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PERIOD1 is an Anti-apoptotic Factor in Human Pancreatic and Hepatic Cancer Cells

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PERIOD1 (PER1) is a clock gene. We examined the effect of knockdown of PER1 on apoptosis in pancreatic cancer (MIA PaCa-2 and PANC-1) and hepatocellular carcinoma (HepG2) cells. Transfection of siRNA against PER1 into these cells increased the cleaved forms of caspases and poly-ADP-ribose-polymerase and induced apoptosis in all three cell lines. In the two pancreatic cancer cell lines, PER1 knockdown resulted in upregulation of Bax and downregulation of Bcl-2. Expression of p53 was not altered in the two pancreatic cancer cell lines containing mutated p53, but was upregulated in the HepG2 cells containing wild-type p53. Cell proliferation of MIA PaCa-2 and HepG2 was inhibited by PER1 knockdown. We also examined, by immunohistochemical staining, the expression of PER1 in pancreatic cancer tissue and found that PER1 was strongly expressed in pancreatic cancer cells. These results indicate that PER1 acts as an anti-apoptotic factor in pancreatic cancer cells.

Key words: apoptosis, clock gene, knockdown, pancreatic cancer, PERIOD1.

Abbreviations: PER1, PERIOD1; PARP, poly-ADP-ribose-polymerase; PBS, phosphate-buffered saline; siRNA, short interference RNA.

Circadian rhythms are daily oscillations of multiple biological processes and are tightly regulated by clock genes (*1*). Mammalian circadian rhythms are regulated by molecular clock systems based on negative feedback loops in normal and tumor cells (*2*, *3*). *Clock* and *brain-muscle-arnt-like-protein 1/2* (*Bmal 1/2*) are clock genes that positively regulate the expression of target genes, while, *PERIOD 1/2/3* (*PER1/2/3*), *cryptochromes 1/2* (*Cry1/2*) and *differentiated embryo-chondrocyte 1/2* (*DEC1/2*), which have also been designated as clock genes, negatively regulate target genes (*4*, *5*).

Recently, the clock genes have been shown to correlate with the circadian machinery in cancer cells. Sahar and Sassone-Corsi (*6*) suggested the possibility that clock may acetylate *estrogen receptor-α gene* in breast cancer cells, and DEC2 negatively regulates vascular endothelial growth factor (VEGF) expression in human oral cancer cells (*7*). These findings suggest that the clock genes are involved in the pathogenesis of cancer.

Apoptosis is programmed cell death and is regulated by the balance between pro-apoptotic and anti-apoptotic factors (*8*). Dysregulation of the apoptosis system often leads to the resistance of tumor cells against chemotherapy and radiation therapy (*9–11*). Gery *et al*. (*12*, *13*) reported that PER1 and PER2 play an important role in tumor suppression and the response to DNA damage in colon cancer and lung carcinoma cells. The expression of clock genes is reported to be related to apoptosis in several types of cell lines (*14*, *15*). However, the details about the relationship between clock genes and apoptosis have not been well elucidated.

In this study, we investigated the role of PER1 in apoptosis using the RNA-interference technique in two pancreatic cancer (MIA PaCa-2 and PANC-1) and one hepatocellular carcinoma (HepG2) cell lines.

MATERIALS AND METHODS

Cell Culture—MIA PaCa-2, PANC-1 and HepG2 cells were obtained from the American-Type Culture Collection (Manassas, VA, USA). MIA PaCa-2 cells were cultured in Dulbecco's Modified Eagle's Medium-high glucose (DMEM) (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) and 2.5% horse serum. PANC-1 cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS. HepG2 cells were cultured in DMEM supplemented with 10% FBS. The cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Short Interference RNA—Short interference RNA (siRNA) against PER1 was synthesized by QIAGEN

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(Hilden, Germany). The sequences for the sense and antisense PER1 siRNA were 5′-r (CGCUCGCCCUGGCCAAUAA) d (TT)-3′ and 5′-r (UUAUUGGCCAGGGCGAGCG) d (TT)-3′. The siRNA was transfected into the cells using the Lipofectamine 2000 reagent (Invitrogen). After transfection, the cells were incubated for 72 h.

