Adenoviral Therapy Is More Effective in Gemcitabine-resistant Pancreatic Cancer than in Gemcitabine-sensitive Cells

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Abstract. *Background: Although gemcitabine is the standard treatment for pancreatic cancer, this particular type of cancer develops rapidly and has intrinsic chemoresistance. Chemoresistance plays a critical role in tumor progression, invasion and migration. Nevertheless, the effect of adenoviral therapy on chemoresistant cancer cells has not been studied. In this study, we compared the efficacy of adenoviral therapy in parental and chemoresistant pancreatic cancer cells. Materials and Methods: To establish gemcitabine-resistant cells, pancreatic cancer SUIT2 cells were exposed to increasing concentrations of gemcitabine. Both parental and chemoresistant cells were infected with adenoviruses expressing either green fluorescent protein (Ad-GFP) or the hepatocyte growth factor antagonist, NK4 (Ad-NK4). To investigate the transduction efficacy, GFP expression and NK4 concentrations were measured and an invasion assay was used to investigate the efficacy of the adenoviral therapy. Results: The 50% inhibitory concentration of gemcitabine was <10 nM in the parental SUIT-2 cells, while it was >1 μM in gemcitabine-resistant cells. A large number of gemcitabine-resistant cells were GFP-positive compared with only a small number of parental cells ($p<0.05$). The NK4 expression level was significantly higher in gemcitabine-resistant cells than in parental cells ($p<0.05$). The supernatant from Ad-NK4-infected gemcitabine-resistant cells significantly inhibited the invasion of cancer cells compared with that from Ad-NK4-infected parental cells ($p<0.05$). Conclusion: Both the efficiency of transduction and the therapeutic efficacy of adenoviral therapy were higher in gemcitabine-resistant cells than in parental cells, suggesting that adenoviral gene therapy is more effective in patients with gemcitabine- resistant pancreatic cancer.*

Pancreatic ductal adenocarcinoma (PDAC) is one of the most fatal type of human cancer. The prognosis of patients with PDAC is extremely poor, with an overall 5-year survival rate of only 4.4% (1). The limited efficacy of conventional systemic therapies is one reason for this poor prognosis. Gemcitabine, a pyrimidine nucleoside analogue, is currently the standard treatment for pancreatic cancer. Although gemcitabine yields great clinical benefit in patients with advanced pancreatic cancer, the response rates and survival benefits when gemcitabine is used alone are very low (2). Therefore, many therapeutic regimens that combine gemcitabine with other cytotoxic agents such as 5-fluororacil (3, 4), irinotecan (5), exatecan (6), cisplatin (7, 8), and oxaliplatin (9) were developed and evaluated in clinical trials; however, these combination therapies have not improved overall survival (2). One reason for the low efficacy of gemcitabine and gemcitabine-based combination therapies is chemoresistance. PDAC is either intrinsically chemoresistant, or rapidly becomes tolerant to gemcitabine. Recent studies show that chemoresistance plays a critical role in tumor progression, invasion and migration, and the malignant potential of chemoresistant cells is higher than that of chemosensitive cells (10, 11). Therefore, new therapeutic approaches are needed.

Adenovirus-based cancer gene therapy is a novel approach for treating tumors that are resistant to established therapies (12). Adenoviral therapy shows promising results both *in vitro* and *in vitro*, and many clinical trials have been conducted (13-17). Nevertheless, the relationship between adenoviral therapy and chemoresistance has not been studied. In this study, we established gemcitabine-resistant pancreatic cancer cells and compared the efficiency of transduction and the efficacy of adenoviral therapy in both chemoresistant and chemosensitive pancreatic cancer cells.

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Materials and Methods

Cell lines and the establishment of gemcitabine-resistant cells. The human pancreatic cancer cell line SUIT-2 was a kind gift from Dr. H. Iguchi (National Shikoku Cancer Center). The human fibroblast cell line, MRC-5, which secretes biologically active hepatocyte growth factor (HGF), was obtained from the RIKEN Cell Bank (Ibaragi, Japan). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with streptomycin (100 μg/ml), penicillin (100 U/ml) and 10% fetal bovine serum (FBS) at 37˚C in a humidified atmosphere of 90% air. To establish a gemcitabine-resistant pancreatic cancer cell line, SUIT2 cells were exposed to increasing concentrations of gemcitabine as previously described (18).

