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Suppressive effect of modified dominant negative RAS mutant on human cancer by gene transfer with non-viral vector

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Abbreviations and acronyms

N116Y-Cdel1 (C-terminal deletion mutant of N116Y 1)

N116Y-Cdel2 (C-terminal deletion mutant of N116Y 2)

MOI (multiplicity of infection)

HVJ (hemagglutinating virus of Japan)

Abstract

N116Y, H-RAS mutant, has dominant negative activity in the RAS function and a suppressive effect on the growth of various types of cancer cells. However, a replication error of N116Y is of potential concern for carcinogenesis in clinical application. To decease the concern, we constructed modified N116Y by deleting the carboxyl terminus, which is necessary for the oncogenic function of Ras. One of the C-terminal deletion mutants of N116Y, N116Y-Cdel2 showed a growth-suppressing effect on various human cancer cell lines *in vitro*: the cervical cancer cell line HeLa, the pancreatic cancer cell line PCI43, the colon cancer cell lines SW480 and LoVo, and the tongue cancer cell line SAS. In addition, the suppressive effect of N116Y-Cdel2 on LoVo cells was also observed *in vivo* using a non-viral gene transfer vector, HVJ envelope. Our experiments suggest that the modified N116Y is a potential candidate gene for human cancer gene therapy.

Running title

Suppressive effect of dominant negative RAS on cancer

Introduction

Deaths from cancer are increasing every year. Using combined therapeutic strategies such as surgical treatment, chemotherapy, radiotherapy, the curability of advanced cancer remains insufficient. Therefore, the development of new concepts for cancer treatment is important. Gene therapy is a promising therapeutic option for cancer therapy[1]. The purpose of cancer gene therapy is to kill the cancer cells with various strategies [2-7].

RAS, a proto-oncogene product, regulates survival, proliferation, differentiation, and cell motility. With RAS mutation, oncogenic RAS is observed in various types of cancer, so RAS suppression is one of the promising options for cancer gene therapy. We originally constructed a dominant negative RAS mutant N116Y [8]. The N116Y gene is a mutant derived from v-H-RAS oncogene by substituting the codon for asparagine-116 with tyrosine. We already reported that N116Y is a potential candidate for cancer gene therapy which has a suppressive effect on pancreatic cancer, esophageal cancer and bladder cancer in vitro and in vivo [8-14]; however, replication errors or reversion of N116Y are potential concern for carcinogenesis in clinical application because N116Y is derived from the v-H-RAS oncogene. We constructed a C-terminal deletion dominant negative RAS mutant and investigated its suppressive effect on cancer *in vitro* and *in*

vivo.

Materials and Methods

Construction of modified dominant negative RAS mutant

To construct serial C-terminal deletion mutants, the N116Y gene [15] in a retrovirus vector pZIP was digested by *Eco*RI and a fragment was removed from 413 bp to 567 bp of the 3' terminus. Several lengths of v-H-RAS gene fragments were ligated to the digested N116Y gene. N116Y-Cdel1 and N116Y-Cdel2 were constructed by ligation with a v-H-RAS gene fragment deleted by 12bp (4 aa) or 72bp (24 aa) from 3' terminus. The N116Y gene and modified N116Y genes in pZIP vector were used as templates in polymerase chain reaction (PCR) with each primer. The forward primer for N116Y, N116Y-Cdel1 and N116Y-Cdel2 was 5'-TAT GGA TCC GCG ATG ACA GAA TAC AAG-3'. The reverse primers for N116Y, N116Y-Cdel1 and N116Y-Cdel2 were 5'-ATA CTC GAG TCA GGA CAG CAC ACA-3', 5'-ATA CTC GAG TCA CTT GCA GCT CAT G-3' and 5'-ATA CTC GAG TCA CTG CCG AAT CTC-3' respectively. The amplified N116Y or modified N116Y gene was inserted into pCMV-3xFLAG vector. pCMV-3xFLAG contains the neomycin resistance gene and the fusion protein of N116Y or modified N116Y and 3 x FLAG tag is expressed under the transcriptional regulation of CMV promoter. Moreover,

Green Fluorescent Protein (GFP), β-galactosidase or modified N116Y genes were inserted into pAd-Track-CMV vector (kindly provided by Dr. Bert Vogelstein of The Johns Hopkins University) [16].

Cell lines

The human colon cancer cell lines SW480 and LoVo, and the human tongue cancer cell line SAS were obtained from the Japanese Collection of Research Bioresources. The human cervical cancer cell line HeLa was obtained from the Division of Biochemical Oncology and Immunology, Institute for Genetic Medicine, Hokkaido University. The pancreatic cancer cell line PCI43 was obtained from the Division of Pathophysiological Science and Division of Cancer Medicine, Hokkaido University Graduate School of Medicine. These cells were maintained in Dulbecco's Modified Eagle's Medium (Nissui, Tokyo, Japan) containing 10 % FCS (Invitrogen, Carlsbad, CA, USA) and 0.05 % glutamine (Invitrogen) in a 37 ^{o}C incubator with 5 % CO_2.

Gene transfection *in vitro*

1. Adenovirus-mediated gene delivery

Replication-defective adenovirus vectors AdGFP, AdN116Y-Cdel1, and AdN116Y-Cdel2 were synthesized using the AdEasy system with pAd-Track-CMV inserted in GFP, N116Y-Cdel1 or N116Y-Cdel2, respectively

[28]. 2 x 10^4 HeLa and PCI43 cells were plated in six-well plates and infected with 100 MOI (HeLa) or 800 MOI (PCI43) of AdGFP, AdN116Y-Cdel1 or AdN116Y-Cdel2. These cells were harvested and counted by hemocytometer, using trypan blue dye exclusion to measure viability at days 1, 3, 5 and 7 after infection. The average of triplicate experiments is presented.

