Clinical Significance of CD155 Expression in Human Pancreatic Cancer

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Abstract. *Background/Aim: CD155 is expressed in many types of human cells and has diverse functions. We herein evaluated the clinical importance of CD155 in pancreatic cancer. Materials and Methods: We investigated CD155 expression in 134 patients with pancreatic cancer, and evaluated the correlations of CD155 with prognosis, tumor immunity and angiogenesis. Furthermore, CD155 functions were examined. Results: CD155 expression was abundant in pancreatic cancer tissues. Patients with high CD155 expression had poorer postoperative prognosis than those with low expression. Multivariate analysis indicated that CD155 expression had a significant independent prognostic value. Tumor CD155 expression inversely correlated with the presence of tumor-infiltrating lymphocytes. Furthermore, it significantly positively correlated with vascular endothelial growth factor expression and intratumoral microvessel density. In addition, silencing of CD155 inhibited proliferation, and induced cell-cycle arrest at G_2/M phase in pancreatic cancer cells. Conclusion: CD155 may play a critical role through both immunological and non-immunological mechanisms in pancreatic cancer and may be a therapeutic target for this intractable malignancy.*

Pancreatic cancer is one of the most deadly types of cancer in humans, and the number of patients has been increasing. Complete surgical resection offers the only chance for cure or long-term survival for patients with pancreatic cancer (1). However, the disease is so aggressive that more than half of patients already have distant metastasis at the time of diagnosis and surgery is currently indicated for only about 20% of patients (2). In addition, although several therapeutic improvements including new reagents and regimens of chemotherapy have recently been introduced, the survival benefit is modest and the median survival time for patients with metastatic pancreatic cancer is still as low as less than one year (2-6). Thus, the identification of novel targets and development of new therapeutic approaches are required to improve patient prognosis.

CD155, originally identified as human poliovirus receptor, is a type I transmembrane glycoprotein belonging to the immunoglobulin superfamily, also termed NECL5 or TAGE4 (7-9). It has been reported to be expressed on many types of cells and to exert diverse functions (7, 10-15). One of major roles of CD155 is its immunoregulatory function in a variety of immune cells. It has recently been reported that the interaction of CD155 with its ligand T-cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT) on T-cell and natural killer (NK) cell induces a direct inhibitory effect on cell proliferation and cytotoxicity (11-13, 15, 16). These effects relate to decrease in expression of T-box expressed in T cells (T-BET), GATA binding protein 3, interferon (IFN) regulatory factor 4, and retinoic acid-related orphan receptor c with inhibition of cytokine production, including IFNγ and TNFα. On the other hand, CD155 is also bound to DNAX accessory molecule-1 (DNAM-1)/CD226 on T-cells and NK cells. This interaction conversely enhances cytotoxic function (17, 18). Therefore, the immunological role of CD155 is complex, and its function may depend on the circumstances.

Besides its immunological roles, CD155 also contributes to various other cellular functions, including cellular adhesion, polarization, differentiation, movement, proliferation, and survival (8, 9, 19, 20). Furthermore, it has been reported that CD155 was highly expressed in several types of human malignant tumors (21-24). Few clinical studies have also demonstrated that there were significant inverse correlations between tumor CD155 expression and prognosis of patients with lung adenocarcinoma and soft-tissue sarcomas (25, 26). In addition, another study showed that CD155 overexpression correlates with malignant phenotype in cutaneous melanoma

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Table I. *Comparison of clinicopathological characteristics according to tumor CD155 expression.*

		CD155 Expression		
	Total	Low	High	*p*-Value
Characteristic	n=134	n=66 (%)	n=68 (%)	
Gender				
Male	78	40 (60.6)	38 (55.9)	0.603
Female	56	26 (39.4)	30 (44.1)	
Age. years				
≤65	52	29 (43.9)	23 (33.8)	0.288
>65	82	37 (56.1)	45 (66.2)	
Histopathological grading				
G1	40	16 (24.2)	24 (35.3)	0.349
G2	75	41 (62.1)	34 (50.0)	
G3	15	8 (12.1)	7 (10.3)	
G4	4	1 (1.5)	3 (4.4)	
Tumor status				
T1	8	4 (6.1)	4 (5.9)	0.913
T2	22	12 (18.2)	10 (14.7)	
T3	95	45 (68.2)	50 (73.5)	
T4	9	5 (7.6)	4 (5.9)	
Nodal status				
N0	57	28 (42.4)	29 (42.6)	0.999
N1	77	38 (57.6)	39 (57.4)	
Metastatic status				
M0	123	61 (92.4)	62 (91.2)	0.999
M1	11	5 (7.6)	6 (8.8)	
UICC stage				
IA, IB	21	12 (18.2)	9 (13.2)	0.591
IIA	35	16 (24.2)	19 (27.9)	
IIB	62	29 (43.9)	33 (48.5)	
III	5	4 (6.1)	1 (1.5)	
IV	11	5 (7.6)	6 (8.8)	

UICC, International Union Against Cancer.

(27). Moreover, CD155 enhances cell invasion and migration in human glioblastoma and lung adenocarcinoma cells *in vitro* (22, 28). However, the precise roles of CD155 in tumor progression and metastasis are not fully elucidated, and its clinical significance is largely unknown.

