# CD133 Expression Pattern Distinguishes Intraductal Papillary Mucinous Neoplasms From Ductal Adenocarcinomas of the Pancreas

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(Citation)
Pancreas,38(8):e207-e214

(Issue Date)
2009-09

(Resource Type)
journal article

(Version)
Accepted Manuscript

# CD133 expression pattern distinguishes intraductal papillary mucinous neoplasms from ductal adenocarcinomas of the pancreas

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Running title: CD133 in IPMNs and pancreatic ductal adenocarcinomas

This study was supported by grants for the Hyogo prefecture Medical Association (to K.S.), by grants-in aid for Scientific Research from The Ministry of Education, Culture, Sports, Science and Technology of Japan (to H.Y. and Y.K.) and by grants for Global Century of Excellence Program for Education and Research on Signal Transduction

Medicine in the Coming Generation “Bring up clinician-scientists in the alliance between basic and clinical medicine” (to Y.K. and T.I.).

**Objectives:** The rate of intraductal papillary mucinous neoplasms of the pancreas (IPMNs) progression is much slower than that of invasive ductal adenocarcinomas. The identification of a clinicopathological marker to distinguish IPMNs from ductal adenocarcinomas is important for understanding the molecular mechanisms of pancreatic cancer.

**Methods:** We examined the expression pattern of the cell surface marker CD133, which has been used to identify putative cancer stem cells from solid tumors, in adult pancreatic ductal adenocarcinomas (n=10) and IPMNs (n=34).

**Results:** CD133 expression was detected in the centroacinar region and intra-lobular ductal cells of normal pancreas. CD133 expression was also observed in ductal adenocarcinomas. In contrast, CD133 expression was not observed in the mucin-producing epithelial cells and carcinoma cells on IPMNs.

**Conclusions:** These results demonstrate that the expression of CD133 is downregulated in IPMNs, suggesting that loss of CD133 expression might be a useful clinicopathological marker distinguishing IPMNs from ductal adenocarcinomas.

**Key words:** intraductal papillary mucinous neoplasm, CD133, cancer stem cell,

pancreas, stem cell

## INTRODUCTION

Pancreatic adenocarcinomas are currently the fourth leading cause of cancer-related mortality. Almost all patients are expected to die from the disease due to the propensity of early metastasis, and because the disease is highly resistant to radiation and chemotherapy. Despite our increasing knowledge in tumor biology, the treatment efficacy of pancreatic adenocarcinomas has not significantly improved over the past decade. Histological evaluation of resected pancreatic adenocarcinomas has facilitated the morphologic classification of dysplastic lesions that represent the putative precursors of invasive carcinoma-pancreatic intraepithelial neoplasias (PanINs) and intraductal papillary mucinous neoplasms (IPMNs). PanINs encompass a spectrum of pancreatic ductal epithelial changes characterized by enhanced mucin production, graded nuclear atypia, and loss of cell polarity, giving rise to ductal adenocarcinomas that account for about ninety percent of pancreatic tumors.[1,2] IPMNs constitute another class of noninvasive precursor lesions that are histologically distinct from, but related to, PanINs.[3] IPMNs are defined as a papillary growth of mucin-producing epithelium of pancreatic duct and produce radiographically identifiable ductal dilatation, which may

predominantly involve the main pancreatic ducts ("main ductal type"), the secondary ducts ("branch ductal type"), or both ("mixed type").[3,4] The frequency of malignancy in the main ductal type is much higher than that in the branch ductal type.[5] Most IPMNs are slow growing and less aggressive compared with conventional, pancreatic ductal adenocarcinomas. Therefore, an important goal in understanding pancreatic cancer progression is to clarify the different molecular mechanisms underlying IPMNs and ductal adenocarcinomas.

Molecular genetic analysis has implicated several genes in the progression of pancreatic cancer including: *K-ras*, INK4A/ARF, and *p53* as well as elements of the EGF, TGFβ, Notch, Hedgehog, and PI-3 kinase signaling pathways.[6-9] Several of these molecules are reported to have different expression levels between in IPMNs and PanINs/ductal adenocarcinomas. For example, SMAD4 levels are reported to decrease in more than 40% of ductal adenocarcinomas, but not IPMNs.[10-12] About 85% of PanIN-3s are reported to express MUC1, but IPMNs rarely do.[13] On the other hand, *K-ras*, *p53* mutations, and Sonic hedgehog expression are observed in both PanINs and IPMNs.[14-16]. However, it remains unclear how these molecules play roles in the

progression of the pancreatic cancer.