2. Establishment of stable clones

Each of 2 x 10^5 SW480, LoVo and SAS cells was transfected with 1.5 μg of pCMV-3xFLAG, 1.5 μg of pCMV-3xFLAG inserted in N116Y gene or 1.5 μg of pCMV-3xFLAG inserted in N116Y-Cdel2 gene by Lipofectamine (Invitrogen). Then SW480, LoVo and SAS cells were cultured in 1.4 mg/ml, 0.6 mg/ml and 1.0 mg/ml neomycin (G418) -containing medium, respectively, for 7 days, and isolated to establish stable clones. The expression of N116Y or N116Y-Cdel2 in each clone was confirmed by Western blot analysis. After establishment, 2 x 10^4 pCMV-3xFLAG control clones, stable clones expressing N116Y and stable clones expressing N116Y-Cdel2 were plated in 12-well plates for growth curve analysis. Three stable clones expressing each gene were used. These cells were harvested and counted by hemocytometer, using trypan blue dye exclusion to measure viability at days 1, 3 and 5. The average of triplicate experiments is presented.

Western blot analysis

Cell lysate was prepared by extracting the cell pellets with 1 x SDS sample buffer and boiled. Cell lysates were separated by 12.5 % SDS-PAGE and electrotransferred to Hybond ECL nitrocellulose membrane (Amersham BioScience, Cardiff, UK). After blocking with 5 % nonfat dry milk in Tris-buffered saline containing 0.05 % Tween-20, the mouse anti-actin IgG monoclonal antibody (CHEMICON, Temecula, CA, USA) and the mouse anti-FLAG M2 IgG monoclonal antibody (Sigma-Aldrich, St. Louis, MO, USA) were used as the primary antibody (1:5,000). Peroxidase-conjugated goat F(ab')2 anti-mouse IgG+IgM (Jackson ImmunoResearch, West Grove, PA, USA) was used as the secondary antibody (1:5,000). The bound antibodies were detected using the ECL system (Amersham BioScience, Cardiff, UK).

Colony inhibition assay

2 x 10^4 LoVo cells in 12-well plates were transfected with 0.6 μg of pCMV-3xFLAG, 0.6 μg of pCMV-3xFLAG inserted in N116Y gene and 0.6 μg pCMV-3xFLAG inserted in N116Y-Cdel2 gene by Lipofectamine, respectively. Transfected cells were harvested, then 1/10 of these cells were cultured in 6 cm dishes with 0.7 mg/ml neomycin-containing medium. Ten days later, the number of neomycin colonies (colonies with a diameter > 1 mm) was counted.

The average of triplicate experiments is presented.

Gene transfection and analysis of tumor cells *in vivo*

We used the method of gene transfection with the hemagglutinating virus of Japan (HVJ) envelope vector (GenomONE-Neo, Ishihara Sangyo Kaisha, Osaka, Japan). First, 1 x 10^7 LoVo cells were subcutaneously injected into the back of nine nude 5-week-old female KSN/Slc mice (Japan SLC, Shizuoka, Japan). Tumors were allowed to develop for 2 days. Tumor volumes were measured every 2 days according to the formula $V = a \times b^2/2$ (a, largest superficial diameter; b, smallest superficial diameter). Three and 10 days after transplantation, intratumoral injections were given to three mice within each group. The first group was injected with PBS (phosphate-buffered saline), the second group was injected with a mixture of pAd-Track-CMV inserted in β-galactosidase gene and HVJ envelope vector, and the third group was injected with a mixture of pAd-Track-CMV inserted in N116Y-Cdel2 gene and HVJ envelope vector. From each plasmid, 75 μg was used.

Results

Construction of modified dominant negative RAS mutant

We deleted the C-terminal region of N116Y to construct modified N116Y (Fig. 1A). N116Y-Cdel1 does not have a CAAX box and N116Y-Cdel2 does not have a CAAX box and hypervariable region. We confirmed that the N116Y-Cdel1 and N116Y-Cdel2 coding regions amplified by PCR are shorter than the original N116Y by agarose gel electrophoresis (Fig. 1B). In addition, the nucleotide sequence of N116Y-Cdel1 and N116Y-Cdel2 was confirmed by ABI Prism 310 Genetic Analyzer (PE Applied Biosystems, CA, USA).

Suppressive effect of N116Y-Cdel2 on HeLa and PCI43 cells with adenovirus vector *in vitro*

HeLa and PCI43 cells were infected with AdGFP, AdN116Y-Cdel1 or AdN116Y-Cdel2, and harvested and counted (Fig. 2). As a result, only AdN116Y-Cdel-2 showed a suppressive effect on both HeLa and PCI43 cells. The average cell number of HeLa cells infected with AdN116Y-Cdel2 was less than 25 % of that of HeLa cells infected with AdGFP at 7 days after infection. The average cell number of PCI43 cells infected with AdN116Y-Cdel2 was less than 50 % of that of PCI43 cells infected with AdGFP at 7 days after infection.

Establishment of stable clones of SW480 and SAS cells

SW480, LoVo and SAS cells were transfected with pCMV-3xFLAG,

pCMV-3xFLAG inserted in N116Y gene or inserted in N116Y-Cdel2 gene, and isolated to establish stable clones. The expression of N116Y or N116Y-Cdel2 in these cells was examined by Western blotting (Fig. 3). As a result, three pCMV-3xFLAG control clones, three stable clones expressing N116Y and three stable clones expressing N116Y-Cdel2 were obtained from SW480 cells. Three pCMV-3xFLAG control clones and three stable clones expressing N116Y-Cdel2 were also obtained from SAS cells; however, stable clones expressing N116Y-Cdel2 were not obtained from LoVo cells.

Morphological change of stable clones expressing N116Y-Cdel2

The morphological change of stable clones was examined (Fig. 4). Little or no difference was apparent in pCMV-3xFLAG control clones compared to parental SW480 cells and control clones showed crisscross patterns of growth. Whereas, like stable clones of SW480 cells expressing N116Y, stable clones expressing N116Y-Cdel2 were morphologically changed and did not show crisscross patterns. Similar morphological change was also observed in stable clones of SAS cells expressing N116Y-Cdel2.