Western Blotting—The cells were seeded at 5×10^4 cells per 35-mm well and lysed using M-PER lysis buffer (PIERCE, Rockford, IL, USA). Protein concentrations were determined using BCA assay. The lysates (20 μg protein) were subjected to SDS/PAGE, and obtained proteins were transferred to PVDF membranes. The membranes were incubated with specific antibodies against PER1 (Trans Genic Inc., Hyogo, Japan; 1 : 1000), PARP (1 : 1000), caspases-3 (1 : 500), caspases-7 (1 : 3000), caspases-9 (1 : 3000), Bcl-2 (1 : 5000) and Bid (1 : 20000), which were purchased from Cell Signaling Technology, Inc.; Bax (1 : 5000) and c-Myc (1 : 2000), which were purchased from Santa Cruz Biotechnology, Inc., p53 (Abcam, Cambridge, UK; 1 : 50000), or actin (Sigma; 1 : 30000) followed by a horseradish peroxidase-conjugated secondary antibody (1 : 5000). Immunoreaction-enhancer solution 1 (TOYOBO, Osaka, Japan) was used for dilution of the primary antibody. An ECL-plus or advanced-Western Blotting Detection kit (Amersham, Uppsala, Sweden) was used for detection.

Immunofluorescent Staining—Cells were seeded in a four-chambered glass slide. The cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 30 min. The cells were permeabilized with 0.2% Triton-X-100 in PBS for 10 min. Hoechst 33258 staining was used to examine nuclear condensation. The cells were observed using confocal laser scanning microscopy (Leica, Solms, Germany) and the numbers of cells positively stained with Hoechst 33258 were counted.

Immunohistochemistry—Human pancreatic cancer tissues were obtained around at 12 : 00 or 13 : 00. The expression of PER1 in moderately differentiated pancreatic cancer cells ($n = 5$) was examined by immunohistochemistry using serial sections of deparaffinized tissue. Immunoreactivity was detected using the DAKO ENVISION Kit/HRP (DAB) (Dako Cytomation, Kyoto, Japan). The sections were pretreated with L.A.B. solution (Polysciences, Inc., Warrington, PA, USA) for 5 min for antigen retrieval. This was followed by incubation overnight at 4°C with anti-PER1 antibody (1 : 400) diluted in Can Get Signal immunostain solution B (TOYOBO). The sections were then incubated with the HRP-conjugated secondary antibody. Finally, the sections were counterstained with Mayer's hematoxylin. Histological specimens were retrieved from the archives of Hirosaki University Hospital under the guidelines produced by the Japanese Society of Pathology.

MTS [3-(4,5-Dimethylthiazol-2-yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2H-Tetrazolium] Assay—MIA PaCa-2, PANC-1 or HepG2 cells were seeded in 96 well plates. The cells were transfected with control siRNA or siRNA against PER1. After 48, 72 or 96 h of transfection, the cells were added to each well along with Cell Titer 96 AQueous One Solution Reagent (Promega, Madison, WI, USA) and incubated at 37°C for an additional 1 h. The absorbance at 490 nm was measured using a 96 well plate reader.

RESULTS

Apoptosis was Induced by PER1 Knockdown in Pancreatic Cancer MIA PaCa-2 Cells—MIA PaCa-2 cells were transfected with control siRNA or siRNA against PER1. After 72 h of transfection, the cells were fixed and observed in differential interference contrast (DIC) or stained with Hoechst 33258. The cell density was lower in the cells transfected with PER1 siRNA than in the control cells (Fig. 1A). To examine if siRNA against PER1 induced apoptosis, the cells were stained with Hoechst 33258. The cells, which were stained with Hoechst 33258, were significantly increased by