Construction of recombinant adenoviruses. A recombinant adenoviral vector expressing human HGF antagonist NK4 (Ad-NK4) and a control vector expressing the bacterial β-galactosidase gene (Ad-lacZ) were constructed as previously described (19-21). In brief, Ad-NK4 was generated by homologous recombination of the pJM17 plasmid and the shuttle plasmid vector pSV2+ containing an expression cassette and the CMV early promoter/enhancer, followed by human *NK4* cDNA and a polyadenylation signal. An adenovirus expressing green fluorescent protein (Ad-GFP) was generated, amplified and titrated as previously described (22, 23).

Propidium iodide (PI) assay. The 50% inhibitory concentration was evaluated by PI fluorescence intensity. Cells were plated in 24-well tissue culture plates and cultured for 24 hours. Several different concentrations of gemcitabine were used and the cells were incubated for a further 48 hours. PI (30 μM) and digitonin (600 μM) were then added to each well. Fluorescence intensity, corresponding to the total number of cells, was measured using an Infinite F200 (Tecan Trading AG, Switzerland) apparatus fitted with 535 nm excitation and 620 nm emission filters. The results were converted to survival rates by comparing treated cells with untreated cells. All experiments were performed in triplicate wells.

Assessment of transgene distribution by evaluation of GFP expression. Parental and gemcitabine-resistant cells were seeded in 6-well plates and cultured in DMEM supplemented with 10% FBS for 24 hours. Cells were infected with Ad-GFP at 10 multiplicities of infection (MOI). The culture medium was replaced with fresh medium 1 hour after transfection. After 24, 48 or 72 hours of infection, the GFP-positive and GFP-negative cells were observed and counted under a fluorescence microscope.

Real-time PCR and reverse transcription-PCR assays. The adenovirus DNA content of the infected cells was determined using real-time PCR, SYBR® Premix Ex Taq 2 (Takara, Tokyo, Japan) and a Chromo4™ System (Bio-Rad, Hercules, CA, USA). PCR conditions were as follows: 40 cycles at 95.5˚C for 5 seconds, 60˚C for 20 seconds, with +0.1˚C/second up to 95˚C for melting analysis. Each sample was run in triplicate. The primers used for the *NK4* gene were: 5'-GCAATTAAAACATGCGCTGA-3' and 3'-ATTGACAGTGC CCCTGTAGC-5' (24). The number of viral DNA copies was calculated from a standard curve obtained for the purified adenovirus vector and was further adjusted according to the protein concentration of each lysate. The mRNA levels for the Coxsackie virus and adenovirus receptor (CAR), β3-integrin, β5-integrin, clathrin, and dynamin 2 were quantified by real-time reverse transcription-PCR using a QuantiTect SYBR Green reverse transcription-PCR kit (Qiagen, Tokyo, Japan), 10 ng of total RNA, and primers specific for CAR, 5'-GGCGCTCCTGCTGTGC-3' and 5'-CTTTGGCTTTTTC AATCATCTTC-3'; β3-integrin, 5'-GAGGATGACTGTGTCGTCAG-3' and 5'-CTGGCGCGTTCTTCCTCAAA-3'; β5-integrin, 5'-CCTGTCCATGAAGGATGACTTG-3' and 5'-CTCATTGAAGCT GTCCACTCTG-3'; clathrin, 5'-CGGTTGCTCTTGTTACGG-3' and 5'-CGGTTGCTCTTGTTACGG-3'; and dynamin 2, 5'-AGGAGTACT GGTTTGTGCTGACTG-3' and 3'-GTGCATGATGGTCTTTGGCA TGAG-5'. The reaction mixture was first incubated at 50˚C for 30 minutes to allow reverse transcription. PCR was initiated with one cycle of 95˚C for 15 minutes to activate the modified Taq polymerase, followed by 40 cycles at 94˚C for 15 seconds, 55˚C for 30 seconds, 72˚C for 30 seconds, and one cycle at 60˚C for 15 seconds, with +0.1˚C/second up to 95˚C for melting analysis. Each sample was run in triplicate. The levels of CAR, β3-integrin, β5-integrin, clathrin, and dynamin 2 mRNA were normalized to those of 18S rRNA amplified using the specific primers 5'-GTAACCCGTTGAACCCCATT-3' and 5'-GCGATGATGGCTAACCTACC-3', and expressed as a ratio compared with untreated controls.

