of CSCs has been confirmed in NETs (35). However, no evidence is available on the expression levels of the CSC markers CD44 and CD133 in PNETs and their effect on the prognosis in patients with PNET.

In the present study, data from 71 patients with PNET were obtained to examine the significance of CD44 and CD133 as prognostic markers for survival. Immunohistochemical analysis revealed that both CD44 and CD133 were expressed in most PNET tissues and revealed a tendency toward co-expression. Overall, CD44 exhibited a higher positive rate and stronger staining intensity compared with CD133. Survival analysis demonstrated that CD133 and/or CD44 upregulation may predict an unfavourable prognosis in patients with PNETs.

CSC populations are primarily responsible for tumour initiation, growth and metastasis (42). To date, studies on CSCs in NETs have been rare. Gaur *et al* (35) identified and characterized neuroendocrine CSCs from a midgut carcinoid cell line (CNDT2.5) using ALDH as a surface marker, revealing that CSCs are present in NETs. However, tumour biological characteristics and stem cell markers may differ between

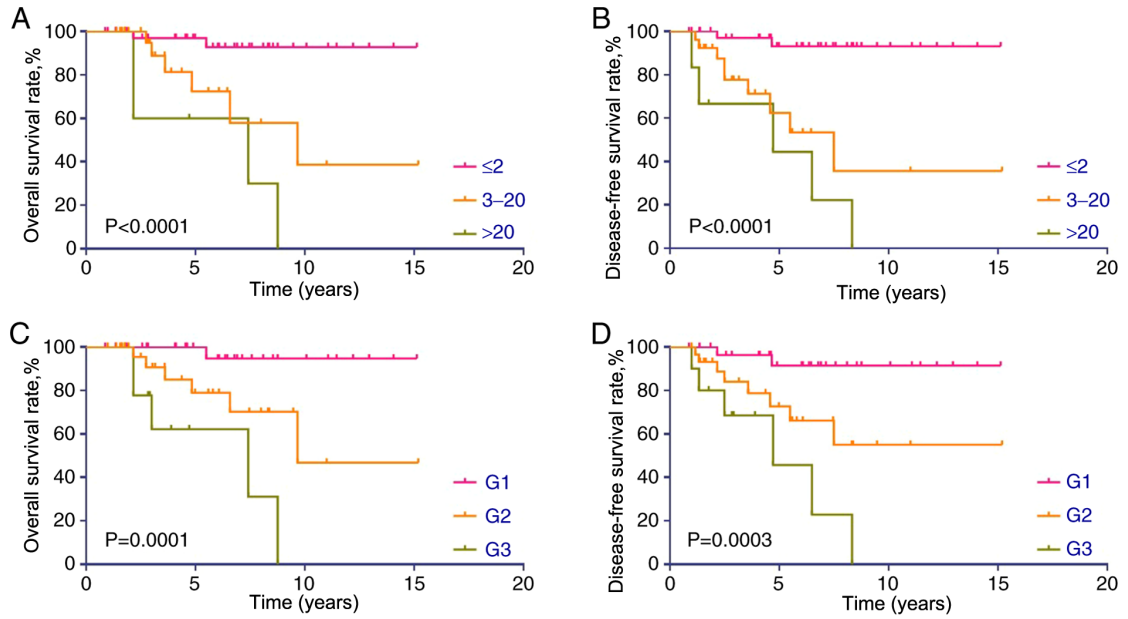

Figure 4. Kaplan-Meier survival curves for OS and DFS stratified by Ki-67 index and histological grade. Increased Ki-67 index was associated with decreased (A) OS and (B) DFS. High histological grade was associated with decreased (C) OS and (D) DFS. P-values were calculated using the log-rank test. OS, overall survival; DFS, disease-free survival.

