# Analysis of invasion-metastasis in pancreatic cancer: Correlation between the expression and arrangement of tight junction protein-2 and cell dissociation in pancreatic cancer cells

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Abstract. High frequency of invasion and metastasis is one of the key characteristics of pancreatic cancer. In our recent study, tight junction protein-2 (Tjp-2) was identified as a differentially expressed gene related to invasion-metastasis in highly (PC-1.0) and weakly (PC-1) invasive and metastatic pancreatic cancer cells by cDNA microarray analysis. Changes in the structure and function of tight junctions are correlated with carcinogenesis and tumour development. In this study, RT-PCR, Western blotting and immunocytochemistry were used to study the correlation between the expression and localisation of Tjp-2 and cell dissociation in pancreatic cancer. Tjp-2 mRNA and protein were differentially expressed in PC-1.0 and PC-1 cells. Furthermore, the addition of dissociation factor (DF) or U0126 (a MEK inhibitor) significantly induced changes in the mRNA expression and protein intracellular localisation of Tjp-2, and in the simultaneous cell dissociation of PC-1.0 and PC-1 cells. However, protein expression of Tjp-2 was not affected by DF or U0126 treatment. The current results indicate that Tjp-2 is involved in the regulation of cell dissociation in pancreatic cancer cells through changes in gene expression and intracellular localisation. Tjp-2 may serve as a new target for molecular therapies that prevent the invasion and metastasis of pancreatic cancer.

### Introduction

High frequency of invasion-metastasis, which confers an extremely poor prognosis, is one of the most important characteristics of pancreatic cancer. The literature currently reports that

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the detachment of tumour cells from the primary site is the first and pivotal step in the tumour invasion-metastasis process (1). However, the molecular mechanisms by which this detachment occurs are still unclear. Elucidating the molecular mechanisms of cell dissociation and subsequent invasion will contribute to an understanding of the mechanism of tumour invasion-metastasis, as well as help to develop new clinical therapies that may improve the prognosis for pancreatic cancer patients.

In our previous study, dissociation factor (DF), which induced the cell dissociation of weakly invasive and metastatic pancreatic cancer cells (PC-1), was isolated from the culture medium of highly invasive and metastatic pancreatic cancer cells (PC-1.0) (2,3). In further investigations, DF was found to activate the mitogen-activated protein kinase kinase 2 (MEK2)/extracellular signal-regulated kinase 2 (ERK2) signal pathway, which was identified as a pivotal signalling pathway related to cell dissociation in PC-1.0 and PC-1 cells (4-6).

In our recent study, cDNA microarray analysis identified tight junction protein-2 (Tjp-2), also named Zonula occludens-2 (ZO-2), as a differentially expressed gene related to invasionmetastasis in PC-1.0 and PC-1 cells (7). There are three tight junction proteins (Tjps): Tjp-1 (ZO-1), Tjp-2 (ZO-2) and Tjp-3 (ZO-3). Tjps belong to a family of membrane-associated guanylate kinase (MAGUK) homologs that are involved in the organisation of epithelial and endothelial intercellular junctions. Tjps bind to the cytoplasmic C termini of junctional transmembrane proteins and link them to the actin cytoskeleton and components of the signalling pathway (8). To date, marked progress has been made in understanding Tjp structure and physiological functions (8,9), but the involvement of Tjp-2 in tumour invasion and metastasis, especially with regard to cell dissociation, remains unclear.

In this study, we analysed the correlation between changes in Tjp-2 expression as well as intracellular distribution and cell dissociation in pancreatic cancer cells in order to clarify the involvement of Tjp-2 in the cell dissociation of pancreatic cancer cells.

## Materials and methods

*Cell lines and cell culture*. Two hamster pancreatic cancer cell lines were used, the weakly invasive and rarely metastatic

cell line PC-1, and the highly invasive and metastatic cell line PC-1.0. The PC-1 cell line was established from pancreatic ductal/ductular adenocarcinomas induced by BOP in a Syrian golden hamster (10). The PC-1.0 cell line was established from a subcutaneous tumour produced after the inoculation of PC-1 cells (11). These two cell lines exhibited different growth morphology *in vitro*: the PC-1 cells formed island-like cell colonies, whereas PC-1.0 cells mainly grew as single cells (12).

These cell lines were incubated in RPMI-1640 (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (Bioserum, Victoria, Australia), 100 U/ml penicillin G and 100  $\mu$ g/ml streptomycin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. The cells were serum starved overnight prior to the experiments.