Measurement of NK4 expression levels. After infection with Ad-NK4, the conditioned media were collected and changed every 24 hours. The HGF concentration in the conditioned media was measured by using a human HGF ELISA Kit (IMMUNIS HGF EIA; Institute of Immunology, Tokyo, Japan) according to the manufacturer's recommendations.

Invasion assay. The invasiveness of pancreatic cancer cells was quantified as the number of cells invading through Matrigel-coated transwell inserts (Becton Dickinson) as previously described (25). In brief, transwell inserts (8 μm pores) were coated with Matrigel (20 μg/well; Becton Dickinson). Gemcitabine-sensitive and -resistant SUIT-2 cells were infected with Ad-lacZ or Ad-NK4 at MOI of 100, and culture media were collected 2 days after infection. Fresh, untreated gemcitabine-sensitive SUIT-2 cells were seeded into the upper chambers of the 24-well plates at a density of $1\times10^5/cm^2$ in 250 μl of DMEM supplemented with 10% FBS and cultured in 750 μl of conditioned media from the gemcitabine-sensitive SUIT-2 cells or gemcitabine-resistant SUIT-2 cells infected with Ad-lacZ or Ad-NK4. MRC-5 cells (1×10^5 cells/well), which secrete HGF, were seeded into the lower chambers of the 24-well plates. After 48 hours of incubation, any cells that had invaded to the lower surface of the Matrigel-coated membrane were counted in three randomly selected fields under a light microscope.

Statistical analysis. Values were expressed as the mean±SD. Comparisons between all groups were made using one-way ANOVA, and Student's *t*-test was used for comparisons between two groups. The level of statistical significance was set at $p<0.05$. To confirm the induction results, experiments were repeated at least three times.

Results

Establishment of gemcitabine-resistant cells. SUIT2 cells were exposed to increasing concentrations of gemcitabine to establish a gemcitabine-resistant pancreatic cancer cell line. As shown in Figure 1A, gemcitabine-resistant cells showed a round morphology when compared with the parent

SUIT-2 cells. The 50% inhibitory concentration for gemcitabine was <10 nM in the parental SUIT-2 cells, while it was >1 μM in gemcitabine-resistant cells (Figure 1B).

GFP expression in Ad-GFP-infected parental cells and gemcitabine-resistant cells. To investigate differential expression of transgenes delivered by the adenoviral vector between parental and gemcitabine-resistant cells, we examined the expression levels of GFP in Ad-GFP-transfected cells. Parental and gemcitabine-resistant SUIT-2 cells were cultured for 24 hours after seeding and then infected with Ad-GFP at 10 MOI. At 24, 48 or 72 hours after infection, the GFP-positive and GFP-negative cells were observed and counted under a fluorescence microscope. Figure 2A shows that a large number of gemcitabine-resistant SUIT-2 cells were GFP-positive, while only a low number of parental SUIT-2 cells were GFP-positive on each day after infection (Figure 2B, $p<0.05$). This suggests that the expression level of adenovirus-delivered transgenes in gemcitabine-resistant cells was higher than that in the parental cells.

NK4 expression in Ad-NK4-infected parental and gemcitabine resistant cells. We next used Ad-NK4 to investigate differences in the efficacy of the adenoviral therapy between parental and gemcitabine-resistant cells. NK4 acts as a competitive HGF antagonist and inhibits pancreatic cancer cell migration and invasion (26, 27). After infection with Ad-NK4, we collected the conditioned media every 24 hours and measured the NK4 expression levels by ELISA. The NK4 levels in gemcitabine-resistant cells were significantly higher than those in gemcitabine-sensitive cells at 1, 2 and 3 days after infection (Figure 3A, $p<0.05$).

Efficacy of the adenoviral therapy in Ad-NK4-infected parental cells and gemcitabine resistant cells. SUIT-2 cells were seeded in the upper chambers of a transwell plate, and MRC-5 cells, which secrete HGF, were seeded in the lower chambers with the culture supernatant from Ad-NK4 or Ad-LacZ-infected parental or gemcitabine-resistant cells, and invasive cells were counted 48 hours later. As shown in Figure 3B, the number of cells that invaded across the membrane after culture with the supernatant from Ad-NK4-infected cells was lower than that of cells cultured with the supernatant from untreated or Ad-LacZ-infected cells, which is consistent with the previous data (28). The number of cells that invaded across the membrane after culture with the supernatant from Ad-NK4-infected gemcitabine-resistant cells was lower than that of cells cultured with the supernatant from Ad-NK4-infected parental cells (Figure 3C, $p<0.05$).