Recently we demonstrated that CD133 is a marker of putative pancreatic progenitor cells during embryogenesis.[17] CD133 is expressed on the apical membrane of ductal cells in the normal adult pancreas and in carcinoma cells on pancreatic ductal adenocarcinomas.[18] The subpopulation of the pancreatic ductal adenocarcinoma cells expressing both CD133 and CXCR4 is involved in tumor metastasis.[19] However, the precise expression of CD133 in IPMNs has not yet been elucidated.

In this study, we examined the expression pattern of CD133 in ductal adenocarcinomas and IPMNs. We confirmed that CD133 was expressed in centroacinar region and intra-lobular ductal cells in adult pancreas. During carcinogenesis, CD133 was expressed in carcinoma cells on ductal adenocarcinomas. In contrast, CD133 expression was not detected in mucin-producing epithelial cells or carcinoma cells on IPMNs. These results suggest that downregulation of CD133 expression may be a useful clinicopathological marker distinguishing IPMNs from ductal adenocarcinomas.

## MATERIALS AND METHODS

**Tissue Specimens.**

Tissue specimens from tumors, including 10 pancreatic ductal adenocarcinomas and 34 IPMNs resected in the period 2001-2008, were retrieved from the archives of the Department of Pathology, Kobe Medical Center and Kobe University Hospital. Pathologists reviewed the slides to ensure that the cases were consistent with pancreatic ductal adenocarcinomas and IPMNs according to the WHO classification.[20] Clinical records and radiological reports were reviewed by a surgeon (Y.H.). Tissue samples were fixed in 10% phosphate-buffered formalin for overnight and embedded in paraffin. The study was performed according to Institutional Review Board-approved guidelines.

**Immunohistochemistry.**

Immunohistochemical staining to detect CD133 expression was done on 3-4 μm sections from formalin-fixed, paraffin-embedded tissues, placed on coated glass slides, and dried at room temperature for overnight. Sections were dewaxed in xylene, rehydrated according to standard procedures. For antigen retrieval, the tissue sections were boiled for 40 min in 1x Target Retrieval Solution (Dako, Carpinteria, CA). The

samples were then cooled in 1x Target Retrieval Solution at room temperature for 20 min and rinsed with distilled water three times, followed by peroxidase block with 3% $H_2O_2$ in methanol for 5 min. After rinse with distilled water twice, the samples were immersed in TBST buffer (25 mM Tris-HCl; pH 7.4, 75 mM NaCl, 0.1% TritonX-100) for 5 min and then incubated with primary antibody (a mouse anti-human CD133/1 monoclonal antibody (mAb) (clone AC133) from Miltenyi Biotech (Bergisch Gladbach, Germany) diluted 1:50 in TBST buffer) for overnight at 4℃. The samples were then rinsed with TBST buffer three times. The primary antibody detection was performed, in accordance with the manufacturer's instructions (Dako ENVIION kit/HRP), with the mouse HRP polymer probe (ChemMate Detection Kit, peroxidase/3,3'-diaminobenzidine, mouse, Dako) for 30 min at room temperature, followed by rinse with TBST buffer twice. The signal was developed with diamino-benzidine DAB+ (Dako) for 10 min. The samples were rinsed with distilled water three times, counter-stained with hematoxylin for 1 min, dehydrated in alcohol solutions and xylene, and mounted in Entellan (Merck, Darmstadt, Germany). The cytokeratin 19 (CK-19) detection was performed using a mouse anti-human CK-19

mAb (1:100 dilution in Dako REAL™ Antibody Diluent) from Dako. The MUC1-core, MUC2, and MUC5AC detections were performed using a mouse anti-human MUC1 mAb (clone Ma552, Novocastra Laboratories Ltd., Newcastle upon Tyne, UK, 1:100 dilution in Dako REAL™ Antibody Diluent), a mouse anti-human MUC2 mAb (clone Ccp58, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, 1:100 dilution in Dako REAL™ Antibody Diluent), and a mouse anti-human MUC5AC mAb (clone CLH2, Chemicon International Inc., Temecula, CA, USA, 1:300 dilution in Dako REAL™ Antibody Diluent). Parallels were stained with H&E for identification of cancerous and normal tissues.