Antibodies. Rabbit polyclonal antibodies raised against amino acid sequences of Tjp-2 and  $\beta$ -actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) of human origin were used as primary antibodies. Horseradish peroxidase-conjugated secondary antibodies and FITC-labelled fluorescence antibodies (Santa Cruz Biotechnology) were used as secondary antibodies for Western blotting and immunofluorescence staining, respectively.

Reverse transcriptase-polymerase chain reaction. Total RNA was extracted from each culture with TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD, USA). Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using the Superscript II One-Step RT-PCR System (Life Technologies, Inc.) in a PTC-200 Peltier Thermal Cycler (MJ Research, Inc., USA). The specific primers used for amplification were: 5'-GCAGAGCGAACGAAGAGTATGG (forward) and 5'-TGACGGGATGTTGATGAGGGT (reverse) for Tjp-2 (245 bp), and 5'-GTGGGGGCGCCCCAGGCACCA (forward) and 5'-CTCCTTAAGTCACGCACGATTCC (reverse) for  $\beta$ -actin (664 bp). The PCR protocol was as follows: 95°C for 5 min, then 30 cycles at 94°C for 20 sec, 63°C for 30 sec and 72°C for 1 min, followed by a final extension at 72°C for 7 min. PCR products were electrophoresed and visualised on 1.5% agarose gel containing ethidium bromide.

*Preparation of cell lysates.* Cells were grown in 90-mm dishes containing 10 ml of RPMI-1640 plus 10% foetal bovine serum.

To evaluate the expression of Tjp-2 protein and the relationship between the expression of Tjp-2 and cell dissociation in pancreatic cancer cells, either the conditioned medium of PC-1.0 cells (DF-CM) or the specific MEK inhibitor U0126 (Cell Signaling Technology, MA, USA) were applied to PC-1 and PC-1.0 cells, respectively (5,13). For activation studies, DF-CM was added to the PC-1 cells at a total concentration of 40%, then cells were incubated for 36 h. For inhibition studies, PC-1.0 cells were incubated with 10  $\mu$ M U0126 for 36 h. Additionally, where noted, PC-1 cells were incubated with 10  $\mu$ M U0126 for 36 h following the incubation with DF-CM.

The cells were lysed on ice for 15 min in 1 ml of ice-cold RIPA buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.5, 1 mM phenyl-methylsulfonyl fluoride, 1 mg/ml leupeptin and 1 mg/ml

aprotinin were added before use). After centrifugation (5 min at 5,000 rpm) at 4°C, the cell lysate supernatant was collected and stored at -80°C.  $\beta$ -actin was used as the internal control.

Western blot analysis. Western blotting was performed as described previously (14). In brief, samples of equivalent total protein (20  $\mu$ g) were run on a 5% polyacrylamide slab gel and transferred to a polyvinilidene fluoride (PVDF) membrane (Bio-Rad, Anaheim, CA, USA). Subsequently, the membranes were incubated with primary antibody diluted in 0.1% Tween-20/PBS overnight at 4°C. The blots were then incubated with horseradish peroxidase-conjugated secondary antibody diluted 1:5000 in 0.1% Tween-20/PBS. Enhanced chemiluminescence (Santa Cruz Biotechnology) was used to detect the signals, developed on Kodak scientific imaging film (Eastman Kodak Company, Rochester, NY, USA).

*Immunofluorescent staining and fluorescence intensity analysis.* Cells were planted on the chamber slides and incubated for 36 h prior to the experiment. To evaluate the redistribution of Tjp-2 protein and the relationship between the expression of Tjp-2 and the cell dissociation of pancreatic cancer cells, DF-CM or U0126 was applied to the cells. Cells were treated as described in *Preparation of cell lysates*.

After incubation with DF-CM or U0126, the cells were fixed with 0.5% paraformaldehyde for 10 min at room temperature. Cells were blocked with 10% normal goat serum for 30 min. Slides were then incubated with polyclonal anti-Tjp-2 antibody (1:200 dilution in 1% bovine albumin in PBS) at 4°C overnight, then with FITC labelled secondary antibody for 2 h at room temperature. The slides were washed three times with PBS between each step. After mounting, immunofluorescence images were captured with a confocal laser scanning biological microscope (FV500-IX; Olympus, Japan). The control slides were prepared as follows: i) slides were processed without a primary antibody; ii) normal goat serum and non-specific goat IgG were used instead of a polyclonal anti-Tjp-2 antibody.