Based on the above reports and a series of our previous studies on T-cell-negative regulatory pathways in tumor immunity (29-32), we hypothesized that CD155 may play certain roles in human pancreatic cancer possibly through its immunoregulatory function. To this end, we tried to clarify the clinical importance of CD155 in human pancreatic cancer. Furthermore, we also addressed the biological roles of CD155 in pancreatic cancer.

Materials and Methods

Patients and specimens. We examined 134 patients with pancreatic cancer who had undergone surgery at the Nara Medical University Hospital, Japan between 1992 and 2010. The median age of the patients was 66.5 years, with a range of 33 to 82 years. No one received preoperative treatment. All tumors were diagnosed as pancreatic ductal adenocarcinoma. Postoperative pathological analysis revealed metastasis in para-aortic lymph nodes in 11 patients and defined as M1, stage IV. These patients were included in this study. Tissues, both cancerous and non-cancerous, were obtained from resected specimens and were then rapidly frozen at −80˚C for storage until use. The remainder of each specimen was fixed in 10% phosphate-buffered formalin and embedded in paraffin. Tumors were classified according to the TNM staging system (33). Primary tumor tissues from these patients were included for histological analysis in this study. Follow-up was until death or October 2014. Written informed consent was obtained from patients to use their surgical specimens and clinicopathological data for research purposes.

Cell lines and culture. The human pancreatic cancer cell lines MIAPaCa-2 and PANC-1 were obtained from RIKEN BioResource Center (Tsukuba, Ibaraki, Japan) and cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum.

Antibodies. Monoclonal antibody (mAb) against human CD155/PVR (mouse immunoglobulin G1) was purchased from R&D Systems (Minneapolis, MN, USA). Anti-cleaved poly (ADP-ribose) polymerase (PARP), anti-phosphorylated extracellular stress regulated kinase (p-ERK), anti-phosphorylated c-JUN NH2-terminal kinase (p-JNK), and anti-phosphorylated p38 (p-p38) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-actin, anti-vascular endothelial growth factor (VEGF), anti-cyclin-dependent kinases 25 in complexes with cyclin B (CDC25C), and anti-p27 mAbs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-CD4, CD8, CD45RO, and CD31 mAbs were purchased from Dako Japan (Kyoto, Japan).

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissues were cut into 5-μm sections, de-paraffinized, and rehydrated in a graded series of ethanol. Antigen retrieval was carried out by heating tissue sections using a Target Retrieval Solution, pH 9.0 (DAKO). To block endogenous peroxidase, sections were immersed in 0.3% solution of hydrogen peroxide in absolute methanol for 5 minutes at room temperature and washed in fresh phosphate buffered saline three times, each for 5 minutes. Purified each mAb was added and sections were incubated overnight at 4˚C. Sections were then washed in phosphate buffered saline three times, for 5 min each, and then EnVision+, Mouse/HRP or Rabbit/HRP (DAKO) was used according to the instructions of the manufacturer. Reaction products were visualized with 3,3'-diaminobenzidine tetrahydrochloride, and the sections were counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene, and coverslipped.

Evaluation of immunostaining. Authorized pathologists who had no knowledge of the patients' clinical status and outcome evaluated immunohistochemistry for CD155/*PVR*. At least 200 tumor cells were scored per field at ×400 magnification. Positive cells were defined as cells with strongly and clearly brown immunostained cytoplasm. Specimens with a 50% or more $CD155^+$ tumor cells were classified as CD155-high and others as CD155-low. Since staining was consistently intense in most cases, the intensity of each sample was not counted in this study. Immunohistochemistry for $CD4^+$, $CD8^+$, and $CD45RO^+$ T-cells were evaluated. An average of at least 50 $CD4^+$, $CD8^+$, and $CD45RO^+$ tumor-infiltrating T-lymphocytes