Suppressive effect of N116Y-Cdel2 on SW480 and SAS cells *in vitro*

The suppressive effect of N116Y-Cdel2 on SW480 and SAS cells was examined by growth curve analysis of stable clones expressing N116Y-Cdel2

(Fig. 5). As a result, like stable clones of SW480 cells expressing N116Y, stable clones expressing N116Y-Cdel2 showed slow growth, and the average cell number of clones was less than 50 % of that of pCMV-3xFLAG control clones at 5 days after beginning culture. The average cell number of stable clones of SAS cells expressing N116Y-Cdel2 was also less than 50 % that of pCMV-3xFLAG control clones at 5 days after beginning culture.

Suppressive effect of N116Y-Cdel2 on LoVo cells *in vitro*

As stable clones of LoVo cells expressing N116Y-Cdel2 were not obtained, the suppressive effect of N116Y-Cdel2 on LoVo cells was examined by the observation of colony inhibition assay (Fig. 6). LoVo cells were transfected with pCMV-3xFLAG, pCMV-3xFLAG inserted in N116Y gene or pCMV-3xFLAG inserted in N116Y-Cdel2 gene, and then the number of neomycin-resistant cells was counted. As a result, about 70 cell colonies transfected with pCMV-3xFLAG were observed; however, cell colonies transfected with pCMV-3xFLAG inserted in N116Y or N116Y-Cdel2 gene were not confirmed.

Suppressive effect of N116Y-Cdel2 on LoVo cells with non-viral vector *in vivo*

We examined the suppressive effect of N116Y-Cdel2 on LoVo cells *in vivo* using the method of gene transfection with HVJ envelope vector (Fig. 7).

Significant suppression of tumor growth was observed in mice treated with the N116Y-Cdel2 gene. The average tumor volume of mice treated with the N116Y-Cdel2 gene was about 30 % of that of mice injected with PBS or transfected with the β-galactosidase gene at 24 days after transplantation of LoVo cells.

Discussion

N116Y-Cdel1 and N116Y-Cdel2 were constructed in this study. As activation of the RAS oncogene product needs to be anchored in the plasma membrane to function, and the RAS oncogene product must have its carboxyl terminus farnesylated to be anchored [17,18], it is highly likely that C-terminal-deleted N116Y has much lower chance to become oncogenic than N116Y. N116Y-Cdel2 showed a growth-suppressing effect on HeLa, PCI43, SW480, LoVo and SAS cells. Morphological change was observed in SW480 and SAS cells expressing N116Y-Cdel2, whereas N116Y-Cdel1 did not show a suppressive effect on HeLa and PCI43 cells, although the deleted region of N116Y-Cdel1 was shorter than that of N116Y-Cdel2. it is highly likely that N116Y-Cdel1 lost the suppressive effect of the dominant negative RAS mutant as a result of its biological structure.

N116Y-Cdel2 was constructed from N116Y, which is constructed by

mutating (Asn116–>Tyr) in v-H-RAS. This amino acid site of 116 is important to bind with GTP; therefore, N116Y loses function to stimulate cell growth [8]. Hwang et al. reported that N116I, which is biochemically identical to N116Y, binds with SDC25C (Saccharomyces cerevisiae SDC25 C-domain gene product) and suppresses the GDP/GTP exchange reaction of H-RAS [19]. It was also reported that the growth of renal cell carcinoma cells, which overexpress hSOS-1 (human GEP), was suppressed by N116Y [20]. N116Y-Cdel2 as well as N116Y has a GEP-binding region. Therefore, it is reasonable to speculate that N116Y-Cdel2 and N116Y competitively inhibit interaction between the RAS oncogene product and GEP. As a result, it is highly likely that signal transmission from the RAS oncogene product was inhibited, and morphological change and suppressive effect were caused, respectively, by the inhibition of RAC activation [21,22] and MAPK family activation [23-26]. Moreover, it was confirmed that N116Y suppresses cancer cell growth and induces apoptosis of bladder cancer, pancreatic cancer and leukemia cells by the inhibition of signal transmission [8-14]. It is also reported phosphorylation of Erk which is important for cell division was inhibited by N116Y [10]. So it is highly likely that suppressive effect of N116Y- Cdel2 is also caused by at least one of the mechanisms or by both, inhibition of proliferation and induction of cell death.

Recently, viral vectors are usually used for gene transfection. In general,

viral vectors are more effective than non-viral vectors for gene delivery into cells. However, viral vectors may present safety concerns due to the concurrent introduction of essential gene elements from the parent virus, leaky expression of viral genes, immunogenicity, and modification of the host genome structure. Consequently, we used the HVJ envelope vector, which is a non-viral vector with high transduction efficiency and safety [27]. Its mechanism of gene induction is by fusion with the cell membrane, not by endocytosis, thus minimizing injury to the cell. Its most important feature is its complete lack of pathogenicity to humans. Transfection efficiency was not affected by repetitive transfection [28]. This vector was used to deriver N116Y-Cdel2 to LoVo cells already transplanted into nude mice. As a result, a suppressive effect of N116Y-Cdel2 on LoVo cells was observed *in vivo.* There are still some obstacles for cancer gene therapy, such as tumor targeting, but the application of modified dominant negative RAS, N116Y might be promising in terms of its efficacy and safety.