Adenoviral mRNA expression and adenoviral DNA content of Ad-NK4-infected parental and gemcitabine-resistant cells. To clarify the mechanisms underlying the increased efficacy of adenoviral therapy in gemcitabine-resistant cells, we investigated the levels of adenoviral mRNA expression and the adenoviral DNA content of Ad-NK4-infected parental and gemcitabine-resistant cells. Parental and resistant cells were infected with Ad-NK4 at 20 MOI, and *NK4* mRNA expression and viral DNA content were quantified 48 hours later. As shown in Figure 4A, the expression level of *NK4* mRNA in gemcitabine-resistant cells was higher than that in parental cells ($p<0.05$). The viral DNA content of gemcitabine-resistant cells was also higher than that of parental cells (Figure 4B, $p<0.05$).

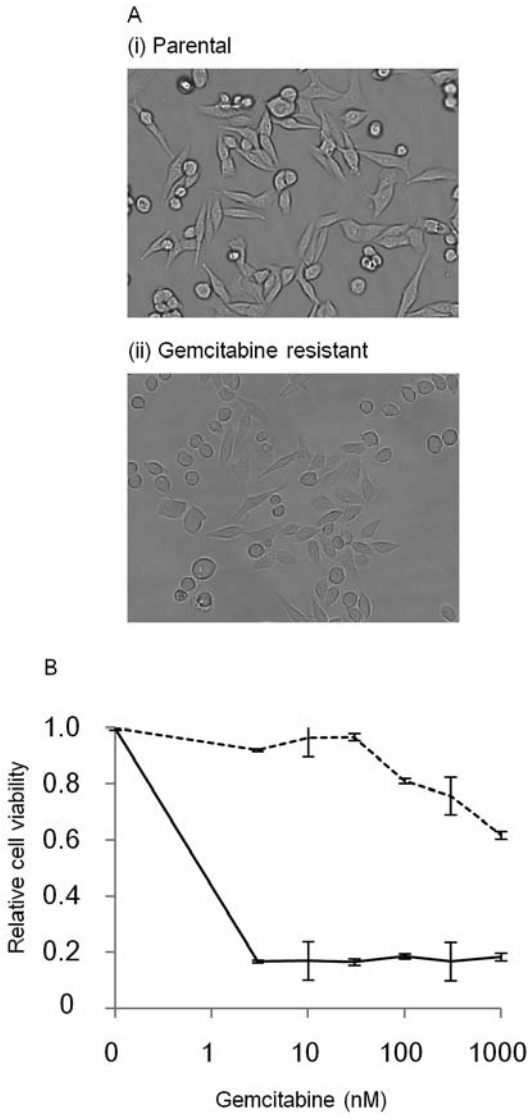

Figure 1. *Gemcitabine-resistant and parental SUIT-2 cells. A: Morphology of (i) parental cells and (ii) gemcitabine-resistant cells (×200 magnification). B: The 50% inhibitory concentration of parental and gemcitabine-resistant cells. Solid line, parental cells; dotted line, gemcitabine-resistant cells.*