**Assessment of Immunohistochemical Staining and Statistical Analysis.**

The quality of CD133 staining was judged in the samples according to the data in the literature about CD133 expression in pancreas.[18] The slides containing pancreatic ductal adenocarcinomas and IPMNs were observed and the number of the cells expressing CD133 in the total cell number in 4 fields at 200x magnification was scored independently by two of the authors (K.S., Y.H.). Results for continuous variables were expressed as means ± standard deviation.

## RESULTS

### CD133 Expression in Adult Pancreas.

To explore the different expression pattern of CD133 between ductal adenocarcinomas and IPMNs, we first examined CD133 expression in the normal adult pancreas using the well-characterized anti-CD133 mAb.[18,19,21,22] CD133 expression was observed in cells that occupied the distinctive centroacinar position as well as in the terminal ductal cells (Figs. 1A, B). The CD133 expression continued in the intra-lobular ducts with staining intensity decreasing towards the larger inter-lobular and main pancreatic ducts, which were negative (Figs. 1C, D). CD133 expression was concentrated on the apical site of the terminal ductal epithelium (Figs. 1A, C). These results are consistent with the previous reports that centroacinar cells and terminal ductal cells express CD133 and that the CD133 expression in ductal cells follows a gradient with weaker intensity and disappears in larger ductal cells.[18]

### CD133 Expression in The Metaplastic Region and Ductal Adenocarcinomas.

We next examined CD133 expression in human ductal adenocarcinomas,

where CD133 was detected in the normal centroacinar and terminal ductal cells at the margin of the tumor (Figs. 2-1A, B). CD133 staining was concentrated in ductal metaplasia of the acinar cells located in the border zone of the tumor (Figs. 2-1C, D). CD133 expression was observed in ductal adenocarcinoma cells (Figs. 2-1E, F), as well as in the metastatic lesion of the lymph nodule (Figs. 2-1G, H). We have also observed cytoplasmic CD133 staining in a minor population of ductal adenocarcinomas cells (Fig. 2-2). CD133 was expressed in all 10 ductal adenocarcinomas examined, and the expression levels did not correlate with the differentiation grade of ductal adenocarcinomas (Table 1). These results indicate ductal adenocarcinoma cells express CD133.

**CD133 Expression in IPMNs.**

We then examined CD133 expression in human IPMNs. Based on imaging studies, including computed tomography and endoscopic retrograde cholangiopancreatography (ERCP), dilatation (diameter $>$ 1 cm) of the main duct with dilatation of the secondary ducts suggested a mixed type of IPMN (Figs. 3A, B). The patient (Table 2, the patient number 1) underwent partial pancreatectomy with second

portion duodenectomy (Fig. 3C). Macroscopic examination confirmed dilatation of the main duct and the secondary ducts (Figs. 3C, D). The IPMN also had carcinoma *in situ* (Fig. 4C). CD133 expression was not detected in either the columnar mucin-producing epithelial cells (Figs. 4A, B) or in the carcinoma *in situ* on the IPMN (Figs. 4C, D). CD133 expression was not detected in invasive carcinoma cells on an IPMN (Table 2, the patient number 10), either (Figs. 4E, F). We observed that CD133-expressing ductal cells connected directly to the columnar mucin-producing epithelial cells that did not express CD133 (Figs. 4H, I). In control staining of the IPMN, the columnar mucin-producing epithelial cells, ductal cells and the carcinoma cells all expressed CK-19 (Fig. 4G and data not shown).

IPMNs are subclassified into a gastric type, an intestinal type, a pancreatobiliary type, and an oncocytic type.[23] We subclassified 25 IPMN samples into 15 gastric type IPMNs, 8 intestinal type IPMNs, a pancreatobiliary type IPMN, and an oncocytic type IPMN, based on histological features and immunohistochemical reactivities with antibodies to specific types of mucin (MUCs) (Table 3). Five of the 15 gastric types and 4 of the 8 intestinal types contained carcinoma *in situ*. Moreover, we

observed 9 IPMNs, each of which contained invasive carcinoma cells, and tentatively classified them as invasive IPMNs in this study. We subclassified the 9 invasive IPMNs into 5 tubular carcinoma type IPMNs and 3 colloid carcinoma type IPMNs. There was an invasive IPMN containing both the two types. The lack of CD133 expression was observed in all the 34 IPMNs examined (Fig. 5 and Table 3). Taken together, these results indicate that IPMNs do not express CD133.

