Nuclear localization of tricellulin promotes the oncogenic property of pancreatic cancer

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Accumulating evidence has shown that dysregulation of tight junctions (TJs) is involved in tumor development and progression. In this study, we investigated the expression and subcellular distribution of tricellulin, which constitutes tricellular TJs, using human pancreatic adenocarcinomas. In well-differentiated pancreatic adenocarcinoma tissues, tricellulin immunostaining was prominent in the cytoplasm and the plasma membrane. In contrast, in poorly differentiated tissues, its immunostaining was predominantly observed in the nuclei and was almost absent in the plasma membrane. The distinct immunostaining of tricellulin successfully distinguished poorly differentiated adenocarcinoma from moderately and well-differentiated adenocarcinomas with high levels of sensitivity and specificity. Nuclear tricellulin expression significantly correlated with lymph node metastasis, lymphatic invasion and poor survival. In pancreatic cancer cell lines, tricellulin localization shifted from the membrane to nucleus with decreasing differentiation status. Nuclear localization of tricellulin promoted cell proliferation and invasiveness possibly in association with MAPK and PKC pathways in pancreatic cancers. Our results provide new insights into the function of tricellulin, and its nuclear localization may become a new prognostic factor for pancreatic cancers.

Pancreatic cancer shows an extremely poor prognosis with a 5-year survival rate of less than 5% due to its highly invasive and metastatic phenotypes1-3. Thus, an understanding of the regulatory mechanisms that control the aggressive behavior of this malignancy is needed.

Tight junctions (TJs) are the apicalmost components of intercellular junctional complexes in epithelial and endothelial cells. They primarily inhibit solute and water flow through the paracellular space4,5. They also separate the apical from the basolateral cell surface domains to establish cell polarity6. TJs are formed by not only integral membrane proteins such as claudins but also a variety of subcellular scaffolding proteins7-9. Accumulating evidence has shown that these components are signaling molecules that have functions in receiving or transmitting signals7-9. Morphological examinations of TJ proteins demonstrated that various human tumors10-16, including pancreatic cancer17,18, show aberrant expression and localization of TJ components. TJs are frequently disassembled in carcinomas with poor differentiation19,20. These findings suggested that dysregulated or disordered TJs are involved in tumor development and progression.

Tricellulin, which is encoded by the TRIC gene, is a transmembrane protein that predominantly localizes at tricellular TJs, where the corners of three epithelial cells meet21,22. Results of previous studies have revealed that this protein is a possible factor contributing to pancreatic neoplasia23,24, and this possibility is supported by the results of our previous study showing that tricellulin regulates epithelial TJ integrity of pancreatic duct epithelial cells via the c-Jun N-terminal kinase pathway25. In addition, immunohistochemical analyses have revealed that well-differentiated pancreatic adenocarcinomas highly express tricellulin in contrast to poorly differentiated carcinomas26. However, the potential role of tricellulin in carcinogenesis remains to be clarified.

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In the present study, we examined tricellulin expression in human pancreatic cancers in association with its subcellular localization, and we evaluated possible correlations with several clinicopathological variables. We also investigated whether tricellulin expression and its subcellular localization are responsible for the aggressive behaviors of cancer cells such as proliferation and invasiveness. Our results suggest that aberrant nuclear localization of tricellulin confers immature histology and oncogenic properties of pancreatic malignancy.

**Results**

**Tricellulin localization alters depending on differentiation levels in human pancreatic cancer tissues.** In normal pancreatic tissues, tricellulin was expressed in the intercellular boundary of pancreatic ductal cells (data not shown). In adenocarcinomas, tricellulin immunoreactivities were more prominent than in normal regions, and the subcellular distribution of tricellulin varied depending on the degree of differentiation (Fig. 1a): In well-differentiated carcinoma components, localization of tricellulin was prominent in the cytoplasm and the plasma membrane. In contrast, in poorly differentiated carcinoma components, localization of tricellulin was predominantly observed in the nuclei with various mixed patterns of cytoplasmic staining, whereas membranous staining was hardly observed. In cases with mixed differentiation, tricellulin was localized at the cytoplasm and plasma membrane in areas with irregularly arranged lumen formation, and tricellulin was localized in nuclei in poorly differentiated areas characterized by lack of tubule formation (Fig. 1b). For semi-quantitative analysis of the nuclear immunoreactivity of tricellulin, total numbers of immunopositive nuclei were counted in ten high-power microscopic fields that were randomly selected (Table 1a). The maximal score for the nucleus was significantly higher in poorly differentiated tissues.