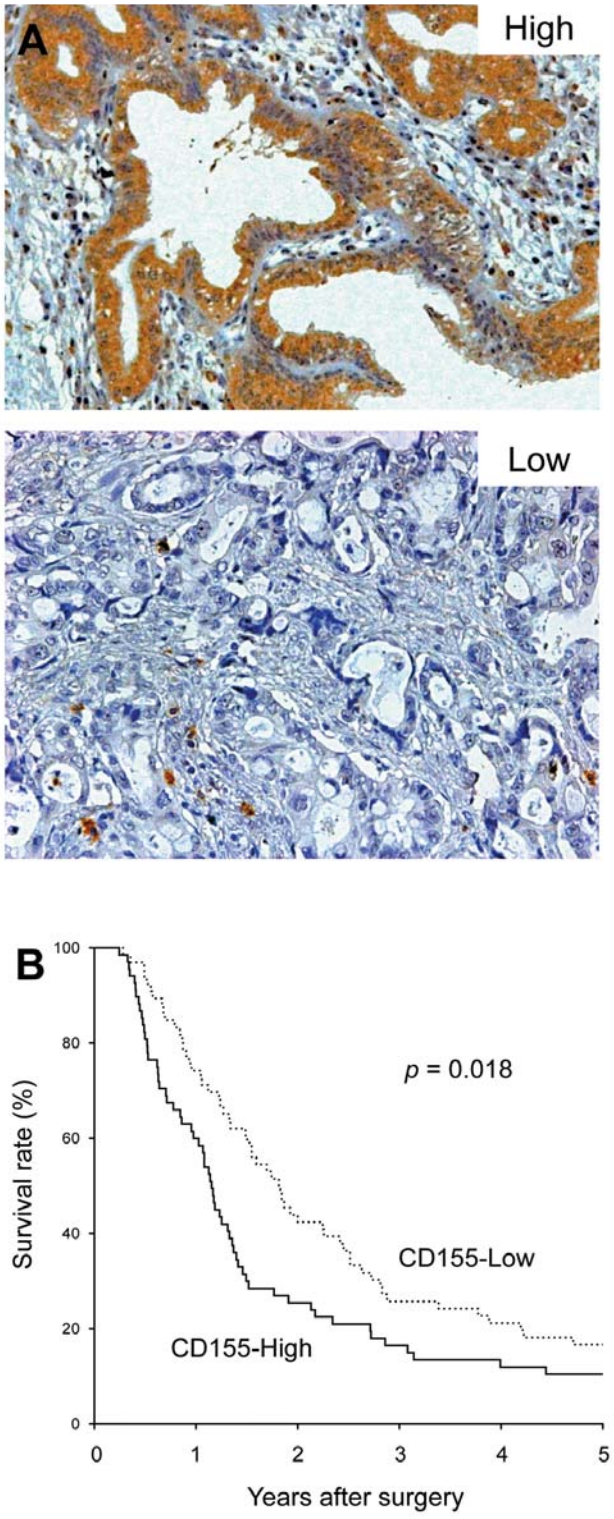

Figure 1. *CD155 expression in human pancreatic cancer tissues. A: Representative case of high positivity (high) and low positivity (low) for CD155. Original magnification, ×200. B: Overall survival of 134 patients with pancreatic cancer in relation to tumor CD155 status. Patients with tumors with high CD155 expression had significantly poorer prognosis compared with those with low expression (p=0.018).*

(TILs) per field at ×200 magnification were scored in five fields. The mean numbers of $CD4^+$, $CD8^+$, and $CD45RO^+$ T-cell counts were 165.0, 199.5, and 205.9, respectively. Immunostaining of VEGF was evaluated as previously described (34).

For microvessel counting, the five most highly vascularized areas were counted at ×200 magnification, and the average counts of CD31 positive cells were recorded (34, 35). The mean microvessel count of these tumors was 55.9.

Extraction of total RNAs and real-time reverse transcriptase polymerase chain reaction (PCR) analysis. Total RNA was isolated using RNAspin Mini (GE Healthcare, Tokyo, Japan) and first-strand cDNA was synthesized from 1 μg RNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. Real-time quantitative PCR analysis was carried out using an ABI Prism 7700 sequence detector system (Applied Biosystems). All primer/probe sets were purchased from Applied Biosystems. PCR was carried out using the TaqMan Universal PCR Master Mix (Applied Biosystems) using 1 μl of cDNA in a 20 μl final reaction volume. The PCR thermal cycle conditions were as follows: initial step at 95˚C for 10 minutes, followed by 40 cycles of 95˚C for 15 seconds and 60˚C for 1 min. The expression level of the housekeeping gene β2-microglobulin was measured as an internal reference with a standard curve to determine the integrity of template RNA for all specimens. The ratio of the mRNA level of each gene was calculated as follows: (absolute copy number of each gene)/(absolute copy number of β2-microglobulin).

Transfection of small interfering RNA for CD155/PVR. For our transfection analyses, MIAPaCa-2 and PANC-1 cells were seeded in 6-well plates and transfected either with control RNA (Santa Cruz Biotechnology) or with 10 nM of small interfering RNA (siRNA) of CD155/PVR. Transfections were carried out using the Lipofectamine system (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol when cells achieved about 30% confluence. The CD155/PVR siRNA duplexes, generated with 30-dTdT overhangs and prepared by QIAGEN (Venlo, Netherlands), were chosen against the DNA target sequence 5'-TCCTGTGGACAAA CCAATCAA-3'.

Cell viability assay. Cell viability was determined using the Celltiter 96 aqueous one solution cell proliferation assay kit, according to the instruction manual (Promega Corporation, Madison, WI, USA). Briefly, aliquots of 1×10^3 cells per well were cultured in 96-well plates with CD155 or control siRNA for 72 h. After incubation, 3-(4,5-dimethyl-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium (MTS), inner salt reagent (Promega Corporation, Madison, WI, USA) was added to each well and plates were incubated for an additional one hour. The absorbance at 492 nm was recorded with a 96-well plate reader. Each experiment was performed in triplicate and repeated at least thrice.

Cell-cycle analysis. For cell-cycle analysis by flow cytometry, we used the Cycle TEST™ PLUS kit (BD Biosciences, Franklin Lakes, NJ, USA). We trypsinized the cells, fixed them in 70% ethanol, resuspended them in Dulbecco's phosphate-buffered saline containing RNase A (50 μg/ml), 0.1% sodium citrate, propidium iodide (50 μg/ml) and 0.1% Triton X-100. We then analyzed the samples by a FACScalibur using Cell Quest Pro software (BD Biosciences). Under each set of experimental conditions, 20,000 events were analyzed. All experiments were performed at least thrice in duplicate.