## DISCUSSION

In this study we found that CD133 was expressed in the centroacinar and terminal ductal cells in the normal pancreas as well as in ductal adenocarcinomas. In contrast, we found that CD133 was not expressed in IPMNs. These results suggest that the lack of CD133 expression can be used as a clinicopathological marker to distinguish IPMNs from ductal adenocarcinomas.

Our result that CD133 expression in the normal ductal cells follows a gradient with weaker intensity in larger ductal cells is consistent with the previous report.[18] In addition we found that mucin-producing epithelial cells in IPMNs that lacked CD133

were directly connected to CD133-expressing ductal cells. CK-19, an epithelial cell marker,[24] was observed in the columnar mucin-producing epithelial cells, and the carcinoma cells on IPMNs as well as in normal ductal cells. These results allow us to provide at least three possibilities: (1) larger ductal cells (main pancreatic and inter-lobular ductal cells) or some specific epithelium among pancreatic duct which do not express CD133 might trans-differentiate to mucin-producing hyperplasia in IPMNs, followed by an adenoma-dysplasia-carcinoma sequence; (2) smaller ductal cells (terminal and intra-lobular ductal cells) might down regulate CD133 expression resulting in a trans-differentiation to mucin-producing epithelium in IPMNs; and (3) IPMNs might be derived from extra-ductal tissue, including exocrine or endocrine cells, which do not express CD133, and acquire an epithelial character(s). Further investigations will be required to resolve these possibilities.

PanINs have pancreatic ductal epithelial changes characterized by enhanced mucin production, graded nuclear atypia, and loss of cell polarity, giving rise to ductal adenocarcinoma.[1-3] Surprisingly we could not observe CD133 staining in PanIN-1As and -1Bs, PanIN-2s, or PanIN-3s (data not shown). We assume that larger ductal cells

that do not express CD133 might trans-differentiate to mucin-producing hyperplasia in PanINs and that CD133 is not a marker distinguishing IPMNs from PanINs.

Metaplasia (the widespread inter-conversion of one cell type into another) has been associated with pancreatic cancer in both humans and animal models and a metaplasia-ductal adenocarcinoma sequence has been proposed for carcinogenesis in pancreas.[7,9,25,26] Acinar-to-ductal metaplasia has been generally regarded as a transdifferentiation and premalignant state.[26-29] Centroacinar cells have been reported to be involved in the metaplasia-ductal adenocarcinoma sequence.[7,9] Here we demonstrated that not only centroacinar cells but also the ductal metaplasia of the acinar cells located in the border zone of ductal adenocarcinomas expressed CD133. Although it remains unknown if the ductal metaplasia of acinar cells is consistent with premalignant acinar-to-ductal metaplasia, our results suggest that CD133 may be one of the molecules involved in this process. In contrast, IPMNs did not express CD133, suggesting that the adenoma-dysplasia-carcinoma sequence in IPMNs may be different from the metaplasia-ductal adenocarcinoma sequence.

CD133 is considered as a universal marker of tissue-specific stem cells or

tumor-initiating cells. We and other groups have demonstrated that CD133 is a marker of putative pancreatic progenitor cells.[17,30] A recent report has described that CD133 is not restricted to stem cells or tumor initiating cells in adult pancreas and that CD133-expressing cells in the pancreas include at least two subpopulations: 1) a main population expressing CD133 at the cell surface which represents a particular stage in cell differentiation connected to the formation of lumina and ducts and 2) a minor cell population that predominantly exhibits cytoplasmic CD133 staining, which represents less than 1% of epithelia cells, both in normal pancreas and in ductal adenocarcinomas.[18] We have also observed cytoplasmic CD133 staining in a minor population of ductal adenocarcinomas cells (Fig. 2-2). The functional significance of this cytoplasmic staining remains unclear. We demonstrated that CD133 staining was observed in all of the ductal adenocarcinomas examined in this study, however the CD133 staining was not observed in all of the ductal adenocarcinoma cells (Table 1). The reason for this is not known, but one possibility is that the microenvironment around ductal adenocarcinoma cells might affect CD133 expression level and/or subcellular localization. Further studies are necessary to clarify the function of CD133

in pancreatic ductal adenocarcinomas.