Lastly, 10 images were chosen at random for each of the following areas: nucleus, cytoplasm, membrane and the whole cell. Tjp-2 protein expression in each image was measured for immunofluorescent staining and fluorescence intensity (FI) analysis with Fluoview 500 software (version 3.3; Olympus).

*Statistical analysis.* The average FI of Tjp-2 expression in 10 sites of the cell nucleus, cytoplasm, membrane and whole cell in various experimental groups was examined, and statistical significance between the groups was determined by an unpaired Student's t-test with the StatView statistical program (SAS Institute, Inc., Cary, NC, USA). A probability value of <0.05 was considered significant.

#### Results

Changes in Tjp-2 mRNA expression in highly (PC-1.0) and weakly (PC-1) invasive and metastatic pancreatic cancer cells. Tjp-2 mRNA was constitutively expressed in the highly invasive and metastatic pancreatic cancer cell line PC-1. Moreover, the constitutive expression of Tjp-2 mRNA in these cells was significantly inhibited after 24 h treatment with U0126 (Fig. 1A).

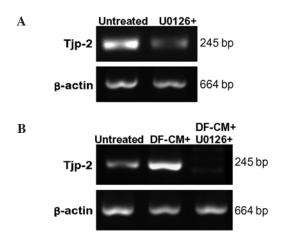


Figure 1. Changes in Tjp-2 mRNA expression in the highly (PC-1.0) and weakly (PC-1) invasive and metastatic pancreatic cancer cells. (A) PC-1.0 cells; (B) PC-1 cells.

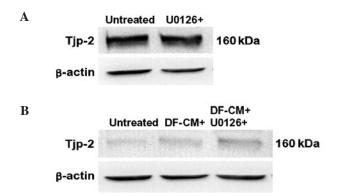


Figure 2. Western blotting of Tjp-2 protein in the highly (PC-1.0) and weakly (PC-1) invasive and metastatic pancreatic cancer cells. The expression of Tjp-2 protein was strong in PC-1.0 cells (A). By contrast, Tjp-2 expression was weak in PC-1 cells (B). No change in Tjp-2 expression was observed after a 36-h treatment with DF-CM or U0126 in either the PC-1.0 or PC-1 cells (A and B, respectively). The molecular marker is indicated.

By contrast, the expression of Tjp-2 mRNA was much lower in the weakly invasive and metastatic pancreatic cancer cell line PC-1. However, Tjp-2 mRNA expression was induced in these cells after a 24-h treatment with DF-CM. Furthermore, Tjp-2 mRNA expression was not induced by a 24-h treatment with DF-CM in PC-1 cells that had undergone pre-treatment with U0126 for 12 h (Fig. 1B)

Western blotting of Tjp-2 protein in highly (PC-1.0) and weakly (PC-1) invasive and metastatic pancreatic cancer cells. Western blotting data showed that Tjp-2 protein was strongly expressed in the highly invasive and metastatic pancreatic cancer cell line PC-1.0 (Fig. 2A). In contrast to the mRNA expression, the expression of Tjp-2 protein was not suppressed in these cells by treatment with U0126 for 36 h (Fig. 2A). On the other hand, Tjp-2 protein was expressed at much lower levels in the weakly invasive and metastatic pancreatic cancer cell line PC-1 (Fig. 2B). However, unlike the expression of Tjp-2 mRNA, DF-CM treatment did not induce the expression change of Tjp-2 protein in PC-1 cells (Fig. 2B).

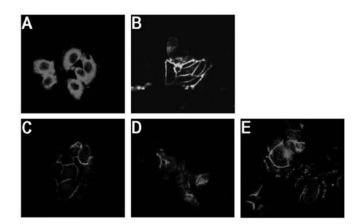


Figure 3. Rearrangement of Tjp-2 localisation in the highly (PC-1.0) and weakly (PC-1) invasive and metastatic pancreatic cancer cells. U0126 treatment significantly induced the localisation of Tjp-2 protein at the sites of cell-cell contact in PC-1.0 cells (B) as compared to untreated PC-1.0 cells (A). In contrast, untreated PC-1 cells showed localisation of Tjp-2 at the sites of cell-cell contact (C). Tjp-2 protein localisation at the sites of cell-cell contact was disrupted by DF-CM treatment for 36 h (D), but was restored by subsequent treatment with U0126 cells for 36 h (E). Immunofluorescence staining; original magnification x400.

Redistribution of Tjp-2 protein in highly (PC-1.0) and weakly (PC-1) invasive and metastatic pancreatic cancer cells. In untreated PC-1.0 cells (Fig. 3A), Tjp-2 protein was found mainly in the cytoplasm; only faint expression was detected at the cellular periphery and in the nucleus. After treatment with U0126 for 36 h, there was a marked increase in Tjp-2 at the sites of cell-cell contact in PC-1.0 cells (Fig. 3B).