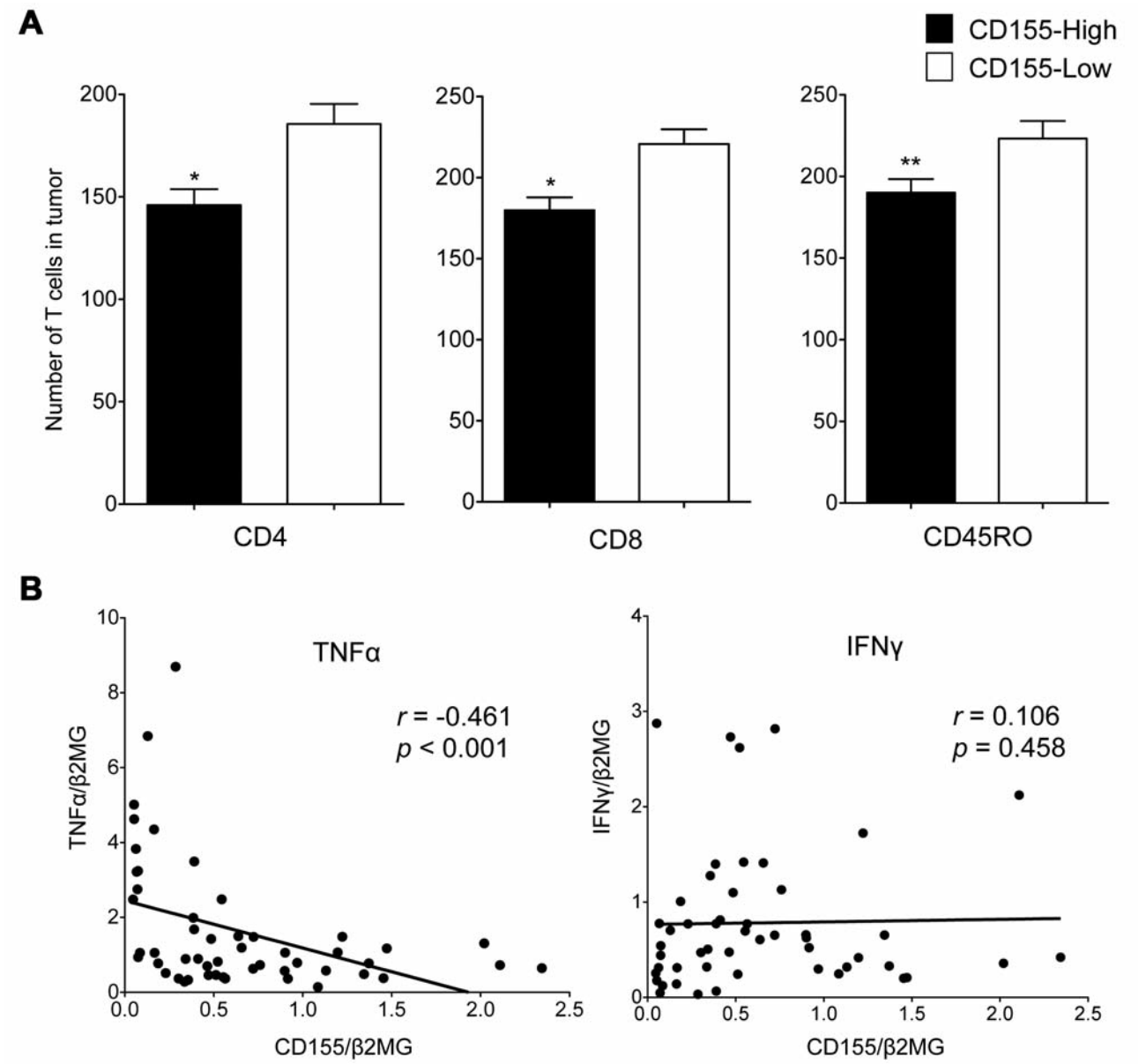

Figure 2. *Inverse correlation between tumor CD155 status and tumor-infiltrating T-lymphocytes. A: The number of each tumor-infiltrating T-lymphocyte subset according to CD155 status as evaluated by immunohistochemistry. *p<0.01, **p<0.05. B: Pearson's correlation of coefficient of CD155 with TNFα and IFNγ gene expression by quantitative real-time polymerase chain reaction (n=51).*

Preparation of cell lysates and western blot analysis. We resolved the cell lysates in sodium dodecyl sulfate polyacrylamide gels and transferred them onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA), which were then blocked in 5% skim milk at room temperature for one hour. The membranes were incubated with the indicated primary antibody overnight at 4˚C, and then incubated with horseradish peroxidase-conjugated IgG (Santa Cruz Biotechnology). We detected peroxidase activity on X-ray films using an enhanced chemiluminescence detection system.