**ACKNOWLEDGMENTS**

The authors thank Drs. Shuho Semba, Masayuki Fujita, and Mr. Okito Hashimoto for helpful discussion. We are grateful to Dr. Aaron M. Zorn (Cincinnati Children's Hospital Medical Center) for his proofreading and critical comments on this manuscript.

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**Figure legends**

**FIGURE 1.** CD133 expression in the normal adult pancreas. (A) CD133 staining in centroacinar epithelium (arrow) and terminal ductal cells (arrowhead). Note that the apical/endoluminal staining for CD133 are positive in these cells. (B) CD133 staining in centroacinar cells surrounded by acinar cells in the high fold magnification. An arrow points to a cell in the centroacinar position. (C) CD133 staining in terminal and small ductal epithelium. (D) CD133-negative larger ductal epithelium. Original magnification was 200x (A, C), 400x (B), 100x (D).

**FIGURE 2.** (1) CD133 expression in normal acinar tissue, metaplasia, and ductal adenocarcinomas. (A, B) Normal acinar tissue adjacent to ductal adenocarcinomas. (C, D) Metaplasia lesion. A lobule that has undergone metaplastic transformation is shown adjacent to the normal acinar tissue. (E, F) Carcinoma cells on the ductal adenocarcinoma. (G, H) Carcinoma cells on the lymph node metastasis lesion. (A, C, E, G) H-E staining. (B, D, F, H) CD133 immunostaining. Original magnification was 100x; bars, 200 μm. (2) Cytoplasmic CD133 staining in carcinoma cells on ductal adenocarcinomas. An arrow indicates a carcinoma cell expressing cytoplasmic CD133.

Original magnification was 400x.

**FIGURE 3.** Imaging studies and macroscopy of IPMNs. (A) Computed tomography. An arrow indicates the dilated main pancreatic duct and an arrowhead indicates the dilated secondary pancreatic ducts. (B) ERCP image. Arrows indicate the dilated main duct. (C, D) Macroscopy of the resected specimen. Arrows indicate resected pancreatic head attached to duodenum in (C). An asterisk indicates dilation of the main pancreatic duct in (D).

**FIGURE 4.** CD133 expression in IPMNs. (A, B) No expression of CD133 in mucin-producing epithelium on IPMNs. (C, D) No expression of CD133 in carcinoma *in situ* on IPMNs. (E, F) No expression of CD133 in invasive carcinoma cells on IPMNs. (A, C, E) H-E staining. (B, D, F) CD133 immunostaining. (G) Expression of CK-19. Asterisks indicate mucin-producing epithelium on IPMNs. Arrows indicate normal ductal epithelium. (H, I) The expression level of CD133 at the transition area from ductal epithelium to columnar mucin-producing epithelium. (H) The columnar mucin-producing epithelium cells (an asterisk) do not express CD133, but ductal epithelium cells express CD133. (I) The columnar mucin-producing epithelium cells

(arrows) bound to the ductal epithelium cells (arrowheads) do not express CD133. Original magnification was 200x (A-F), 100x (G), 400x (H, I).

**FIGURE 5.** CD133 expression in subtypes of IPMNs. (A-E) Gastric type. (F-J) Intestinal type. (K-O) Panreatobiliary type. (P-T) Oncocytic type. (A, F, K, P) H-E staining. (B, G, L, Q) CD133 immunostaining. (C, H, M, R) MUC1 immunostaining. (D, I, N, S) MUC2 immunostaining. (E, J, O, T) MUC5AC immunostaining. Original magnification was 200x.