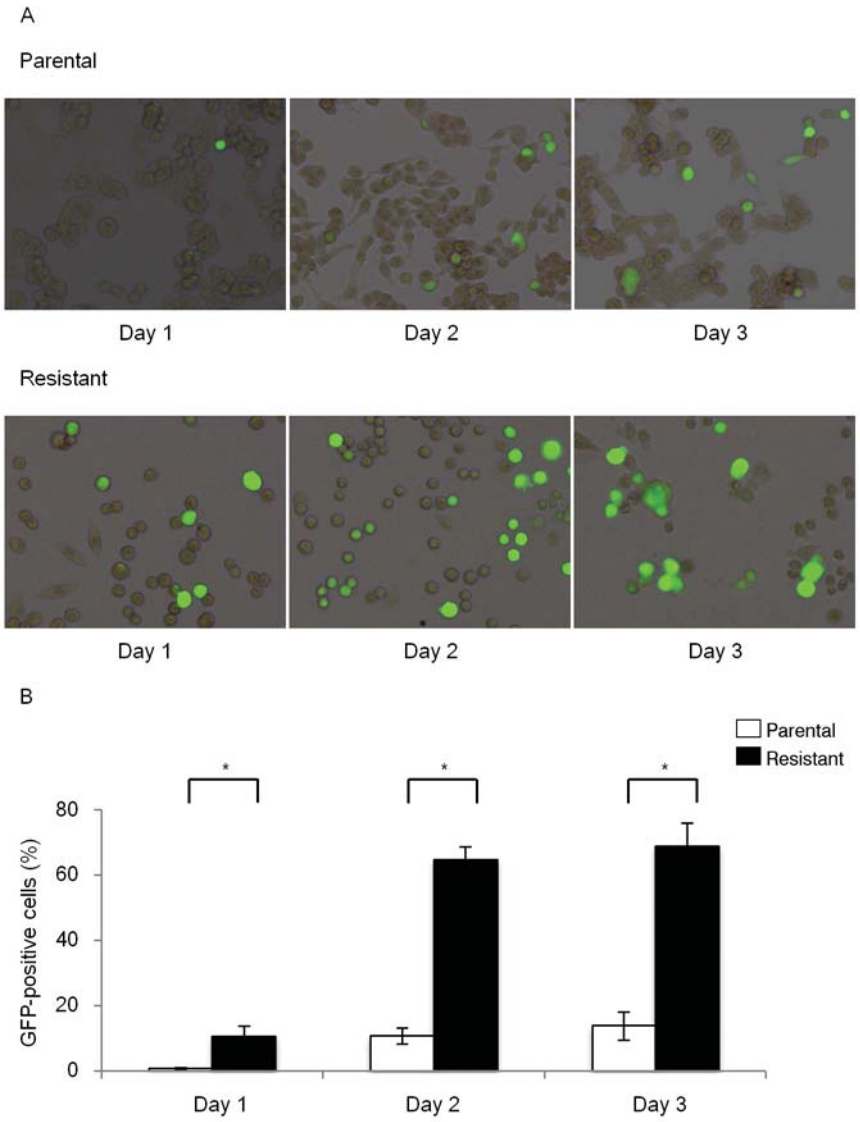

Figure 2. *GFP expression in Ad-GFP-infected parental and gemcitabine-resistant cells. Parental and gemcitabine-resistant cells were seeded and cultured for 24 h. Cells were infected with Ad-GFP at 10 multiplicities of infection (MOI). At 24, 48 or 72 h after infection, the GFP-positive and GFP-negative cells were observed and counted under a fluorescence microscope. A: GFP-positive and GFP-negative cells observed by fluorescence microscopy (×200 magnification). B: The percentage of GFP-positive cells. Each value represents the mean±SD of triplicate measurements. *p<0.05.*

Levels of CAR, β3-integrin, β5-integrin, clathrin and dynamin 2 mRNA in parental and gemcitabine-resistant cells. The viral DNA and mRNA expression levels in gemcitabine-resistant cells were higher than in those in parental cells. Therefore, to investigate the efficiency of adenoviral cell attachment and endocytosis in gemcitabine-resistant and parental cells, we quantified the levels of CAR, β3- and β5-integrin, clathrin and dynamin 2 mRNA using qRT-PCR (Figure 4C). These molecules are required for adenoviral cell attachment and endocytosis. We found no difference in the expression levels of any of these mRNAs between gemcitabine-resistant and parental cells.

Discussion

The results of this study show that: i) the uptake of adenoviral genes and the efficiency of transduction were higher in gemcitabine-resistant cells than in gemcitabine-sensitive cells; ii) adenoviral gene therapy is more effective against gemcitabine-resistant cells than against gemcitabine-sensitive cells; and iii) the levels of CAR, β3-integrin, β5-integrin, dynamin and clathrin mRNA expression were not different between gemcitabine-resistant cells and gemcitabine-sensitive cells.