We next performed ROC curve analysis of the nuclear immunoreactive score to determine whether it can distinguish the differentiation status of pancreatic adenocarcinomas (Supplemental Fig. 1). At a cutoff value of 21, poorly differentiated adenocarcinoma was successfully distinguished from moderately and well-differentiated adenocarcinomas with high levels of sensitivity and specificity (Table 1b), indicating that nuclear localization of tricellulin in poorly differentiated pancreatic adenocarcinoma is significantly different from that in moderately and well-differentiated adenocarcinomas.

**Nuclear localization of tricellulin is a prognostic factor of pancreatic cancers.** We next examined the possible relationships between nuclear staining score of tricellulin and clinicopathological parameters. At a cutoff value of 11, nuclear tricellulin expression was significantly correlated with lymph node metastasis (pN category, p = 0.004), lymphatic invasion (p = 0.059), and vessel invasion (p = 0.057, Table 2). In contrast, nuclear localization of tricellulin was not associated with sex, age, pT status, UICC stage, vessel invasion, neural invasion or tumor location. Kaplan-Meier analysis demonstrated that patients whose nuclear staining scores were 10 or less had significantly better survival than that of patients whose scores were greater than 10 (Fig. 2, 95\% CI: 1.3–8.1, log-rank P = 0.010, hazard ratio: 3.2). These findings indicated that nuclear localization of tricellulin is likely to be a prognostic factor of pancreatic cancers.

**Tricellulin accumulates in the nucleus of a poorly differentiated pancreatic cancer cell in vitro.** To determine whether tricellulin nuclear localization is directly involved in the malignant behaviors of cancer cells, we performed in vitro experiments using two human pancreatic cancer cell lines.

In HPAC, a cell line derived from moderately differentiated adenocarcinoma, tricellulin immunoreactivity was basically observed along the intercellular boundary just like a TJ marker protein, occludin, and was especially concentrated at a tricellular TJ, as its name means (Fig. 3a). In contrast, in the poorly differentiated cell line PANC-1, tricellulin immunoreactivity was predominantly observed in the nucleus with weak staining at the intercellular boundary (Fig. 3a). In confocal scanning laser microscopic analysis of PANC-1 cells, tricellulin immunoreactivity (Fig. 3b, green) was observed in the nucleus and cytoplasm, including perinuclear areas. It was also seen at the cell borders between adjacent cells. Tricellulin immunoreactivity was co-localized with DAPI (Fig. 3b, blue), a nuclear staining marker. Tricellulin immunoreactivity was observed throughout the cell, including the cytoplasm and cell borders, but was strongly detected in heterochromatin of the nucleus and nuclear membrane by immuno-transmission electron microscopic analysis with the anti-tricellulin antibody (Fig. 3c). Nuclear localization of tricellulin was not obvious in HPAC cell by either of the analyses (data not shown). Subcellular fractionation analysis was performed to compare the subcellular localizations of tricellulin in the two cell lines. In the poorly differentiated cell line PANC-1, tricellulin was detected in the nuclear and membrane fractions but was not detected in the cytosolic fraction (Fig. 3d). On the other hand, in HPAC cells, tricellulin was detected in the membrane fraction and a small amount of tricellulin was observed in the nuclear fraction. Tricellulin in the membrane fraction must correspond to immunoreactivity in the cytoplasm and plasma membrane because the membrane fraction contains the plasma membrane and membranous organelles according to the manufacturer’s instructions. PANC-1 cells showed a pronounced propensity for localization in the nucleus compared to HPAC cells by using the N/M ratio, a signal intensity ratio of tricellulin in the nuclear fraction (N) to that in the membrane fraction (M), which was estimated on the basis of Western blot analysis of biochemical fractionation (Fig. 3d). These results indicated that tricellulin localization shifts from the membrane to the nucleus with decreasing differentiation status in vitro.