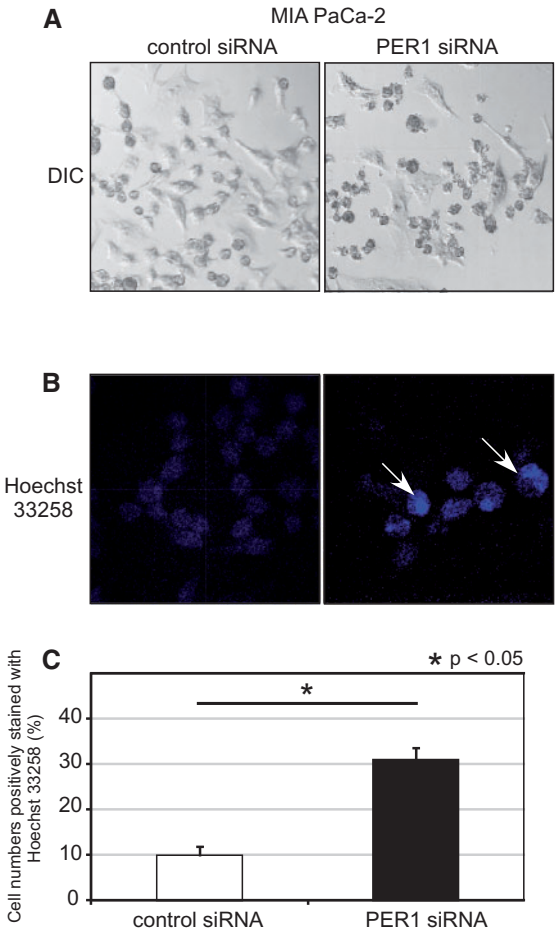

Fig. 1. **Apoptotic cells were observed after PER1 knockdown.** (A) MIA PaCa-2 cells were seeded in a four-chambered glass slide and incubated overnight. The cells were transfected with scrambled siRNA (control siRNA) or specific siRNA against PER1 (PER1 siRNA). After 72 h of transfection, the cells were fixed and observed in DIC (A) or stained with Hoechst 33258 (B). The arrows show the nuclear condensation. (C) The cells positively stained with Hoechst 33258 were counted in six random microscopic fields at ×40 magnification. Percentage of positive cells were shown, and each value represents the mean ± SE (bars) of three independent experiments $^*P < 0.05$, according to the *t*-test.

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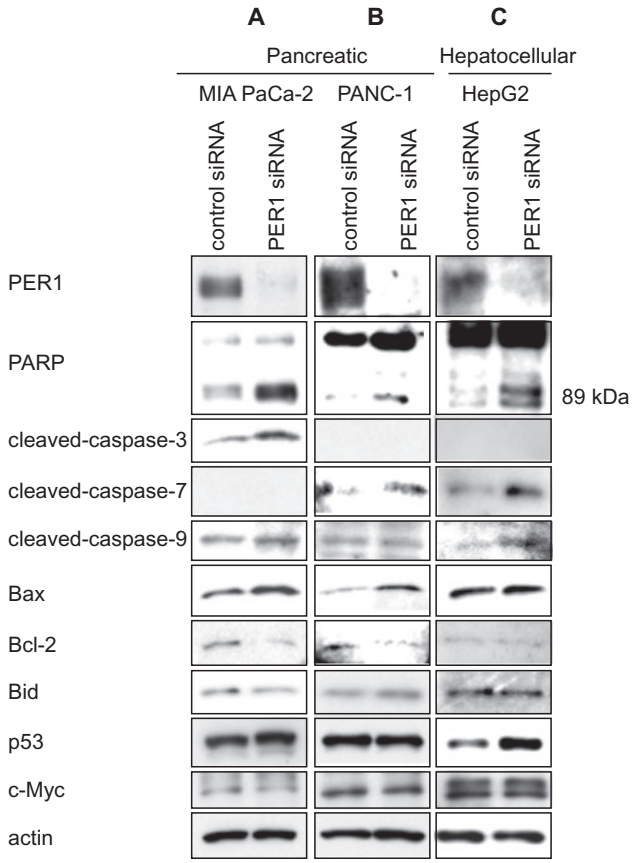

Fig. 2. **PER1 knockdown affects apoptosis-related proteins in MIA PaCa-2, PANC-1 and HepG2 cells.** (A) MIA PaCa-2, (B) PANC-1 or (C) HepG2 cells were transfected with control siRNA or siRNA against PER1. After transfection, the cells were lysed and the lysates were subjected to western blot analyses for PER1, PARP, cleaved caspases-3, cleaved caspases-7, cleaved caspases-9, Bax, Bcl-2, Bid, p53, c-Myc or actin.

transfection of PER1 siRNA (Fig. 1B and C). Hoechst 33258 stains nuclear condensation and nuclear condensation is one of the features of apoptosis. In addition to positive staining with Hoechst 33258, knockdown of PER1 increased the cleaved PARP and cleaved caspases-3. Therefore, it was suggested that knockdown of PER1 resulted in the apoptosis of MIA PaCa-2 cells. Knockdown of PER1 protein was confirmed by western blot analysis (Fig. 2A).