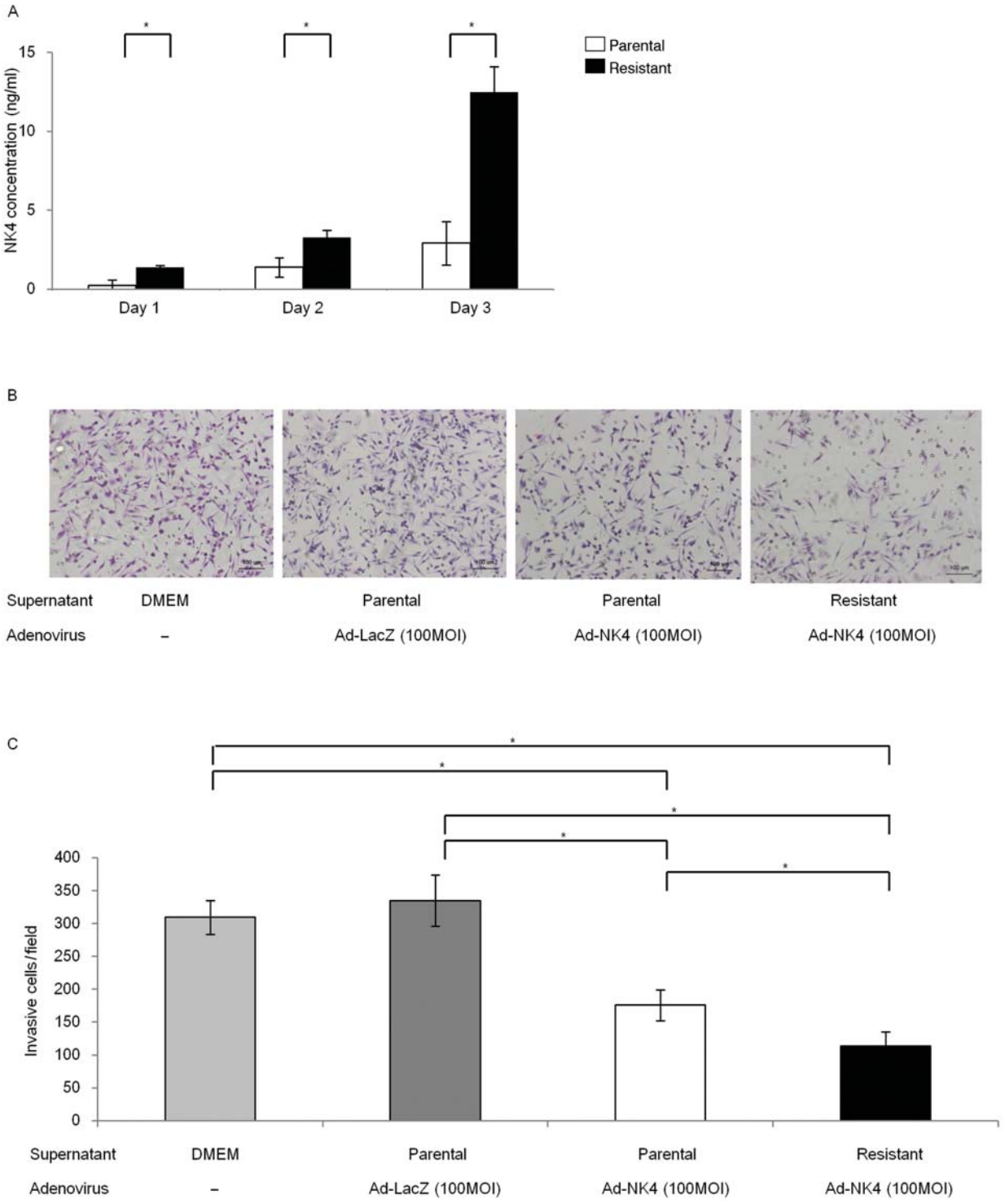

Figure 3. *Differences in the efficacy of the adenoviral therapy between parental and gemcitabine-resistant cells. A: The concentration of NK4 in each cell type. Parental and resistant cells were infected with Ad-NK4 at 20 MOI, and 48 hours after infection, the supernatants were collected and NK4 levels in culture media were measured by ELISA. Each value represents the mean±SD of triplicate measurements. *p<0.05. B, C: Invasion assay. Parental and gemcitabine-resistant cells were infected with Ad-lacZ or Ad-NK4 at MOI of 100 and culture media were collected 2 days after infection. Fresh, untreated parental SUIT-2 cells were seeded into the upper chamber of a transwell and cultured with each of the conditioned media. MRC-5 cells (1×10^5 cells/well), which secrete HGF, were seeded in the lower chamber. After 48 h, cells that had invaded to the lower surface of the Matrigel-coated membrane were counted. Each value represents the mean±SD of triplicate measurements. *p<0.05.*