In PANC-1 cells, exogenously transfected tricellulin was also localized in the nucleus. We constructed a vector expressing N-terminally GFP-tagged full-length tricellulin (GFP-tricellulin) and transfected it in PANC-1 cells to monitor the localization of exogenously expressed tricellulin (in live cells). Confocal scanning laser microscopic analysis revealed co-localization of GFP-tricellulin and Hoechst33258, a nuclear staining marker, at 48 hours after transfection (Fig. 4a), indicating that tricellulin accumulates in the nucleus of live PANC-1 cells. In Western blot analysis with anti-tricellulin antibody, we confirmed the GFP-tricellulin band (90.6 kDa) in addition to the endogenous tricellulin band (63.6 kDa) in the nuclear fraction from GFP-tricellulin-transfected PANC-1 cells.
Figure 1. HE staining and immunohistochemical staining for tricellulin in human pancreatic adenocarcinoma tissues. (a) Expression of tricellulin in surgical specimens of pancreatic adenocarcinoma. Tricellulin was localized at the plasma membrane and cytoplasm in well- and moderately differentiated pancreatic adenocarcinoma, whereas it assembled in the nuclei in poorly differentiated adenocarcinoma. Bar: 30 μm. (b) In cases with mixed differentiation, tricellulin was localized at the cytoplasm and plasma membrane in areas with irregularly arranged lumen formation (arrows), and tricellulin was localized in the nuclei in poorly differentiated areas characterized by lack of tubule formation (arrowheads). Bar: 30 μm.
On the other hand, after overexpression of tricellulin in HPAC cells, membrane and cytoplasmic localization of tricellulin was observed by immunofluorescence microscopy, but nuclear localization of tricellulin was not obvious (Supplemental Fig. 2).

Nuclear localization of tricellulin promotes proliferation and invasiveness of poorly differentiated pancreatic cancer cells in vitro. Our preliminary observations suggested that nuclear localization of tricellulin has some roles in tumor progression of poorly differentiated pancreatic cancer cells. To clarify the possible relationship between nuclear localization of tricellulin and malignant behavior in pancreatic neoplasia, tricellulin was overexpressed or its expression was suppressed in PANC-1 cells. Overexpression and suppressed expression of tricellulin were examined by Western blot analysis (Fig. 4a and Supplemental Fig. 3) at 48 hours after transfection of the GFP-tricellulin vector and small interfering RNA (siRNA) for tricellulin, respectively. Nuclear localization of overexpressed GFP-tricellulin was confirmed by confocal scanning laser microscopic analysis as described above (Fig. 4b).

The proliferating ability of the cells was evaluated by using the BrdU incorporation assay at 48 hours after transfection (Fig. 5a,b). Tricellulin-overexpressing cells exhibited a significant increase (159%) of BrdU-labeled

### Table 1. Immunoreactivity of tricellulin in pancreatic adenocarcinoma tissues.

| Differentiation | Median | Inter-quartile range | P value |
|-----------------|--------|----------------------|---------|
| Sex             | F      | 29                   | 4–32    | 0.092 (Mann-Whitney test) |
|                 | M      | 36                   | 9–200   |                      |
| Age (median)    | <69    | 33                   | 4.5–145 | 0.909 (Mann-Whitney test) |
|                 | 70+    | 32                   | 8–74    |                      |
| Size (median)   | <30    | 35                   | 4–48.5  | 0.161 (Mann-Whitney test) |
|                 | 31+    | 30                   | 8.25–123.5 |        |
| pT category     | 1      | 5                    | 0–8.5   | 0.124 (1 vs 2) |
|                 | 2      | 2                    | 2–200   | 0.023 (1 vs 3) |
|                 | 3      | 11                  | 6.5–104 | 0628 (2 vs 3) |
| pN category     | 0      | 17                   | 2.5–18.75 | 0.004 (Mann-Whitney test) |
|                 | 1      | 48                   | 9.75–134.5 |        |
| UICC stage      | 1      | 2                    | 7–9     | 0.279 (Kruskal-Wallis test) |
|                 | 2      | 22                  | 5–200   |                      |
|                 | 3      | 13                  | 2–4.95  |                      |
|                 | 4      | 21                  | 6–118   |                      |
| Lymphatic invasion | negative | 13             | 2.75–23.5 | 0.058 (Mann-Whitney test) |
|                 | positive | 14              | 6–118   |                      |
| Vessel invasion | negative | 16              | 4–19.75 | 0.057 (Mann-Whitney test) |
|                 | positive | 49              | 6.5–134.5 |        |
| Neural invasion | negative | 4               | 21–286.5 | 0.071 (Mann-Whitney test) |
|                 | positive | 61              | 5–69.75 |                      |
| Tumor location  | Pb     | 8                   | 4–155.5 | 0.143 (Kruskal-Wallis test) |
|                 | Pb(U)  | 3                   | 2.5–13.5 |                      |
|                 | Ph     | 38                  | 6.75–118 |                      |
|                 | Phb    | 3                   | 18.5–118 |                      |
|                 | Phbt   | 5                   | 18–161.5 |                      |
|                 | Pr     | 7                   | 1–21    |                      |
|                 |        |                     | 6.5–81.5 |                      |