**Table 1 Summary of patients and their clinical characteristics**

| Pat.no. | Sex | Age (years) | Pathol. subtype | Grading | Metastasis | CD133 expression | % CD133(+) cells[a] |
|---|---|---|---|---|---|---|---|
| 1. | M | 63 | Tub. | M. | Liver | (+) | 31% ± 8 |
| 2. | F | 54 | Tub. | M. | ( - ) | (+) | 47% ± 4 |
| 3. | M | 66 | Tub. | M. | Liver | (+) | 42% ± 20 |
| 4. | M | 66 | Tub. | P. | Liver | (+) | 31% ± 14 |
| 5. | M | 71 | Tub. | M. | Liver | (+) | 19% ± 12 |
| 6. | F | 78 | Tub. | M. | Ascites | (+) | 16% ± 6 |
| 7. | F | 77 | Tub. | M.-P. | ( - ) | (+) | 23% ± 6 |
| 8. | M | 78 | Tub. | W. | ( - ) | (+) | 49% ± 12 |
| 9. | F | 56 | Tub. | W.-M. | ( - ) | (+) | 21% ± 14 |
| 10. | M | 73 | Tub. | W.-M. | ( - ) | (+) | 18% ± 5 |

NOTE: All 10-tissue samples were pancreatic ductal adenocarcinomas isolated from patients.

Pat. no., Patient number; Pathol. subtype, Pathological subtype; Tub., Tubular adenocarcinoma; M., Moderately differentiated; P., Poorly differentiated; W., Well differentiated.

[a] The number of the cells expressing CD133 in the total cell number was scored as described in Materials and Methods.

**Table 2 Summary of patients and their clinical characteristics**

| Pat.no | Sex | Age (years) | Pathological subtype | Metastasis/ invasion | Location | CD133 expression | % CD133(+) cells[a] |
|---|---|---|---|---|---|---|---|
| 1. | M | 69 | IPMC (mixed) | ( - ) | Head | ( - ) | 0 |
| 2. | F | 68 | IPMA (main) | ( - ) | Body | ( - ) | 0 |
| 3. | M | 56 | IPMA (branch) | ( - ) | Head | ( - ) | 0 |
| 4. | F | 53 | IPMA (branch) | ( - ) | Uncs | ( - ) | 0 |
| 5. | F | 73 | IPMA (mixed) | ( - ) | Uncs+Head | ( - ) | 0 |
| 6. | M | 68 | IPMC (branch) | ( - ) | Head | ( - ) | 0 |
| 7. | F | 60 | IPMA (branch) | ( - ) | Tail | ( - ) | 0 |
| 8. | M | 75 | IPMC (mixed) | ( - ) | Head | ( - ) | 0 |
| 9. | F | 56 | IPMA (mixed) | ( - ) | Head | ( - ) | 0 |
| 10. | M | 64 | Invasive IPMN [b] | ( + ) | Head | ( - ) | 0 |

NOTE: All 10-tissue samples were IPMNs isolated from patients.

Pat. no., Patient number; IPMC, intraductal papillary mucinous carcinoma which has carcinoma *in situ*; IPMA, intraductal papillary mucinous adenoma; main, main ductal type; branch, branch ductal type; mixed, mixed type.

[a] The number of the cells expressing CD133 in the total cell number was scored as described in Materials and Methods.

[b] Invasive IPMN; an IPMN containing invasive carcinoma cells.

**Table 3 Summary of CD133 expression in IPMN subtypes**

| Pathological subtype | Number of samples | CD133 expression | % CD133(+) cells[a] |
|---|---|---|---|
| Gastric type | 15 | 0 | 0 |
| (carcinoma in situ) | (5) | (0) | (0) |
| Intestinal type | 8 | 0 | 0 |
| (carcinoma in situ) | (4) | (0) | (0) |
| Pancreatobiliary type | 1 | 0 | 0 |
| Oncocytic type | 1 | 0 | 0 |
| Invasive IPMN | 9 | 0 | 0 |
| Tubular carcinoma | (5) | (0) | (0) |
| Colloid carcinoma | (3) | (0) | (0) |
| Tubular & colloid[b] | (1) | (0) | (0) |
| | Total 34 | | |

NOTE: All 34-tissue samples were IPMNs isolated from patients.

[a] The number of the cells expressing CD133 in the total cell number was scored as described in Materials and Methods. [b] Tubular & colloid: both of tubular carcinoma and colloid carcinoma were observed in the same invasive IPMN.