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## **SUPPRESSION OF Erk ACTIVATION AND IN VIVO GROWTH IN ESOPHAGEAL CANCER CELLS BY THE DOMINANT NEGATIVE Ras MUTANT, N116Y**

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### **Abstract**

Our previous studies demonstrated that introduction of a dominant negative H-ras mutant, N116Y, inhibits the growth of various types of cancer cells in vitro. In this study, we tested the efficacy of N116Y in blocking the growth of esophageal cancer cells using an adenoviral vector. Infection with N116Y adenovirus, (AdCMV-N116Y), in which N116Y expression is driven by the cytomegalovirus promoter, significantly reduced the in vitro growth of all esophageal cancer cell lines studied. Esophageal cancer cells that contained wild-type K-ras and H-ras (TE8, SGF3, SGF7) were more sensitive to AdCMV-N116Y than HEC46 cells that expressed mutant K-ras protein. Most importantly, direct injection of AdCMVN116Y into TE8-or SGF3-induced tumors in nude mice suppressed their growth significantly. To examine the suppressive mechanism of N116Y, cell cycle profile and the activation of extracellular signal-regulated kinase 2 (Erk2) were examined by flow cytometry and Western blot analysis, respectively. In TE8 cells, progression into S phase was clearly blocked after infection with AdCMV-N116Y. Infection with AdCMV-N116Y did not strongly suppress the activation of Erk2 after EGF stimulation in serum-starved HEC46 cells, whereas it completely suppressed activation in TE8, SGF3 and SGF7 cells. Our observations suggest that N116Y reduces growth of human esophageal cancer cells and suppresses the activation of Erk2; they also indicate that N116Y is a potential candidate gene for human esophageal cancer gene therapy.

### **Introduction**

Esophageal cancer ranks among the 10 most frequently diagnosed cancers in the world (Montesano et al., 1996). In Japan, it is the 6th most common cancer in males. The number of persons who die of esophageal cancer is increasing, with the age-adjusted mortality rate being relatively constant since 1950 in the range of 6.67-7.76/100,000 (Nishihira et al., 1993). Esophageal cancers generally have a poor prognosis. Because of the lack of serous membrane in the outer surface of the esophagus, esophageal cancers rapidly invade surrounding tissues. Furthermore, they often metastasize to regional lymph nodes and distant organs. Therefore, despite intensive therapy using surgery, radiation and chemotherapy, 5-year survival rates are only 10-34% (Montesano et al., 1996; Nishihira et al., 1993), underscoring the need to develop new approaches for treatment. One approach showing promise is gene therapy in which antisense oligonucleotides to oncogenes, tumor suppressor

genes and other related genes are used therapeutically to eradicate cancer cells (Liu et al., 1994).

Many studies have already identified a number of alterations of oncogenes and/or tumor suppressor genes in esophageal cancer. Ras proteins are key transducers of extracellular stimuli from the plasma membrane to the nucleus. The Ras pathway is activated by an epidermal growth factor receptor (EGFR) that is found at high levels in esophageal cancers (Montesano et al., 1996). These findings suggest that disruption of the cellular Ras function may be an effective approach in the treatment of esophageal cancer.

To abolish cellular Ras function, we used a dominant negative H-ras mutant, N116Y, in this study. It was derived from the v-H-ras oncogene by substituting asparagine with tyrosine at codon 116, the GTP-binding consensus sequence (Clanton et al., 1986). The action of Ras is regulated by several guanine-nucleotide exchange factors and GTPase activating proteins. Another H-ras mutant, N116I, which is biochemically identical to N116Y, formed a stable but catalytically inactive complex with a guanine-nucleotide exchange factor and inhibited the H-ras p21 guanine-nucleotide exchange reaction (Hwang et al., 1993). Therefore, N116Y is thought to prevent production of the GTP-bound form of the endogenous ras p21 by consuming free guanine-nucleotide exchange factor, inhibiting the signaling pathway of ras p21. Our previous studies demonstrated that transfection of an expression vector of N116Y by the lipofection procedure inhibited the colony formation of various human tumor cell lines including A431 (vulva, wild-type [wt] ras), PC3 (prostate, wt ras), T24 (bladder, wt ras), MCF7 (breast, wt ras), NKPS, TMK1 (stomach, wt ras) and PCI 35 (pancreas, K-ras mutation) in selection medium (Ogiso et al., 1994; Shichinohe et al., 1996). These observations suggested that N116Y may be applicable for gene therapy for human tumors.