Table 2. Nuclear distribution of tricellulin and clinicopathological features of pancreatic adenocarcinomas.
cells, whereas knockdown of tricellulin significantly reduced the BrdU incorporation ratio to 32.9% of that of control cells. The WST-1 assay also showed that tricellulin overexpression significantly increased cell proliferation, whereas knockdown of tricellulin reduced proliferation of PANC-1 cells (Fig. 5c,d). These results indicated that cell proliferation is positively associated with the amount of nuclear localized tricellulin because overexpressed GFP-tricellulin was clearly observed in the nucleus as described above. In HPAC cells, in which nuclear localization of tricellulin was not obvious, we confirmed that knockdown of tricellulin expression did not affect cell proliferation in a WST-1 assay (data not shown).

To assess the invasive properties, we performed a Matrigel invasion assay (Fig. 5e,f). At 24 hours after plating, there was a significant difference in infiltration efficiency between control and tricellulin knockdown cells, 33% and 46.6%, respectively. In contrast, overexpression of tricellulin dramatically increased the efficiency to 242%, suggesting that nuclear accumulation of tricellulin plays an important role in invasiveness.

Finally, we investigated signal transduction pathways that mediate the effects of tricellulin expression on malignant behaviors (Fig. 6). Tricellulin overexpression enhanced phosphorylation of PKC and ERK1/2 in PANC-1 cells but not in HPAC cells. Phospho-Akt level was not affected by tricellulin overexpression in either of the cell lines. These results suggested that tricellulin is preferentially associated with PKC and MAPK signaling pathways that can promote pancreatic neoplasia.

Discussion

In the present study, we found that nuclear localization of tricellulin is significantly associated with clinicopathological parameters such as lymph node metastasis and shorter survival time. We also demonstrated that nuclear localization of tricellulin promotes malignant behaviors of pancreatic carcinoma cells. Immunohistochemical analyses revealed that immunoreactive intensity of tricellulin was decreased in poorly differentiated adenocarcinomas compared with that in well- and moderately differentiated adenocarcinomas. Furthermore, changes in tricellulin immunostaining were not only its staining intensity but also its localization, from the plasma membrane and cytoplasm (well- and moderately differentiated) to the nucleus (poorly differentiated).

It is plausible that with progression of pancreatic cancer tricellulin alters its expression level and localization as a component of TJ structures in association with destruction of TJs, one of the characteristic phenomena during dedifferentiation. In addition to increased nuclear staining of tricellulin, we also observed that membranous staining was decreased in poorly differentiated adenocarcinoma compared to that in well-differentiated adenocarcinoma, as was reported by Korompay et al. It has been shown that the expression of TJ-associated proteins (TJPs) disperses from the original location in the apical membrane fraction to the cytoplasm in association with progress of epithelial-mesenchymal transition (EMT) or dedifferentiation in vivo and in vitro. For example, expression of claudin-1 was decreased in pancreatic cancer cell lines compared with that in normal human pancreatic duct epithelial cells, and its expression was observed in the cytoplasm in poorly differentiated pancreatic adenocarcinoma cells. Since the observations indicated that TJPs are useful immunohistochemical markers for the differentiation status of cancer, our results suggest that tricellulin could be included in such markers.

We clearly observed nuclear localization of tricellulin in poorly differentiated adenocarcinomas, though tricellulin has been shown to be a transmembrane protein in the plasma membrane. This is based on published data showing that CLDN-1 mislocalized from the plasma membrane to the nucleus and cytoplasm in metastatic colon cancer cells and in airway smooth muscle cells. Nuclear distributions of CLDN-2 and E-cadherin were also observed in lung adenocarcinoma cells and pancreatic endocrine tumors, respectively. The presence of tricellulin in the nucleus was previously reported for HCCs and hepatoblastoma, but the authors of those reports stated that nuclear localization of tricellulin was randomly observed in only a few cells and they did not analyze the association between nuclear staining and prognosis. In this study, we also observed nuclear localization of tricellulin in pancreatic adenocarcinoma and we also found that the nuclear staining score was associated
Figure 3. Nuclear localization of tricellulin in poorly differentiated pancreatic cancer cell in vitro.