Statistical analysis. The overall survival time was calculated from the date of surgery to the date of death. Kaplan-Meier survival calculations and the corresponding log-rank tests were carried out to determine differences in survival rates. Multivariate analysis was performed using the Cox regression model. We used the term of residual tumor status as R factor, tumor status as T factor, nodal status as N factor, and metastatic status as M factor in tumor-node-metastasis classification. Results were analyzed using the Student t-test, the chi-square test, Fisher's exact test, or the Mann-Whitney U-test to determine significant differences. Spearman's rank test was

Table II. *Univariate and multivariate prognostic analysis of 134 patients with pancreatic cancer.*

	Univariate analysis			Multivariate analysis		
Characteristic	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value
Age (≤ 65 *vs*. > 65 years)	1.028	0.711-1.486	0.885			
Gender (female *vs*. male)	1.001	0.683-1.468	0.996			
Tumor status (T3-4 *vs*. T1-2)	1.650	1.057-2.575	0.028	1.245	0.766-2.024	0.377
Nodal status (N1 *vs*. N0)	1.708	1.178-2.476	0.005	1.300	0.861-1.964	0.213
Metastatic status (M1 *vs*. M0)	4.571	2.398-8.714	<0.001	2.774	1.417-5.433	0.003
Residual tumor (R1-2 *vs*. R0)	2.168	1.497-3.138	<0.001	1.868	1.261-2.766	0.002
Histopathological grading (G3-4 *vs*. G1-2)	1.488	0.899-2.461	0.122			
CD155 status (high *vs*. low)	1.546	1.075-2.224	0.019	1.476	1.019-2.139	0.040

HR, Hazard ratio; CI, confidence interval.

also used to examine the correlation between two factors. A p-value of less than 0.05 was considered statistically significant. The statistical analyses were performed using the SPSS software program, version 19.0 (SPSS, Chicago, IL, USA).

Results

CD155 expression and prognostic value in human pancreatic cancer. We first evaluated the CD155 expression in 134 actual human pancreatic cancer tissues by immunohistochemistry. CD155 was abundant and expressed mainly in the plasma membrane and cytoplasm of cancer cells (Figure 1A). On the other hand, there was limited expression in non-cancer tissues, including islet cells. Each sample was classified into one of two groups according to more or less than 50% CD155 positivity (Figure 1A). As a result, 68 (50.7%) patients were classified as high and 66 (49.3%) were classified as low for CD155 expression at the protein level. Interestingly, the patients with CD155-high tumor had significantly poorer postoperative prognosis than patients with CD155-low (p=0.018) (Figure 1B). The median survival times were 421 days in patients with CD155-high and 666 days in those with CD155-low.

Next, we evaluated the correlation of the CD155 expression with clinicopathological findings. There were no significant differences between the two groups for any clinicopathological finding, including TNM status and pathological UICC stage (Table I). Furthermore, the multivariate analysis using Cox regression model showed that positive tumor CD155 status was a significant independent negative prognostic factor (HR=1.476, p=0.040). In addition, metastatic status and residual tumor status were defined as independent negative prognostic factors (Table II). These data suggest that CD155 expression may play an important role in tumor progression in pancreatic cancer independently of conventional TNM classification.

Inverse correlation between tumor CD155 status and the presence of TILs. We then analyzed the correlation between tumor CD155 expression and TILs by immunohistochemistry. We found that there were significant inverse correlations between CD155 expression and TILs ($CD4^+$ T-cells, p=0.005; $CD8^+$ T-cells, p<0.001; $CD45RO^+$ cells, p=0.020) (Figure 2A). Furthermore, in order to evaluate local immune activation, we examined cytokines by real-time RT-PCR analysis. We found statistically significant inverse correlation between CD155 expression and TNFα (r=−0.461, p<0.001), although there was no significant correlation with IFNγ (Figure 2B). These data suggest that CD155 expression on human pancreatic cancer cells might inhibit the infiltration of various TILs and local immune activation.

Association of CD155 with VEGF expression and angiogenesis. We then explored the relationship between CD155 and angiogenesis, since CD155 was recently reported to interact with VEGF and induce angiogenesis in human vascular endothelial cells (36). High positivity for CD155 expression was found to correlate significantly with intense expression of VEGF in human pancreatic cancer (p=0.035; Figure 3A). This was also confirmed at mRNA levels, as determined by real-time PCR showing significant positive correlation between CD155 and VEGF expression (r=0.519, p<0.001) (Figure 3B). Furthermore, we examined the intratumoral microvessel density by immunohistochemistry. A single microvessel was defined as any brown immunostained endothelial cells separated from the adjacent microvessels, tumor cells, and other connective tissue elements (Figure 3C). The microvessel count ranged from 20.7 to 115.0, with a mean count of 55.9. There was a significant positive correlation between CD155 expression and intratumoral