In contrast to PC-1.0 cells, in untreated PC-1 cells (Fig. 3C) Tjp-2 protein was exclusively distributed at the peripheral sites of cell-cell contact, and only faint levels of Tjp-2 were detected in the nucleus and cytoplasm. However, this peripheral distribution of Tjp-2 at the sites of cell-cell contact was disrupted and became discontinuous after treatment with DF-CM for 36 h in PC-1 cells (Fig. 3D). Furthermore, when these DF-CM-treated PC-1 cells were treated with U0126 for a subsequent 36 h, the disruption of Tjp-2 distribution was mitigated, and Tjp-2 once again aggregated to the sites of cell-cell contact (Fig. 3E).

Fluorescence intensity of Tjp-2 expression in highly (PC-1.0) and weakly (PC-1) invasive and metastatic pancreatic cancer cells. The results of FI analysis of Tjp-2 expression in PC-1.0 cells and PC-1 cells are shown in Fig. 4A and B, respectively.

The average whole cell expression of Tjp-2 was extremely strong in PC-1.0 cells (average FI 1160.7 $\pm$ 259.1) (Fig. 4A). Tjp-2 protein was mainly localised to the cytoplasm (FI 3273.7 $\pm$ 131.2). Almost no fluorescence was detected in the nucleus (FI 139.0 $\pm$ 115.7) or at the cellular periphery (FI 69.5 $\pm$ 38.0). After U0126 treatment for 36 h, the average whole cell expression of Tjp-2 was not significantly changed in the PC-1.0 cells (FI 1009.4 $\pm$ 257.8, P>0.05). As previously noted, clear Tjp-2 localisation was observed at the sites of cell-cell contact (FI 2814.8 $\pm$ 297.0, P<0.05). There was no apparent change in the amount of Tjp-2 in the nucleus (FI 75.3 $\pm$ 28.1, P>0.05), but Tjp-2 expression in the cytoplasm was markedly reduced (FI 139.5 $\pm$ 47.5, P<0.05).

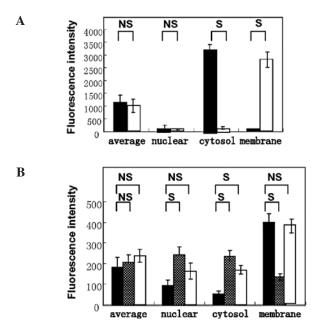


Figure 4. Fluorescence intensity (FI) of Tjp-2 expression in highly (PC-1.0) and weakly (PC-1) invasive and metastatic pancreatic cancer cells. (A) FI values of Tjp-2 expression in PC-1.0 cells. Black bar, untreated PC-1.0 cells; white bar, U0126-treated PC-1.0 cells. (B) FI values of Tjp-2 expression in PC-1 cells. Black bar, untreated PC-1 cells; dotted bar, DF-CM-treated PC-1 cells; white bar, DF-CM and subsequently U0126-treated PC-1 cells. S, significant; NS, not significant.

In the PC-1 cells (Fig. 4B), the average FI of whole cell Tjp-2 expression was 182.3±48.2. Weak Tjp-2 expression was observed in the nucleus (FI 93.6±27.2) and cytoplasm (FI 52.1±16.4), but Tjp-2 expression at the sites of cell-cell contact was strong (FI 401.5±41.3). After treatment with DF-CM for 36 h, the average whole cell expression of Tjp-2 was not significantly changed as compared to untreated cells (FI 207.1±35.4, P>0.05), although Tjp-2 expression in the nucleus (FI 242.7±38.9, P<0.05) and cytoplasm (FI 236.2±27.3, P<0.05), as well as at the sites of cell-cell contact (FI 135.6±15.8, P<0.05), was significantly increased. In addition, after subsequent U0126 treatment for 36 h, the FI of Tjp-2 expression in the cytoplasm (FI 169.1±20.0, P<0.05) was significantly increased in PC-1 cells, whereas no significant change in Tjp-2 FI was detected in the whole cell (FI 238.5±31.6, P>0.05), the nucleus (FI 163.7±37.3, P>0.05) or at the sites of cell-cell contact (FI 382.6±34.1, P>0.05).