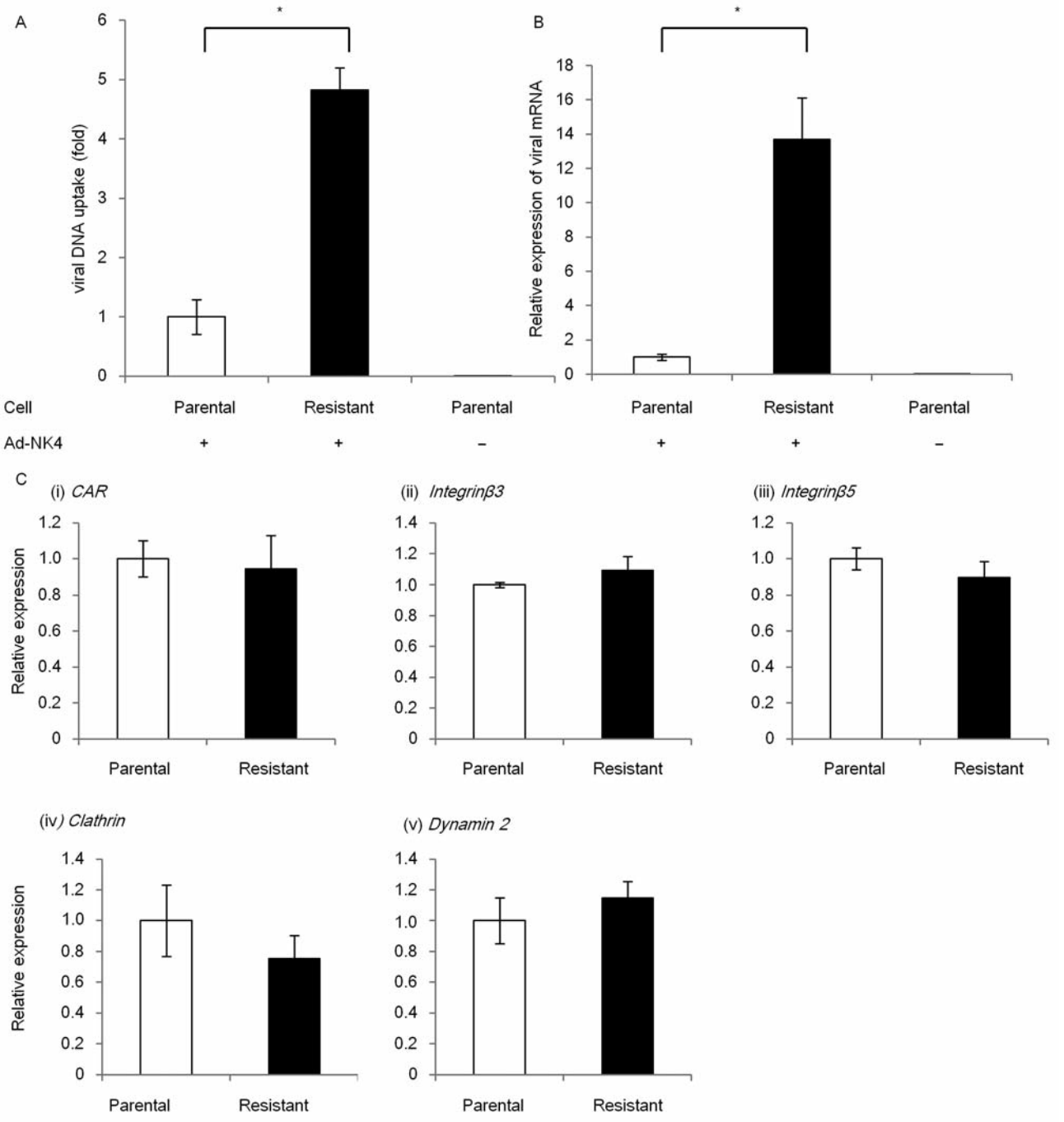

Figure 4. *The adenoviral mRNA levels and adenoviral DNA content of Ad-NK4-infected parental and gemcitabine-resistant cells. Parental and resistant cells were infected with Ad-NK4 at 20 MOI, and NK4 mRNA expression and viral DNA content were quantified 48 h after infection. Each value represents the mean±SD of triplicate measurements. *p<0.05. A: The expression level of adenoviral mRNA. B: Adenoviral DNA content. C: The levels of CAR, β3 and β5integrins, clathrin and dynamin 2 mRNA in parental cells and gemcitabine-resistant cells. Each value represents the mean±SD of triplicate measurements.*