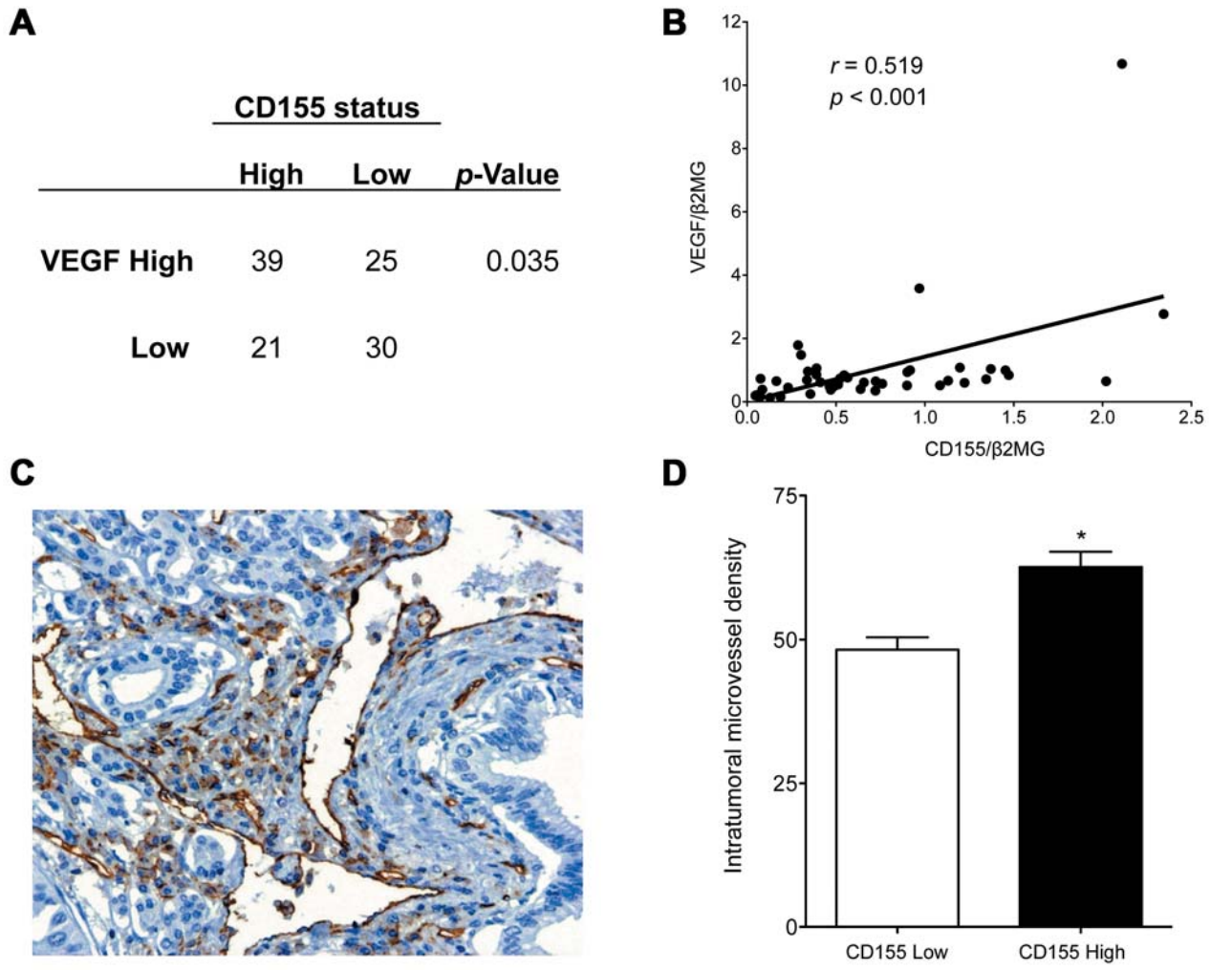

Figure 3. *Relationship between CD155 expression and angiogenesis in human pancreatic cancer. A, B: Positive correlation between CD155 and vascular endothelial growth factor (VEGF) expression in human pancreatic cancer by immunohistochemistry and quantitative real-time polymerase chain reaction. C: Immunohistochemical staining of CD31 in human pancreatic cancer tissues. Original magnification ×200. D: The association between intratumoral microvessel density and CD155 intensity by immunohistochemistry. *p<0.001.*

microvessel density ($p<0.001$) (Figure 3D). These data indicate that CD155 might play an important role in angiogenesis in human pancreatic cancer.

Silencing of CD155 inhibits proliferation of human pancreatic cancer cells. Finally, in order to evaluate the therapeutic potential of targeting *CD155* in pancreatic cancer, we employed the siRNA silencing technique. When transfected with *CD155* siRNA for up to 72 h, both mRNA and protein expressions of CD155 in human pancreatic cancer cell lines MIAPaCa-2 and PANC-1 were substantially reduced (Figure 4A and B). As a result, the cell proliferation was significantly inhibited by *CD155* gene silencing in these cells (Figure 4C).

Furthermore, so as to precisely investigate the effect of *CD155* knockdown in these cells, we performed flow cytometric analysis. The percentage of cells in the G_2/M phase was significantly increased by inhibition of *CD155* knockdown compared to controls (Figure 4D and E). The data demonstrate that the knockdown of *CD155* induces cell-cycle arrest in the G_2/M phase and thereby inhibits proliferation of human pancreatic cancer cells.

To further define the underlying mechanisms, we investigated several pathways in association with CD155 expression. To this end, we examined the effect of knockdown of *CD155* on the expression of VEGF, cyclin, CDK, CDK inhibitor, mitogen-activated protein (MAP) kinases including ERK, JNK, p38, and PARP as apoptosis marker (37, 38). As a result, p27,