Effect of Knockdown of PER1 on Apoptosis-related Proteins in Pancreatic MIA PaCa-2 and PANC-1 Cells—To explore underlying mechanisms of apoptosis caused by PER1 knockdown, we examined whether knockdown of PER1 affects the expression of apoptosis-related proteins in MIA PaCa-2 cells. The cells were transfected with control siRNA or siRNA against PER1. After 72 h of transfection, the cells were lysed and the lysates were subjected to western blot analyses. PER1 knockdown increased the amounts of cleaved PARP (89 kDa) and cleaved caspases-3 in the MIA PaCa-2 cells (Fig. 2A). PER1 knockdown also increased Bax expression, while the expression of Bcl-2 protein was decreased. No obvious change was detected in the levels of cleaved caspases-9, Bid, p53, c-Myc or actin. Cleaved caspases-7 were not detected in this cell line. These data suggest that knockdown of PER1 induces apoptosis in MIA PaCa-2 cells and that alteration of the expression of Bax, Bcl-2 and cleaved caspases-3 is involved in this reaction. We also performed the same experiments in another pancreatic cancer cell line, PANC-1 cells (Fig. 2B). The results were similar to those for MIA PaCa-2 cells; the expression of cleaved PARP, cleaved caspases-7 and Bax was slightly increased by PER1 knockdown, while that of Bcl-2 was significantly decreased.

Knockdown of PER1 Induces Apoptosis in Hepatocellular Carcinoma HepG2 Cells—We also examined the effects of PER1 knockdown on apoptosis in another cell line, HepG2 cells. PER1 knockdown increased the expression of cleaved PARP, cleaved caspases-7, cleaved caspases-9 and p53, while the expression of Bax, Bcl-2, Bid and c-Myc proteins was not changed (Fig. 2C). Cleaved caspases-3 were not detected. This suggests that knockdown of PER1 also induces apoptosis in HepG2 cells, although the expression pattern of apoptosis-related proteins differed among the cell lines tested.

Effects of Knockdown of PER1 on the Proliferation of Pancreatic Cancer MIA PaCa-2/PANC-1 and Hepatocellular Carcinoma HepG2 Cells—To examine the role of PER1 in cell proliferation, we performed an MTS-assay in MIA PaCa-2, PANC-1 and HepG2 cells transfected with siRNA against PER1. MIA PaCa-2 cells transfected with siRNA against PER1 showed a 17 and 32% decrease in proliferation at 72 and 96 h, respectively (Fig. 3A). HepG2 cells transfected with siRNA against PER1 showed a 7, 11 and 10% decrease in proliferation compared with control cells at 48, 72 and 96 h, respectively (Fig. 3C). On the other hand, the proliferation of PANC-1 cells was not affected by PER1 siRNA (Fig. 3B).

PER1 Protein Expression in Normal and Cancer Cells of Pancreas—We further examined the expression of PER1 protein in pancreatic cancer tissues by immunohistochemical staining. PER1 was significantly stained in all of five cancer tissues tested (square T), while it was stained faintly in normal tissues (square N). The photographs of three cases were shown in Fig. 4.

DISCUSSION

Clock genes play a crucial role in regulating circadian rhythms. Various studies have indicated that the expression of clock genes is influenced by both physiological and pathological conditions, including tumor progression, type-2 diabetes, hypertension and familial advanced sleep phase syndrome (*16–20*). It has been reported that expression of PER1/2/3 is involved in the progression of breast cancer (*21–23*). However, the role of clock genes in pancreatic cancer is poorly understood. Therefore, we conducted this study to evaluate the role of PER1 in pancreatic cancer. Our previous report showed that knockdown of PER1 decreases the cell proliferation of MIA PaCa-2 cells (*24*). In the present study,