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Figure legends

Figure 1

(A) Construction of modified N116Y. We constructed modified N116Y by deleting the carboxyl terminus. First, N116Y gene was digested by *Eco*RI and a fragment removed from 413 bp to 567 bp of the carboxyl terminus. Then, v-H-RAS gene fragment with *Eco*RI site was ligated with digested N116Y gene. N116Y-Cdel1 and N116Y-Cdel2 were constructed by ligating with the v-H-RAS gene fragment deleted by 12bp (4 aa) or 72bp (24 aa), respectively, from the 3' terminus. (B) Analysis of modified N116Y by agarose gel electrophoresis. N116Y (N), N116Y-Cdel1 (C1) and N116Y-Cdel2 (C2) were amplified by PCR and the products confirmed by 3.0% agarose gel electrophoresis with a marker (M). N116Y was 567 bp, Cdel1 was 555 bp, and Cdel2 was 495 bp.

Figure 2

Suppressive effect of AdN116Y-Cdel2 on HeLa and PCI43 cells *in vitro*. 2 x 10^4 HeLa and PCI43 cells were plated. Then, cells were infected with 100 MOI (HeLa) or 800 MOI (PCI43) of AdGFP (—◆—), AdN116Y-Cdel1 (—△—) or AdN116Y-Cdel2 (—×—). These cells were harvested and counted at days 1, 3, 5 and 7 after infection. Average of triplicate experiments is presented.

Figure 3

Confirmation of the expression of N116Y and N116Y-Cdel2 in stable clones

by Western blot analysis. The expression of N116Y and N116Y-Cdel2 fused with 3 x FLAG was detected by anti-FLAG tag antibody in pCMV-3xFLAG control clones (Neo), stable clones expressing N116Y (N116Y) and stable clones expressing N116Y-Cdel2 (Cdel2).

Figure 4

Morphological change of stable clones expressing N116Y-Cdel2. pCMV-3xFLAG control clones, stable clones expressing N116Y and stable clones expressing N116Y-Cdel2 were examined in SW480 cells using a phase microscope (x 200). Each cell was grown to 100% confluence.

Figure 5

Suppressive effect of N116Y-Cdel2 on SW480 and SAS cells *in vitro*. 2 x 10^4 pCMV-3xFLAG control clones (—○—), stable clones expressing N116Y (—□—) and stable clones expressing N116Y-Cdel2 (—✱—) of SW480 cells were plated. 2 x 10^4 pCMV-3xFLAG control clones (—○—) and stable clones expressing N116Y-Cdel2 (—✱—) of SAS cells were also plated. These cells were harvested and the average cell number was counted at days 1, 3 and 5 after plating. Average of triplicate experiments is presented.

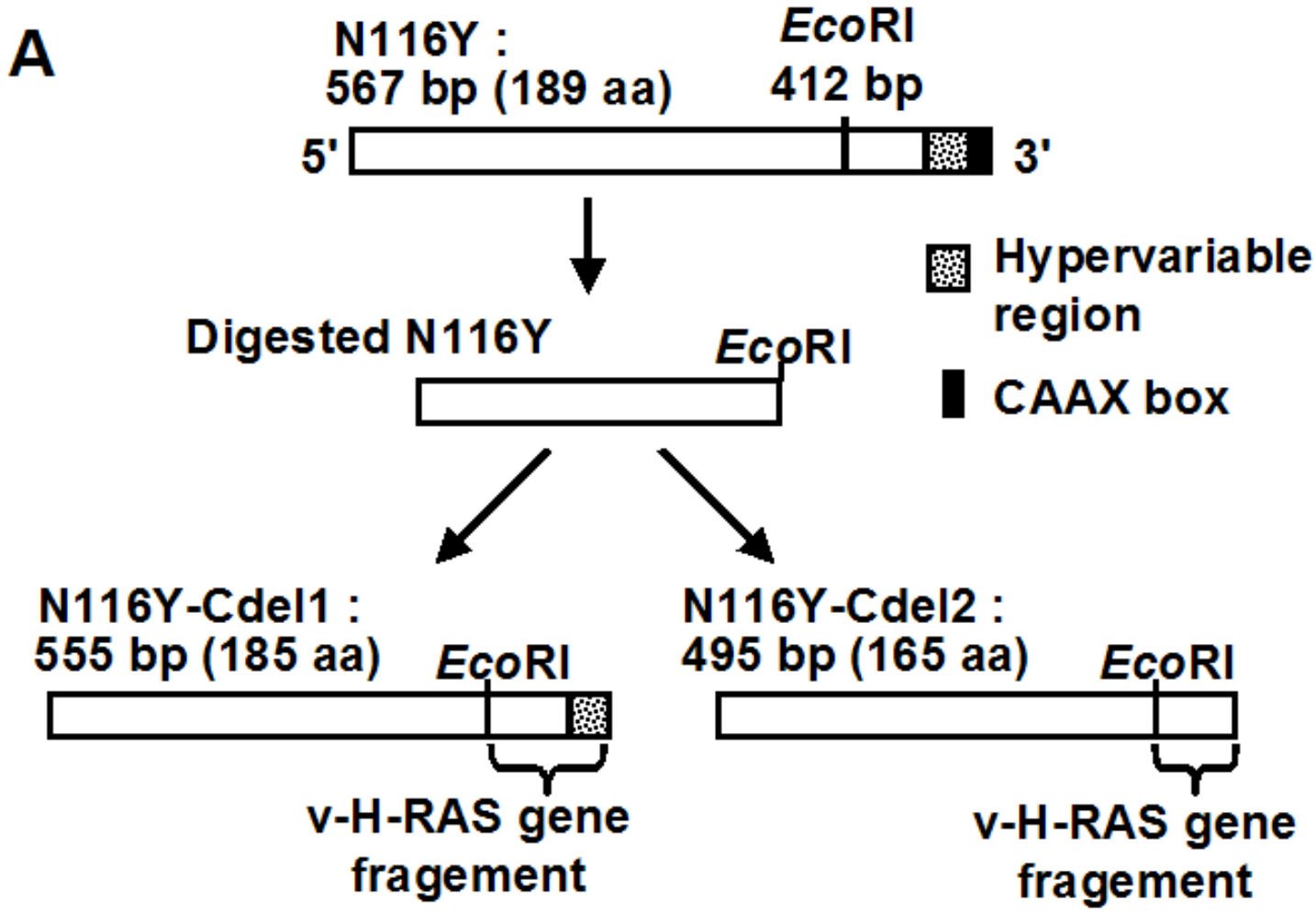
Figure 6

The number of neomycin-resistant colonies of LoVo cells transfected with each gene. Suppressive effect of N116Y-Cdel2 on LoVo cells was confirmed by observation of colony inhibition assay. 2 x 10^4 LoVo cells were transfected with pCMV-3xFlag (Neo), pCMV-3xFLAG inserted in N116Y gene (N116Y) or pCMV-3xFLAG inserted in N116Y-Cdel2 gene (Cdel2). Then, the number of neomycin-resistant colonies (colonies with a diameter > 1mm) was counted. Average of triplicate experiments is presented.