(a) Immunofluorescence labeling of tricellulin (green) and occludin (red). Differentiated HPAC cells showed tricellulin staining predominantly at the tricellular tight junction, whereas poorly differentiated PANC-1 cells showed tricellulin staining in the nucleus. Occludin was localized at the intercellular boundary in both cell lines. (b) Confocal laser microscopic images of tricellulin in PANC-1 cells. Tricellulin co-localized with DAPI in nuclei. (c) Immuno-transmission electron microscopic images with anti-tricellulin antibody in PANC-1 cells. The lower panel is an enlarged view of the square area in the upper panel. Presentative immunogold particles (arrowheads) were detected especially at the nucleus and nuclear membrane. C: cytoplasm, N: nucleus, M: membrane, MV: microvilli, NM: nuclear membrane. Bar: 2 μm (upper panel), 1 μm (lower panel). (d) Subcellular distributions of tricellulin in PANC-1 and HPAC cells. The ratio of tricellulin in the nuclear fraction and that in the membrane fraction (ratio of N/M) was increased in PANC-1 cells compared with that in HPAC cells. W: whole lysate, C: cytosolic fraction, M: membrane fraction, N: nuclear fraction. Data are represented as means ± SD, and statistical significance was assessed by Student’s t test. **p < 0.001. Full-length blots are presented in Supplemental Fig. 4.
with differentiation and prognosis. In addition to immunohistochemistry, we confirmed the nuclear localization of tricellulin through a multiple approach including live cell imaging, immunofluorescence, immunoelectron microscopy and biochemical fractionation by using a cell culture model.

Importantly, a high nuclear tricellulin immunoreactive score was correlated with lymph node metastases and lymphatic invasion and reduced overall survival in pancreatic cancer patients. These results may support the explanation that nuclear localized tricellulin contributes to tumor malignancy, including metastatic ability, in pancreatic cancer. This is based on our observations of nuclear localization of tricellulin in a poorly differentiated cancer cell line (PANC-1), a cell line without intact TJ function (unpublished data), but not in a differentiated cancer cell line (HPAC), a cell line with intact TJ function. In addition, overexpression of tricellulin in the nucleus promoted proliferation and invasiveness of PANC-1 cells. The association between expression level of tricellulin and prognosis has been controversial. It has been reported that poor prognosis is linked to high expression of tricellulin in HCC but to low expression of tricellulin in iCCC and hepatoblastoma. The relationship between expression level of tight junction proteins and prognosis has also been controversial: both increased expression and decreased expression have been reported to be associated with poor prognosis in various cancers. Each of the tight junction molecules, including tricellulin, may play different roles depending on tissue type or tumor grade. For a better understanding of each molecule, its subcellular localization should be evaluated in addition to immunoreactive intensity.

Since it is unlikely to translocate to the nucleus, the question of what mechanism regulates the translocation of tricellulin to the nucleus has remained to be answered. Although underlying mechanisms have not been fully established, several transmembrane proteins of TJs and adherens junctions were observed to be mislocalized to the nucleus in some cancer cells. Unexpectedly, we did not detect any classical nuclear localization signal (NLS) sequences in the human tricellulin sequence by searching with nuclear localization software-based analysis (Nuclear Localization Signal database, http://rostlab.org/services/nlsdb/). We then hypothesized that