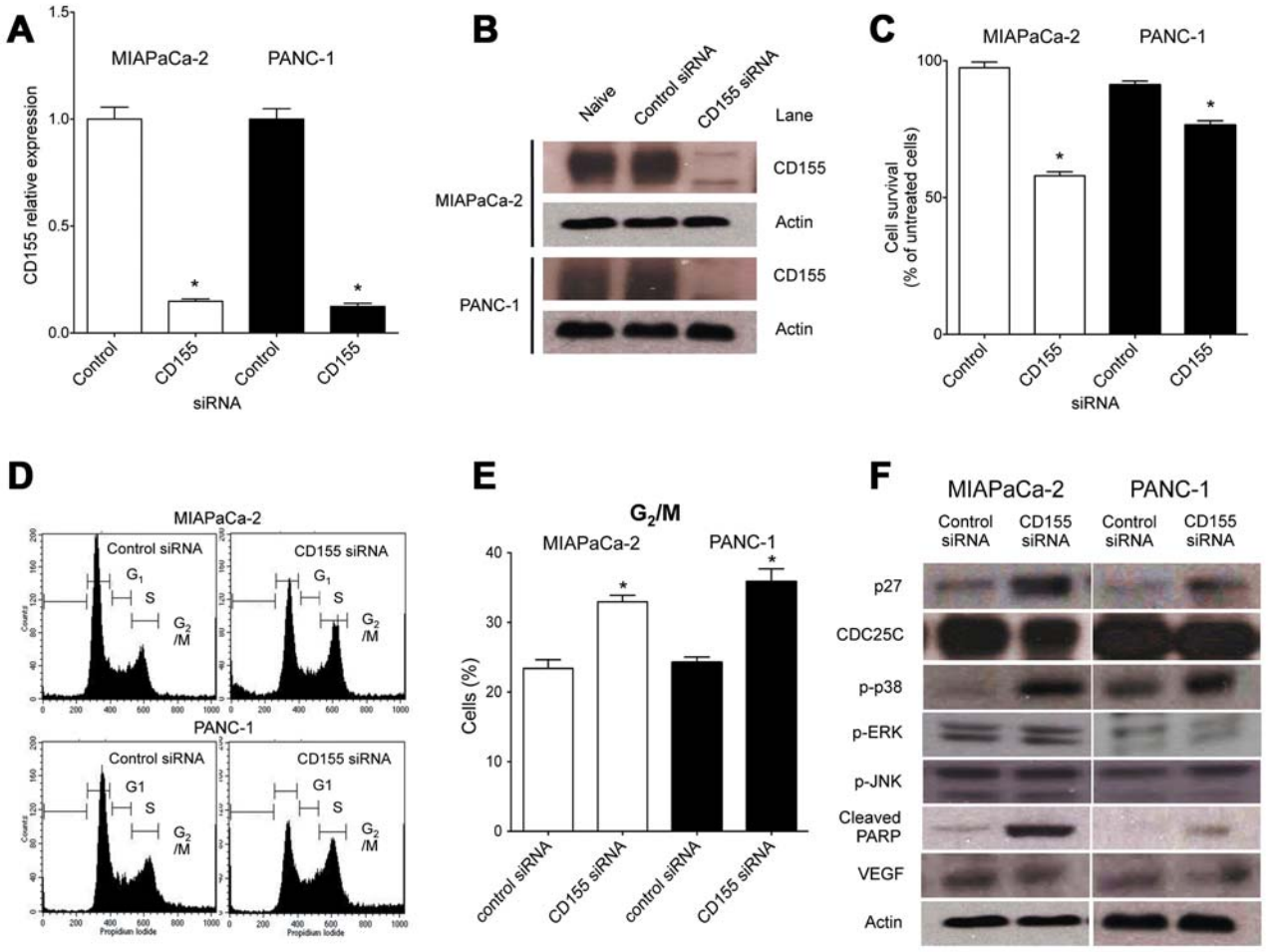

Figure 4. *CD155 blockade inhibits the growth of pancreatic cancer cells. A: MIAPaCa-2 and PANC-1 cells were transfected with CD155 siRNA or control RNA. The relative expression of CD155 was determined by quantitative real-time polymerase chain reaction. The expression of mRNA level of CD155 was reduced in both cell lines when transfected with CD155 siRNA for up to 72 h (n=4 of each group). B: Cells were treated as above. Total protein lysates were extracted and subjected to immunoblotting analysis using antibodies against CD155 and actin. The protein expression of CD155 was also effectively suppressed in both cells. C: After 72 h incubation, cell proliferation was determined by MTS assay. Cell proliferation was significantly inhibited by CD155 gene silencing in both cell lines (n=5 of each group). The figure is representative of at least three independent experiments. *p<0.001 versus control siRNA.*

cleaved PARP and phosphorylated p38 were increased by *CD155* knockdown, while CDC25C was somewhat reduced (Figure 4F). However, knockdown of *CD155* did not affect VEGF, p16, CDC2, cyclin B and D1, and phosphorylated ERK and JNK.

Discussion

Tumor cells escape immune surveillance by expressing several ligands that engage inhibitory T-cell receptors and attenuate T-cell functions within the tumor microenvironment (39, 40). Our group and others have reported that tumor-infiltrating $CD4^+$, $CD8^+$, or $CD45RO^+$ T-cells have a better impact on prognosis in several types of human cancer, including pancreatic cancer (29, 41-45). Therefore, a strategy enhancing TIL infiltration is a potentially promising approach to anticancer treatment. In fact, blockade of immunological checkpoints, including the B7/cytotoxic T-lymphocyte antigen-4 and programmed cell death protein 1, has been evaluated in clinical trials, and demonstrated to induce certain effects and prolong patient survival (46-48). However, the clinical efficacy seems to be limited. Therefore, there is still a need to explore other novel therapeutic targets. In this study, we addressed the clinical significance and functional role of a recently discovered immunoinhibitory ligand, CD155, in pancreatic cancer.