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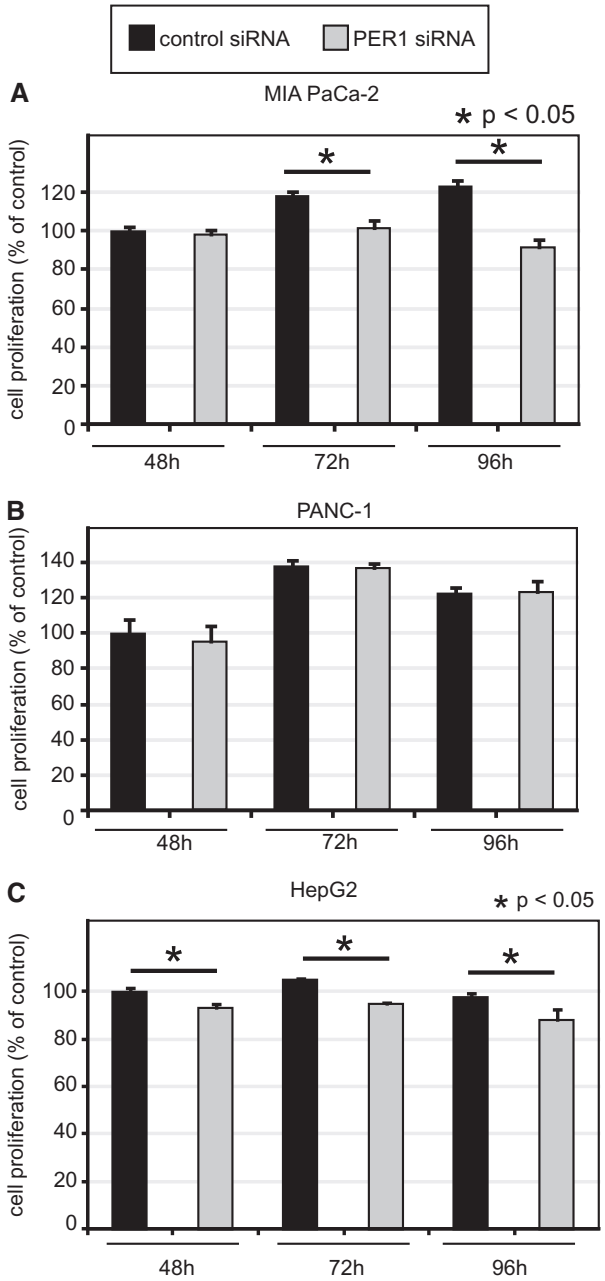

Fig. 3. **Knockdown of PER1 decreased the proliferation of MIA PaCa-2 and HepG2 cells.** (A) MIA PaCa-2, (B) PANC-1 or (C) HepG2 cells were transfected with control siRNA or siRNA against PER1. After 48, 72 or 96 h of transfection, cell proliferation was assessed using an MTS-assay. Each value represents the mean ± SE (bars) of three independent experiments $^{*}P < 0.05$, according to the *t*-test.

we focused on the potential role of PER1 in apoptosis of pancreatic cancer cells. We found that knockdown of PER1, by siRNA transfection, induced apoptosis in MIA PaCa-2 and PANC-1 cells and inhibited cell proliferation in MIA PaCa-2 cells. This suggests that PER1 has anti-apoptotic properties. However, our results are in conflict with previous reports related to other cancers; overexpression of PER1 or PER2 has been reported to induce apoptosis in malignant lymphoma, colon cancer, lung carcinoma and mammary carcinoma in a p53-dependent manner (*12*, *13*, *16*). MIA PaCa-2 and PANC-1 cells used in this study had mutations in their *p53 gene*, so the apoptosis induced by knockdown of PER1 may be p53-independent. In addition, we showed that knockdown of PER1 in MIA PaCa-2 cells significantly decreased cell proliferation in a time-dependent manner, but did not affect c-Myc expression. This suggests that some factors other than c-Myc may be relevant to the regulation of proliferation in this cell line. This observation is also in conflict with previous reports (*12*, *13*), which showed that overexpression of PER1 induces c-Myc expression and inhibits colony formation in colon cancer and lung carcinoma cells (*12*, *13*). These discrepancies may be due to the characteristics of the cell lines used or differences in the experimental procedures (knockdown vs overexpression).

We further tried the knockdown of PER1 in Capan-2 cells, another pancreatic cancer cell line, which possesses wild-type p53. However, we were unable to achieve a high-enough transfection efficiency in this cell line. So, we also used hepatocellular carcinoma HepG2 cells, which also express wild-type p53. We found that knockdown of PER1 induced upregulation of p53 and apoptosis in HepG2 cells. Thus, knockdown of PER1 induced apoptosis in these cell types. However, the apoptosis-related molecules were differentially expressed in these cells. These results suggest that apoptosis can be induced by PER1 knockdown, via different pathways among these cell types. We also examined the expression of the PER1 protein in pancreatic cancer tissue. PER1 was intensely stained in pancreatic cancer cells, but was faintly stained in normal pancreatic cells. Taken together, our results suggest that PER1 has anti-apoptotic properties in pancreatic cancer cells.

We were unable to elucidate the reason for the discrepancies between our findings and those of the previous reports (*12*, *13*). Another clock gene, *DEC1*, is also thought to be related to the regulation of apoptosis. Overexpression of DEC1 was reported to induce apoptosis in thymocytes and NIH3T3 cells (*14*), while it inhibits apoptosis in colon cancer and HEK-293T cells (*25*, *26*). So, we speculate that clock genes such as *PER* and *DEC* regulate the balance between pro-apoptotic and anti-apoptotic reactions. Details of the roles of clock genes in the regulation of apoptosis should be investigated further.