Changes in cell dissociation status in highly (PC-1.0) and weakly (PC-1) invasive and metastatic pancreatic cancer cells. Light microscopic images showed that untreated PC-1.0 cells grew as single cells (Fig. 5A). Of note, the PC-1.0 cells aggregated and formed island-like cell colonies after treatment with U0126 for 36 h (Fig. 5B). In contrast to PC-1.0 cells, untreated PC-1 cells grew as island-like cell colonies (Fig. 5C). However, after a 36-h incubation with DF-CM, the island-like colonies of PC-1 cells became dissociated and the cells exhibited a morphological change, becoming elongated with a high frequency of pseudopodia formation (Fig. 5D), similar to the morphology of PC-1.0 cells. Moreover, the formerly dissociated PC-1 cells re-aggregated into island-like colonies after subsequent incubation with U0126 for 36 h (Fig. 5E).

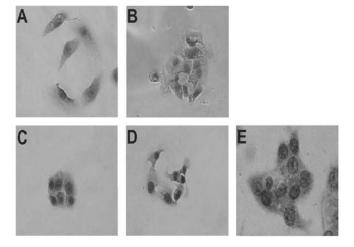


Figure 5. Morphological changes in highly (PC-1.0) and weakly (PC-1) invasive and metastatic pancreatic cancer cells. Untreated PC-1.0 cells (A) aggregated and formed island-like cell colonies after U0126 treatment for 36 h (B). The island-like cell colonies of untreated PC-1 cells (C) were dissociated by DF-CM treatment for 36 h (D), and the formerly dissociated cell colonies re-aggregated after subsequent U0126 treatment for 36 h (E). Papanicolaou's staining, original magnification x400.

## Discussion

In our previous studies, we found that dissociation factor (DF) induces the activation of the MEK2/ERK2 signal transduction pathway as well as cell dissociation in pancreatic cancer cells. By contrast, the MEK inhibitor U0126 inhibits the activation of the MEK2/ERK2 signal transduction pathway and cell dissociation in pancreatic cancer cells (5,6).

In our recent study, Tjp-2 was identified as an invasionmetastasis related gene by comparing highly invasive and metastatic pancreatic cancer cells (PC-1.0) with weakly invasive and metastatic pancreatic cancer cells (PC-1) via cDNA microarrays (7).

In our current study, we found that Tjp-2 mRNA is differentially expressed in the highly (PC-1.0) and weakly (PC-1) invasive and metastatic pancreatic cancer cells. Moreover, DF and U0126 were found to increase or decrease Tjp-2 mRNA expression, respectively. These results demonstrate that Tjp-2 is potentially involved in the regulation of invasion-metastasis in pancreatic cancer cells. Furthermore, the gene expression of Tjp-2 may be regulated by the MEK2/ERK2 signal transduction pathway.

In addition, although the protein level of Tjp-2 was markedly differentiated between PC-1.0 and PC-1 cells, the protein level of Tjp-2 was not affected by DF or U0126 treatment. These results suggest that the involvement of Tjp-2 in the invasion-metastasis of pancreatic cancer is regulated not only by the MEK2/ERK2 signal transduction pathway, but also by other factors. One such factor may be microRNAs (15), which are known to regulate the translation process (16,17).

Our current results indicate that DF and U0126 are able to change the intracellular localisation of Tjp-2 protein. Moreover, the intracellular arrangement of Tjp-2 is closely correlated with the cell dissociation status of pancreatic cancer cells. These results reveal that the proper localization of Tjp-2 to the sites of cell-cell contact may play a role in sustaining the stability of tight junctions and in preventing cell dissociation and subsequent invasion-metastasis in pancreatic cancer cells. Furthermore, it is possible that changes in the intracellular localization of Tjp-2 are involved in the regulatory mechanisms of cell dissociation and invasion-metastasis.

It is also worth noting that Tjp-2 protein can be phosphorylated, thus changes in Tjp-2 phosphorylation may regulate its intracellular localisation (18-20). In addition, since the translocation of Tjp-2 protein between the nucleus, cytoplasm and cell membrane was found to be correlated with the activation (phosphorylation) of MEK2/ERK2 signalling molecules, changes in the phosphorylation of Tjp-2 may be regulated by the MEK2/ ERK2 signal transduction pathway. Further investigation is required to determine the role of Tjp-2 phosphorylation in the regulatory mechanisms of pancreatic cancer.

In conclusion, Tjp-2 is involved in the regulation of cell dissociation in pancreatic cancer cells through changes in gene expression and intracellular localisation. Tjp-2 may serve as a new target for molecular therapies that prevent the invasion and metastasis of pancreatic cancer.

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