Figure 7

Suppressive effect of N116Y-Cdel2 on LoVo cells *in vivo*. 1 x 10^7 LoVo cells were subcutaneously injected into nine nude mice. Tumor volumes were measured every 2 days. Three and 10 days after transplantation, intratumoral injections of vectors were performed. First group (—◆—) was injected with PBS. Second group (—□—) was injected with a mixture of pAd-Track-CMV inserted in β-galactosidase gene and HVJ envelope vector. Third group (—▲—) was injected with a mixture of pAd-Track-CMV inserted in N116Y-Cdel2 gene and HVJ envelope vector.

Fig. 1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EcoRI
412 bp
5'
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N116Y-Cdel1 :
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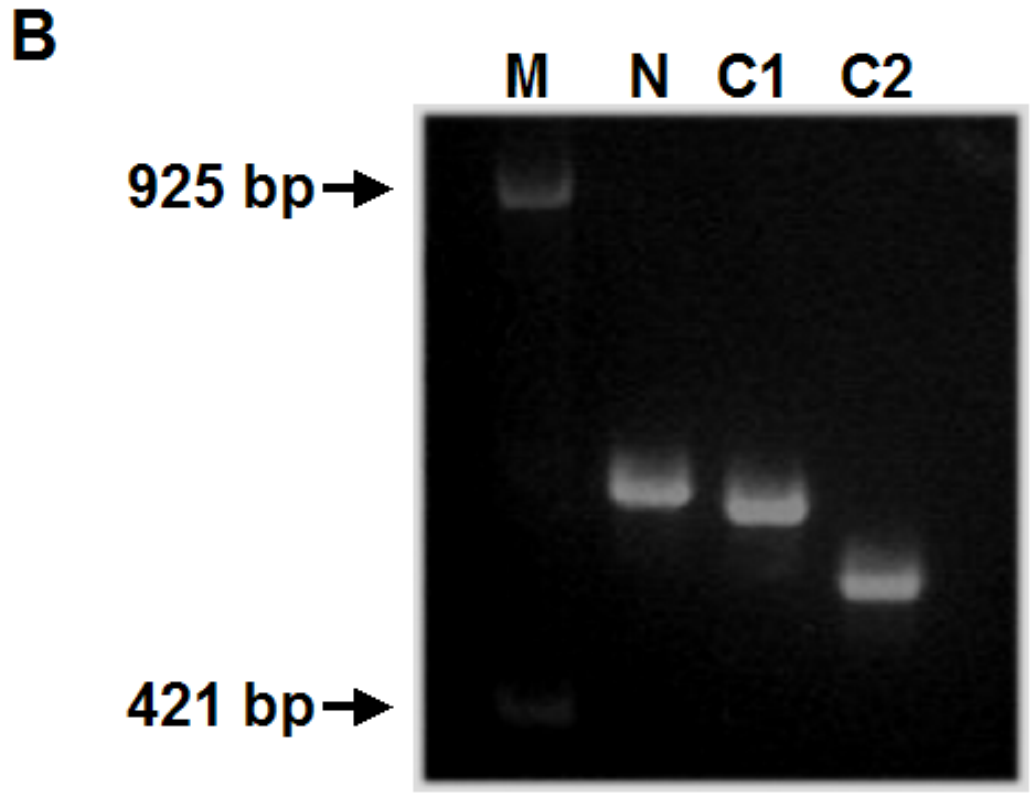
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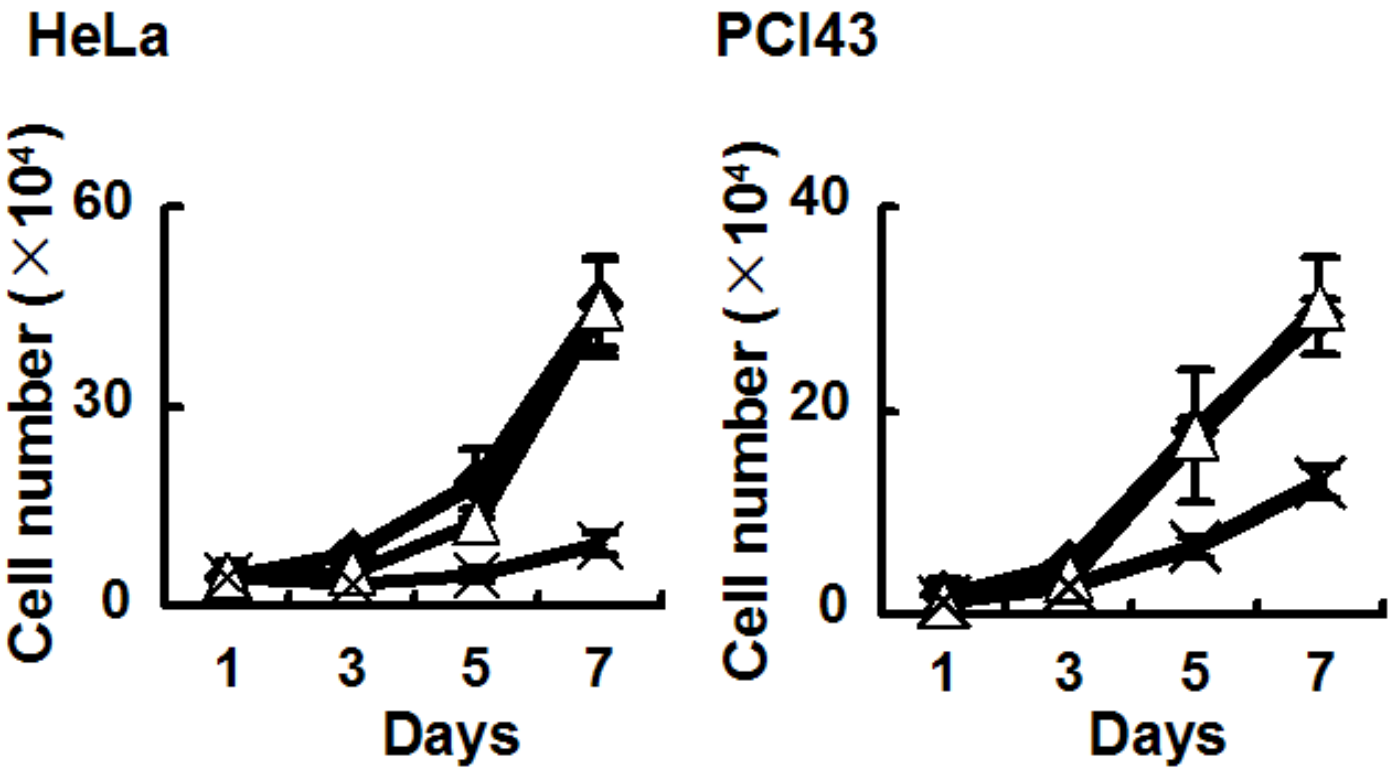
Fig. 2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Days