To date, the precise roles of CD155 in human physiological and pathological conditions have not been fully-elucidated. Especially in tumor biology, its function is largely unknown. In immunological functions of CD155, previous studies have suggested that CD155 might have both stimulatory and inhibitory roles under various conditions. The antitumor effect by the interaction of CD155 and DNAM-1 induced stimulation of these immune cells *in vivo* in a murine T-cell lymphoma (49). In sharp contrast, it was recently shown that TIGIT also binds to CD155, and inhibits T-cell and NK cell activity, including secretion of cytokines such as IFNγ and TNFα (11-13, 16, 50). Furthermore, CD155 expression on human vascular endothelial cells has been demonstrated to attenuate the acquisition of effector functions in $CD8^+$ T-cells (16). Interestingly, TIGIT inhibition has been reported to be dominant over the coacti vation of DNAM-1 on T-cells and NK cells in human tumor cell lines (11, 12, 15). Taken together, experimental data are conflicting, and the functions of CD155 in tumor immunity may depend on each tumor type and each tumor environment.

In this study, we first examined CD155 expression in clinical pancreatic cancer and found that it was abundant in most human pancreatic cancer tissues. Although CD155 has been reported to be expressed in many kinds of cells, our immunohistochemical study showed that there was only limited expression of CD155 in non-cancer tissues of the pancreas, including the interstitial area. Importantly, this study on clinical materials clearly demonstrates that CD155 plays an inhibitory role in tumor immunity in pancreatic cancer. This was confirmed by the systematic analysis of TILs in association with CD155. Furthermore, patients with high CD155 expression had significantly poor prognosis in comparison with patients with low CD155 expression. More importantly, the multivariate analysis demonstrated that tumor CD155 status was as a significant independent prognostic factor for patients with pancreatic cancer. A few previous reports have shown that CD155 expression contributes to the postoperative prognosis of patients with malignant tumors, including lung adenocarcinoma and soft-tissue sarcomas (25, 26). Our data may warrant further investigation of CD155 as a therapeutic target. However, the immunological roles of CD155 are complicated, and many immune cells other than pancreatic cancer cells might be influenced by CD155-targeted therapy. In addition, it may also be possible that TILs suppress or down-regulate *CD155* expression on tumors. Therefore, further careful evaluation is required since acquired immunodeficiency, autoimmune disease or unknown adverse events may be induced by CD155-targeted therapy.

Next, we focused on angiogenesis as another underlying mechanism. Angiogenesis plays a key role in tumor growth and metastasis. VEGF is the dominant proangiogenic growth factor in the tumor microenvironment (51, 52). It was recently reported that CD155 interacts with VEGF receptor 2 and regulates VEGF-induced angiogenesis (36). However, the role of CD155 on tumor angiogenesis in human cancer has not been addressed. In addition, we and others have shown the prognostic importance and significant involvement of angiogenesis in pancreatic cancer (32, 34, 35). Therefore, we investigated the correlation of CD155 with VEGF and angiogenesis, and found that there are indeed positive correlations in clinical pancreatic cancer. On the other hand, the blockade of CD155 did not affect VEGF expression of pancreatic cancer cell lines *in vitro*. This may be consistent with a recent study (36). Tumor CD155 may regulate angiogenesis by controlling the interaction of VEGF receptor with other molecules, including integrin, rather than by directly interacting with VEGF itself. Although further studies are required, our data may provide a new insight into mechanisms of tumor expression of CD155 in association with angiogenesis. Furthermore, anti-VEGF treatment is currently standard therapy for several human malignancies. Therefore, combined treatment targeting CD155 and VEGF may exert synergistic and curative effects on pancreatic cancer.

Finally, we evaluated the therapeutic potential of targeting CD155 for patients with pancreatic cancer. To this end, we employed the siRNA method. As a result, we observed that *CD155* gene silencing induced significant inhibition of cell proliferation of human pancreatic cancer cells. This might be consistent with previous reports on glioblastoma, cutaneous melanoma and lung adenocarcinoma cells (22, 27, 28). The flow cytometric analysis of the cell cycle distribution further clarified that the inhibition of tumor cell proliferation was associated with G_2/M arrest in pancreatic cancer. Moreover, the blockage of CD155 induced the increase of p27 and decrease of CDC25C as shown by western blotting analysis. A few reports on the cell cycle indicated that CD155 shortened the G_0/G_1 phase of the cell cycle in murine NIH3T3 cells (21, 53). Therefore, CD155 may contribute to enhancing cell-cycle progression and its blockade may induce cell-cycle arrest, although further studies are required in various tumor cell types. In addition, we investigated the effect of CD155 blockade on MAP kinase pathways mediated by ERK, JNK, and p38 (37). p38 was activated and cleaved PARP was increased in both MIAPaCa-2 and PANC-1 cells in response to transfection with *CD155* siRNA. These data suggested that p38 activation inhibits cell proliferation, and contributes to apoptosis induced by *CD155* down-regulation. Therefore, CD155 blockade may hold promise for the treatment of pancreatic cancer. Since there were only a few reports about the association between CD155 and MAP kinase (21, 53), further fundamental studies are needed to clarify the underlying molecular mechanisms.

In conclusion, we showed, for the first time, that CD155 may be involved in tumor immunity, cell proliferation, and angiogenesis in human pancreatic cancer. Most importantly, tumor CD155 expression has a significant prognostic value

in patients with pancreatic cancer. This study may provide a rationale for developing a novel therapy targeting CD155 for human malignant diseases.

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