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**Figure 4. Subcellular distribution of overexpressed GFP-tricellulin in PANC-1 cells.** (a) Western blot analysis of subcellular fractions from PANC-1 cells overexpressing GFP-tagged tricellulin. GFP-tagged tricellulin was detected in all subcellular fractions including the nuclear fraction. W: whole lysate, C: cytosolic fraction, M: membrane fraction, N: nuclear fraction. Full-length blots are presented in Supplemental Fig. 4. (b) Live cell image of PANC-1 cells overexpressing GFP-tagged tricellulin using confocal laser microscopy. GFP-tricellulin co-localized with Hoechst33258 in the nucleus.
Figure 5. Nuclear localization of tricellulin promotes proliferation and invasiveness of poorly-differentiated pancreatic cancer cell in vitro. (a,b) Representative images of PANC-1 cells in the BrdU incorporation assay (green: BrdU-labeled nuclei). (c,d) WST-1 assay. (e,f) Representative images of PANC-1 cells in the Matrigel invasion assay. Tricellulin-overexpressed cells (tricellulin) show enhanced proliferation and invasiveness, whereas cells with suppression of tricellulin expression (tricellulin-siRNA) show reduced proliferation and invasiveness. Scr-siRNA: scrambled siRNA. Data are represented as means ± SD, and statistical significance was assessed by Student’s t test. *p < 0.05, **p < 0.001.
tricellulin may interact with an unknown partner molecule(s) with an NLS sequence to go to the nucleus or translocate to the nucleus via some importin-independent system. In the case of claudin-2, its nuclear distribution is up-regulated by dephosphorylation and serves to retain ZONAB and cyclin D1 in the nucleus, resulting in the enhancement of proliferation of lung adenocarcinoma cells\(^5\). We did not observe interaction between tricellulin and ZO-1, a TJP with NLS sequences, by immunoprecipitation analyses (data not shown). Our studies, however, have not fully proven the significance of the nuclear localization of tricellulin in tumor progression, whether it is a causative event or just a bystander. Further studies are needed to clarify the molecular mechanism underlying the nuclear localization of tricellulin in poorly differentiated pancreatic cancer and how its nuclear localization contributes to pancreatic neoplasia via such signaling pathways. The combination of immunoprecipitation and mass spectrometry may reveal a molecule(s) that interacts with tricellulin in the nucleus.

Another question is how nuclear localized tricellulin contributes to tumor malignancy, because there has been no report about tricellulin function in the nucleus. In this study, we observed that nuclear overexpression of tricellulin induced activation of MAPK and PKC pathways, but not the Akt pathway, in a poorly differentiated pancreatic cancer cell line. Activation of MAPK and PKC pathways is generally supported in malignant pancreatic tumors, and a large number of molecules are involved in activation of the pathways\(^1\)–\(^3\). Our results suggested that nuclear localized tricellulin promotes cell proliferation and invasiveness possibly in association with MAPK1 and PKC pathways in pancreatic cancers, although detailed mechanisms remain to be elucidated.

In conclusion, poorly differentiated pancreatic carcinomas show mislocalization of tricellulin from the plasma membrane to nuclei, which is significantly correlated with poor survival and tumor malignancies. Our results provide new insights into the function of the TJP tricellulin, and its nuclear localization may become a new prognostic factor for pancreatic cancers.

**Materials and Methods**

**Antibodies.** Primary antibodies used in this study include the following: rabbit polyclonal anti-tricellulin (c-term), mouse monoclonal anti-occludin (Zymed Laboratories, San Francisco, CA); rabbit polyclonal anti-actin (Sigma-Aldrich, St Louis, MO); goat polyclonal anti-LDH (as a marker enzyme of cytosolic fraction), mouse monoclonal anti-calnexin (as a marker of the membrane fraction) (Abcam, Cambridge, MA); mouse monoclonal anti-PAPR-1 (as a marker of the nuclear fraction) (Calbiochem). Twelve-nm colloidal gold-conjugated anti-rabbit IgG was purchased from Jackson Immuno Research Laboratories (Western Grove, PA). The secondary antibodies used were horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse immunoglobulin (Ig)G (Dako ChemMate, Glostrup, Denmark), Alexa Flour 488 (green)-labeled anti-rabbit or anti-mouse IgG (Invitrogen, Carlsbad, CA), and Alexa Flour 594 (red)-labeled anti-rabbit or anti-mouse IgG (Invitrogen). DAPI was obtained from Sigma-Aldrich and Hoechst 33342 solution was obtained from Dojindo (Kumamoto, Japan).

**Immunohistochemistry for primary pancreatic adenocarcinoma tissues.** A total of 65 cases of operated pancreatic adenocarcinoma were obtained from Sapporo Medical University Hospital, Japan. Written informed consent was obtained from all patients. The study was approved by the ethics committee of Sapporo Medical University. All methods were performed in accordance with the relevant guidelines and regulations of the University. Hematoxylin and eosin-stained slides from each case were reviewed. We determined glandular differentiation of the tumor according to the guidelines of the WHO classification\(^5\). According to the evaluation by three pathologists, 17, 28 and 20 cases were verified as well-, moderately and poorly differentiated adenocarcinoma, respectively.
Immunostaining of tricellulin was performed as described previously\textsuperscript{14} using the primary polyclonal anti-tricellulin antibody. Antigen retrieval was performed by immersing sections in 10 mM Tris - 1 mM EDTA buffer (pH 9.0) and boiling them in a microwave (95 °C, 30 min). Immunostaining was performed using the Dako REAL\textsuperscript{TM} EnVision\textsuperscript{TM} Detection System (Dako ChemMate, Glostrup, Denmark) and diaminobenzidine (Dako anti-tricellulin antibody. Antigen retrieval was performed by immersing sections in 10 mM Tris - 1 mM EDTA mixture at

\[ \text{pH 9.0} \] and boiling them in a microwave (95 °C, 30 min).