Fig. 3

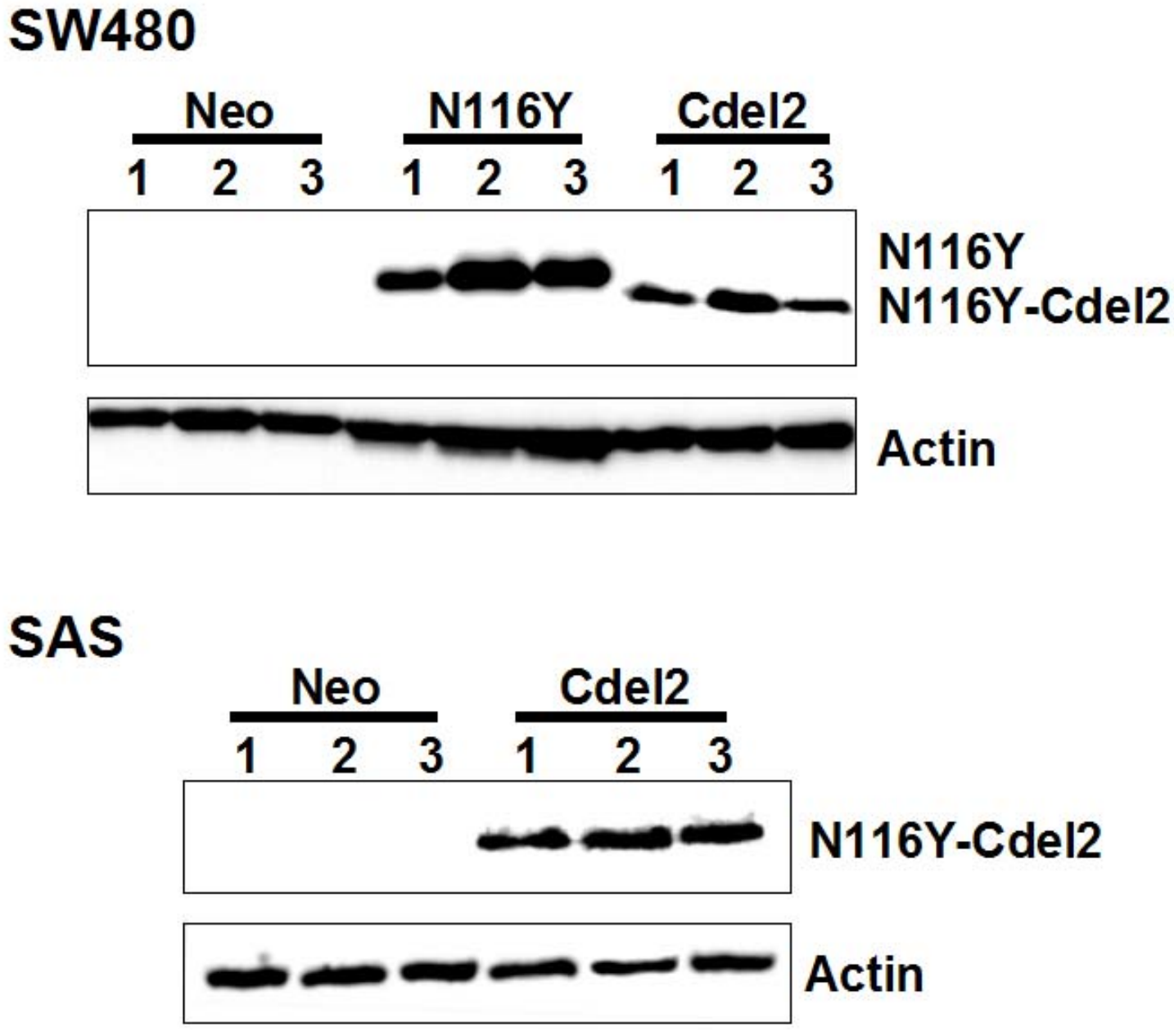

SW480
Neo
N116Y
Cdel2
1 2 3 1 2 3 1 2 3
N116Y
N116Y-Cdel2
Actin
SAS
Neo
Cdel2
1 2 3 1 2 3
N116Y-Cdel2
Actin