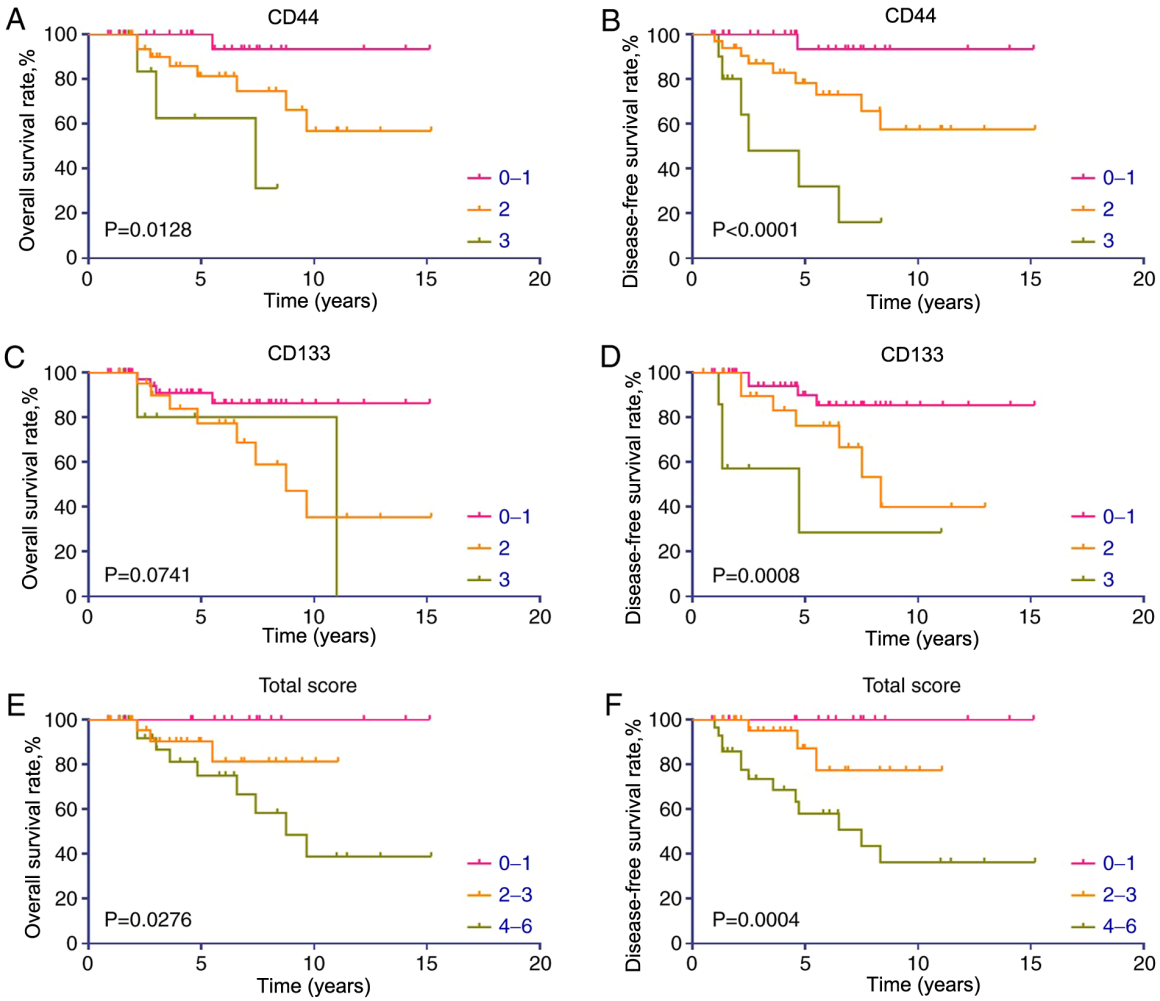

Figure 5. Kaplan-Meier survival curves for OS and DFS stratified by CD44/CD133 expression. Patients with high CD44 expression exhibited significantly decreased (A) OS and (B) DFS. (C) Comparison of OS in patients with different CD133 expression levels. Patients with high CD133 levels tended to have decreased OS, but the difference was not significant. (D) Patients with high CD133 expression exhibited significantly decreased DFS. (E) OS and (F) DFS curves in patients with pancreatic neuroendocrine tumours with different combined immunohistochemical scores according to CD44 and CD133 expression levels.

midgut NETs and PNETs. For example, CD24 is a CSC marker, and its expression is frequently noted in primary and metastatic midgut NETs, but is rarely observed in pancreatic and duodenal NETs (36). In midgut NET cells, Gaur *et al* (35) observed that nearly all CNDT2.5 cells bind to CD44, whereas cells were not labelled with CD133. In the present immunohistochemical assessment of PNET tissues, CD44 and CD133 were co-expressed in PNETs. The significant associations between CD44 and/or CD133 and the prognosis in patients with PNETs suggest that these proteins are important tumour promoters and potential CSC markers in PNETs.

Surgical resection remains the curative treatment for patients with PNET. Most studies on prognostic factors for outcomes after resection of PNET include patients with distal metastases at resection, stage III patients with tumours involving the celiac axis or the superior mesenteric artery, R1 resections or patients with familial syndromes (43-46). In the present study, a selective group of patients with stage I-II PNETs with R0 resection was included, and risk factors for prognosis were analysed. In the present cohort, recurrences were noted in patients with Ki-67 index ≤1 and in G1 patients. However, patients with a CD44/CD133 total combined score ≤1 exhibited no risk of recurrence. By contrast, patients with an increased total score exhibited a significantly increased risk of recurrence. Therefore, the type of follow-up visit may be selected based on different recurrence risks to be more cost-effective. To the best of our knowledge, there is no evidence that evaluates the effects of adjuvant therapy after radical surgical resection of PNETs. In current clinical practice, the decision regarding adjuvant therapy after surgery remains at the discretion of the attending physician. The present findings may help to avoid unnecessary adjuvant treatments in patients with a very low risk of recurrence and to optimize patient selection to investigate the role of adjuvant therapy based on recurrence risk in further clinical research.

There are several limitations to the present study. First, due to the low incidence of PNETs, the sample size of a PNET cohort in a single centre is typically small, as in the current study. Given that a large cohort is required to meet the requirements of Cox regression analysis, it was not possible to perform this analysis. Second, the aforementioned conclusions are limited by the nature of single-centre data; an external validation cohort is required to investigate whether the prognostic value of CD44/CD133 is significant in other populations. Third, there was an extended inclusion period in the present study. During this period, follow-up strategies, surgical resection techniques and systemic treatments have changed, which may have altered patient outcomes. However, these limitations are difficult to overcome given the low incidence and long course of PNETs. Further prospective and multi-centre trials are warranted to discover prognostic factors for PNETs and to reveal the underlying mechanisms involved in the development of this type of tumour.

In conclusion, the present study revealed that CD44/CD133 expression may be a useful biomarker to predict prognosis after surgical resection of PNETs, and it may have a pivotal role in the progression of PNETs. The current study was limited by the nature of a retrospective design, and further prospective studies and laboratory research are required to confirm the present results and provide additional evidence for the role of CD44/CD133 in PNETs.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

BH conceived the present study. ZS and DL drafted the initial manuscript. HW performed the immunohistochemical examination of the sections. ZS was a major contributor to drafting the final version of the manuscript. ZS, DL and BH analysed and interpreted the patient data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Formalin-fixed, paraffin-embedded primary specimens were obtained from all patients with protocols approved by the Medical Ethics Review Committee of Guangdong Provincial People's Hospital (Guangzhou, China; approval no. KY2020-169-01), and written informed consent was provided by all patients. All the work was performed in accordance with the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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