Cells were incubated for 2 h in 35-mm glass-base dishes (Iwaki, Chiba, Japan) coated with rat tail collagen. The cells were incubated for 2 h with 2 N HCl and were then fixed in cold absolute ethanol. After the samples had been incubated with 20 μM BrdU and were then fixed in cold absolute ethanol. After the samples had been incubated with 2 N HCl at room temperature for 20 min, they were incubated with monoclonal anti-BrdU antibody (LSAB2 Kit, Dako A/S, Copenhagen, Denmark). The specimens were fixed with 1.0% glutaraldehyde for 15 min at 4 °C and incubated with 0.05 M HEPES solution. After incubation, they were enhanced with a silver enhancement kit (Jackson Immuno Research Laboratories). The specimens were fixed with 1.0% OsO\textsubscript{4} in PBS at 4 °C for 1 h, dehydrated through a graded ethanol series, embedded in epoxy resin, and cut into ultrathin sections (100 nm) using a ultramicrotome (Diatom, USA). The sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (JEOL Ltd., Tokyo, Japan).

Cells were grown on 35-mm glass-base dishes (Iwaki, Chiba, Japan) coated with rat tail collagen. The cells were incubated for 2 h after treatment with 20 μM BrdU and were then fixed in cold absolute ethanol. After the samples had been incubated with 2 N HCl at room temperature for 20 min, they were incubated with monoclonal anti-BrdU antibody
(Dako, Santa Barbara, CA, USA) (1:100) at room temperature for 1 h and then with Alexa 488 (green)-conjugated anti-mouse IgG antibody (1:100) at room temperature for 1 h. DAPI (Sigma-Aldrich, St Louis, MO) was used for counterstaining of nuclei in the cells. The number of cells with BrdU-labeled nuclei was counted using an epifluorescence microscope (Olympus, Tokyo, Japan). More than 1000 cells were counted per dish, and three dishes were examined per experiment.

PANC-1 cells were seeded in 96-well plates. After 24 h of incubation, cell viability was assessed by using a cell counting kit-8 (Dojindo) according to the manufacturer’s instructions. Absorbance at a wavelength of 450 nm was measured using a Bio-Rad Model 680 microplate reader (Bio-Rad).

For the invasion assay, we used a Corning BioCoat Matrigel Invasion Chamber (Fisher Scientific) according to the manufacturer's instructions. PANC-1 cells in a medium without FBS were plated onto the Matrigel chambers at 48 h after transfection. The lower chamber of the Transwell was filled with medium containing 10% FBS. At 24 h after plating, invading cells were fixed and visualized by 0.04% Crystal violet and 2% ethanol for 10 min. The areas of invading cells were measured using a microscope imaging system (Olympus).

Statistical analysis. In immunohistochemical analysis of human tissues, the measured values are presented as medians and inter-quartile ranges (25–75%). Correlations between number of tricellulin-positive nuclei and clinicopathological variables were assessed by the Mann-Whitney test, Kruskal-Wallis test or Kruskal-Wallis test with post hoc Steel-Dwass test. Survival rates were calculated by the Kaplan-Meier method and compared by the log-rank test. In all other analyses, the measured values are presented as means ± SD. Statistical significance of differences was evaluated using the unpaired Student’s t-test.

All statistical calculations were performed with the use of SPSS statistics ver. 20.

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**Author Contributions**
A.T., M.M. and N.S. designed the study. A.T. and M.M. wrote the main manuscript text and prepared figures. A.T., M.M., K.T. and Y.O. performed the experiments. M.N. was responsible for the statistical design. T. Kono and K.H. collected and analyzed the clinical data. M.O., S.T., T. Kojima and N.S. analyzed and interpreted the data. All authors contributed to the review, edit and approval of the paper. All authors agree to be accountable for the content of this paper.

**Additional Information**

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