Fig. 4

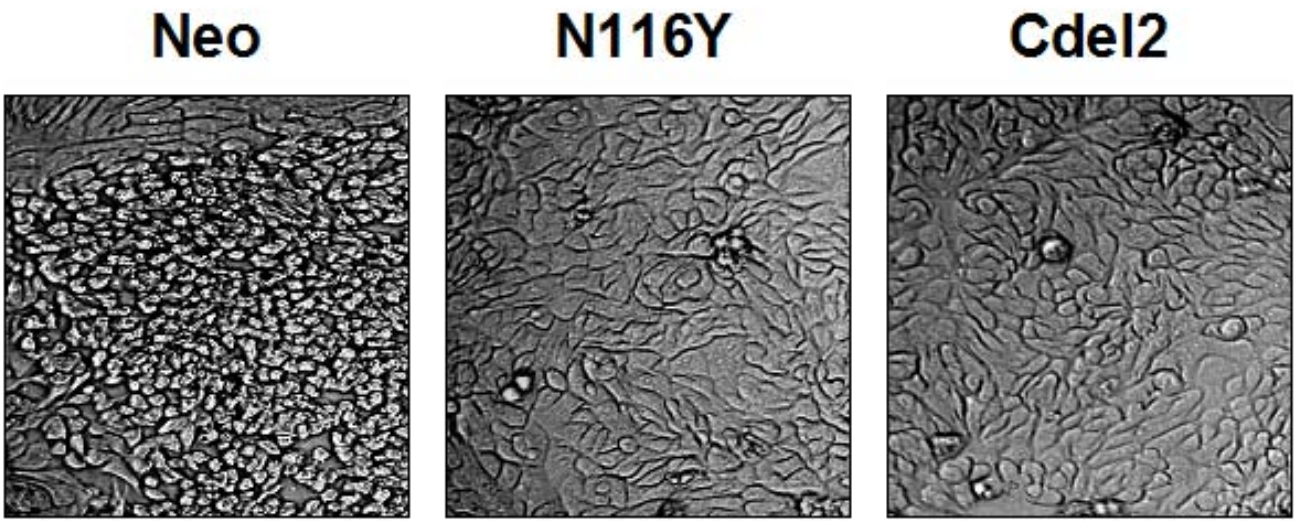


Fig. 5

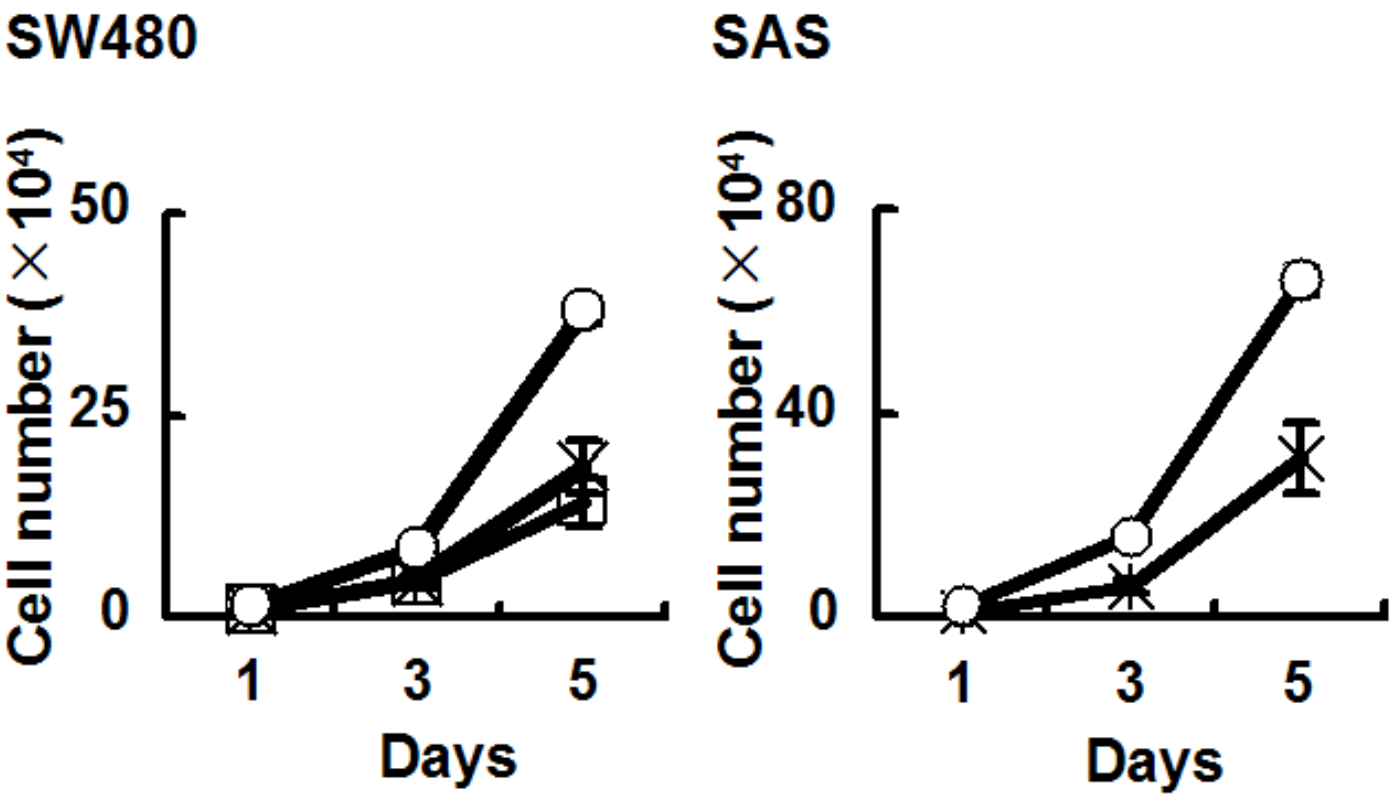

SW480
Cell number (×10⁴)
50
25
0
1
3
5
Days
SAS
Cell number (×10⁴)
80
40
0
1
3
5
Days