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CONFLICT OF INTEREST

None declared.

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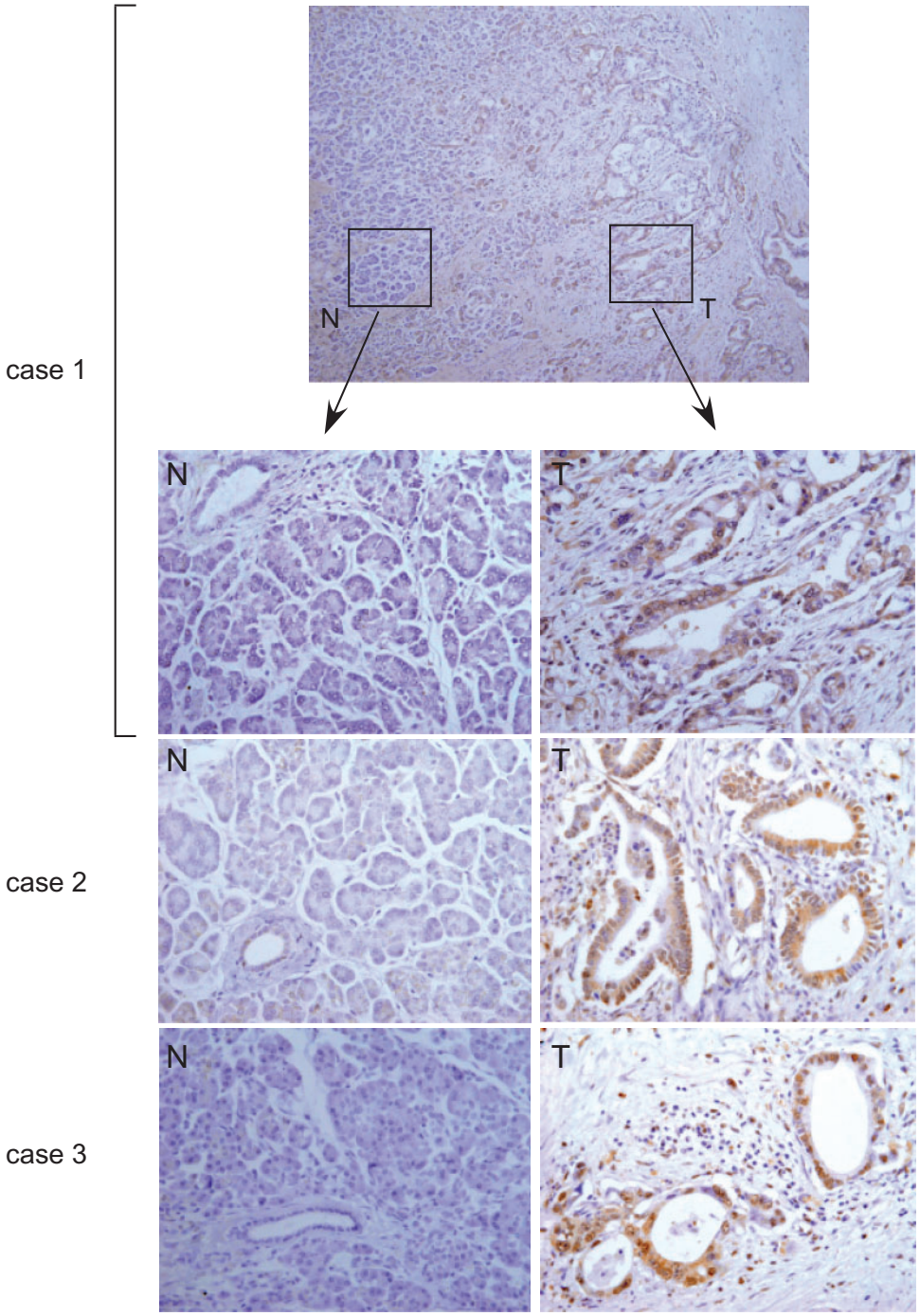

Fig. 4. **Immunohistochemical staining for PER1 protein in pancreatic cancer tissue.** PER1 was significantly stained in pancreatic cancer tissues from three cases (T), while it was faintly stained in normal tissues (N).

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