Although gemcitabine is currently the standard treatment for pancreatic cancer, the response rates and survival benefits are very low. In recent studies, several strains of gemcitabine-resistant cells were established and their characteristics of including molecular markers related to gemcitabine resistance and associated with the acquisition of an epithelial–mesenchymal-like phenotype by cancer cells or cancer stem cells, were investigated (29-31). However, the relationship

between chemoresistance and adenoviral therapy has not been studied. We first investigated this using Ad-GFP to examine the association between chemoresistance and adenoviral therapy. Surprisingly, the expression level of GFP in gemcitabine-resistant SUIT-2 cells was significantly higher than that in the parent cells. We also found that there were significant differences in the efficacy of the adenoviral therapy between parental and gemcitabine-resistant cells. These data suggest that adenoviral gene therapy is a good treatment option for pancreatic cancer that has developed tolerance to gemcitabine.

HGF is a multi-domain glycoprotein first identified as a potent mitogen for adult rat hepatocytes in primary culture and which has high affinity for the c-Met receptor (32), which is frequently overexpressed in pancreatic cancer. The interaction between HGF and the c-Met receptor increased the rate of proliferation, invasion, migration and angiogenesis of cancer cells (33-36). NK4 is composed of the *N*-terminal hairpin and subsequent four-kringle domains of HGF, and it acts as a dose-dependent, competitive HGF antagonist. NK4 inhibits pancreatic cancer cell migration and invasion *in vitro* and suppresses growth, invasion, and metastasis of human pancreatic carcinoma *in vivo* (21, 26, 37). Adenoviruses expressing NK4 have been developed, and these viruses have similar antitumor effects (37, 38). In this study, we used Ad-NK4 to investigate the efficacy of adenoviral gene therapy in parental and gemcitabine-resistant cells. The expression level of the *NK4* transgene delivered by the adenoviral vector was higher in the supernatant of gemcitabine-resistant cells than in the supernatant of parental cells, which is consistent with the results of our experiments using Ad-GFP. In addition, the supernatant from Ad-NK4-infected gemcitabine-resistant cells, which contained a higher concentration of NK4, inhibited pancreatic cancer cell invasion to a greater extent than that from Ad-NK4-infected parental cells. These data also suggest that adenoviral gene therapy is more effective in pancreatic cancer cells that have acquired gemcitabine resistance.

The expression level of *NK4* mRNA and the viral DNA content of Ad-NK4-infected gemcitabine-resistant cells were higher than those of parental cells, suggesting that the difference in transgene expression level between parent and gemcitabine-resistant cells was caused by differences in the efficiency of cellular uptake of adenoviral particles. Attachment and internalization are required for an adenovirus to enter a host cell (39, 40). During the first step, the fiber protein of all adenovirus serotypes (except subgroup B) binds to a primary receptor, CAR. During the second step, the CAR-docked particles activate αvβ3 and αvβ5integrins and their co-receptors, triggering endocytosis. The adenoviral particles are then rapidly internalized. Endocytosis of adenoviral particles is mediated by clathrin and involves the large GTPase, dynamin (39). Although we quantified the levels of CAR, β3 and β5integrins, clathrin and dynamin 2 mRNA in both parental and gemcitabine-resistant cells, we found no differences. Furthermore, we performed additional experiments, including flow cytometry to detect CAR expression on the cell surface and a promoter assay to check the CMV promoter (data not shown), but found no reasonable explanation for this mechanism. Therefore, we were unable to identify the mechanism by which transgene expression in gemcitabine-resistant cells is more efficient than in parental cells. However, there may be some unknown, causal mechanism that awaits future study.

In conclusion, our results show that both the efficiency of transduction and efficacy of adenoviral therapy were higher in gemcitabine-resistant cells than in gemcitabine-sensitive cells. This suggests that adenoviral gene therapy should be more effective in patients with pancreatic cancer that has acquired gemcitabine resistance, and that adenoviral therapy may be a good therapeutic choice, especially for patients who do not respond to 'conventional' gemcitabine therapy.

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