Fig. 6

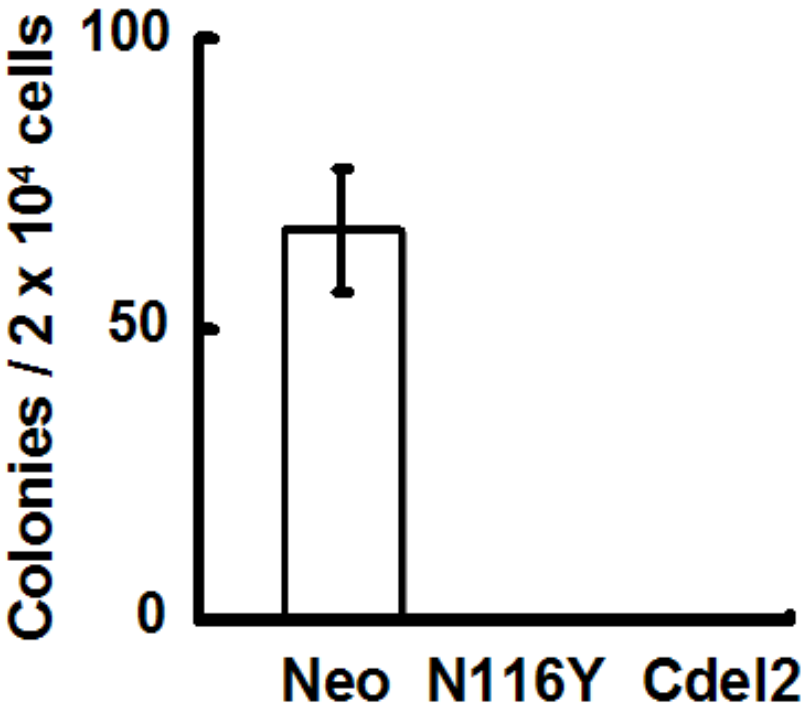
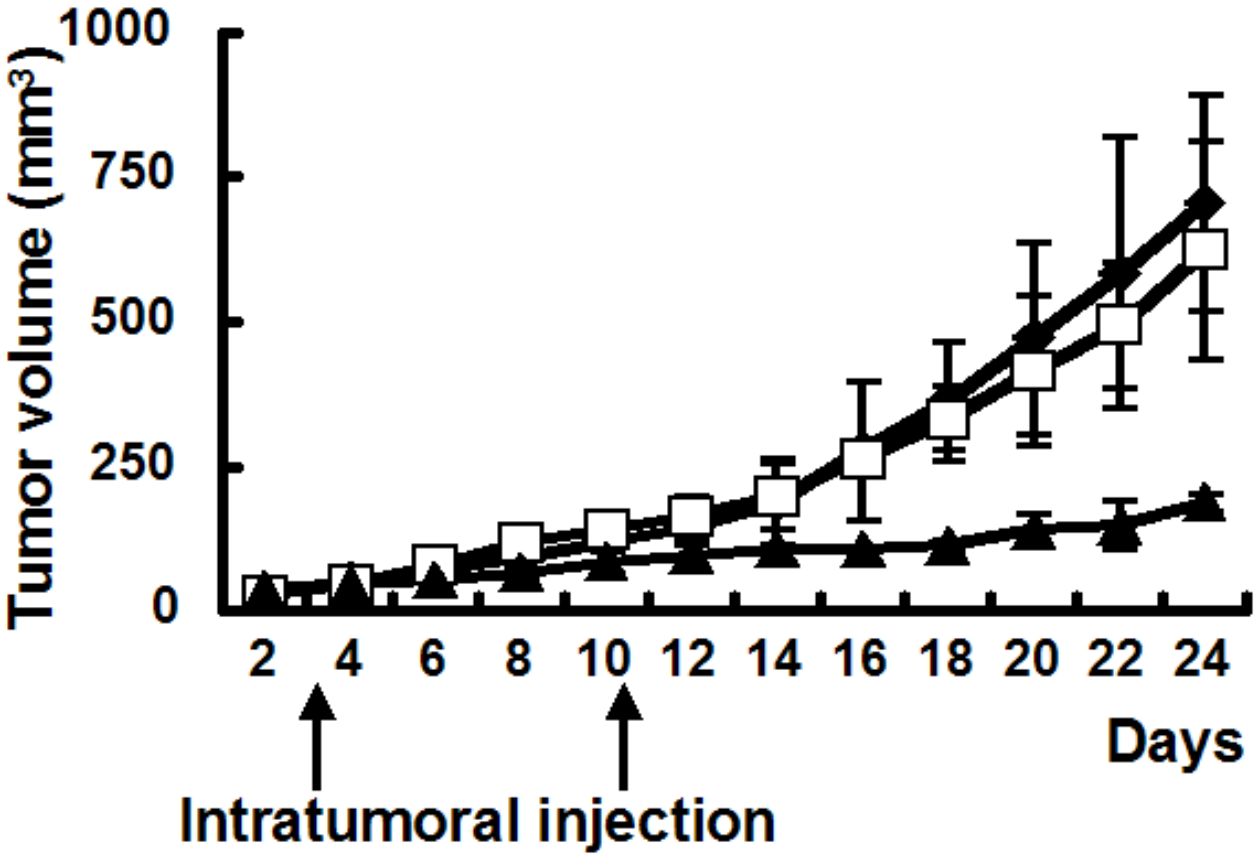


Fig. 7


Tumor volume (mm3)
1000
750
500
250
0
2
4
6
8
10
12
14
16
18
20
22
24
Days
Intratumoral injection