Among the delivery modalities for gene therapy, adenoviral vectors provide many advantages. In general, the titer of adenovirus is 100-to 1,000-fold higher than that of retrovirus. Along with the advantage of producing high-titer viral stocks, adenovirus infection does not result in an integration of the adenoviral DNA into the host genome, and adenovirus can infect virtually all epithelial cells regardless of their cell cycle stage. Adenovirus produces little morbidity and has not been associated with human malignancies (Sokol and Gewirtz, 1996). Using this vector, we examined the growth suppressive effects of the N116Y ras mutant on esophageal cancer cell lines and analyzed its mechanism, particularly the effect on Erk2 activation. Our results indicate that N116Y should be a particularly useful gene for esophageal cancer gene therapy.

## **MATERIAL AND METHODS**

### *Cell lines and culture conditions*

Human squamous cell carcinoma of esophagus cell line TE8 (Nishihira et al., 1993) was generously provided by Dr. Nishihira (University of Tohoku, Japan). HEC46 (Yanagihara et al., 1993) was provided by Dr. Toge (University of Hiroshima, Japan), and SGF3 and SGF7 (Saito et al., 1994) were provided by Dr. Saito (Toyama Medical and Pharmaceutical University, Japan). TE8 and HEC46 were grown in Dulbecco's modified Eagle's medium with 10% heat-inactivated FCS with penicillin/streptomycin at 5% CO<sub>2</sub>. SGF3 and SGF7 were maintained in RPMI/F12 medium (1:1, v:v) with 10% FCS with penicillin/streptomycin.

### *Polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) and sequencing*

To detect point mutations at codons 12, 13 and 61 for K-ras and H-ras in esophageal cancer cell lines, PCR-SSCP was performed as described previously (Shichinohe et al., 1996). The sequencing reactions were carried out using a DNA sequencing kit (Perkin Elmer, Foster City, CA) as suggested by the manufacturer and were analyzed using an ABI PRISM 310 Genetic Analyzer (Perkin Elmer).

### *Recombinant adenoviruses*

A dominant negative H-ras mutant, N116Y, originally derived from the proviral DNA of Harvey murine sarcoma virus, cloned in the pSV2neo plasmid (Clanton et al., 1986), was provided by Dr. Shih (NCI, Frederick, MD). The 2.2 kb BamHI-EcoRI fragment containing N116Y cDNA was inserted into the BamHI-EcoRI site of the CMV early promoter adenovirus shuttle plasmid pCA14 (Hitt et al., 1995, Microbix Biosystems, Toronto, Canada). The shuttle plasmid containing N116Y, pCA14-N116Y and pJM17 (Hitt et al., 1995) plasmid were co-transfected into 293 transformed human embryonic kidney cells by calcium phosphate precipitation to generate the AdCMV-N116Y (Hitt et al., 1995). The control viral vector contains the CMV promoter and the SV 40 polyadenylation signal without a cDNA insert (AdCMV). Recombinant adenovirus was isolated from a single plaque and expanded in 293 cells. Viral stocks were purified by cesium chloride ultracentrifugation. The viral titers were determined by plaque-forming activity in 293 cells (Hitt et al., 1995).

### *Infection conditions*

Infection of the cell line was carried out according to previously reported techniques (Liu et al., 1994). Briefly, cells were infected with AdCMV-N116Y or AdCMV at the indicated multiplicity of infection (MOI) in medium containing 2% FCS. After incubation at 37° C for 60 min, medium containing 10% FCS was added, and cells were incubated at 37° C for the indicated time.

### *$\beta$ -galactosidase expression*

Cells (5  $\times$  10<sup>4</sup>) per well were plated in 6-well plates, and after 24 hr they were infected with AdCMV-LacZ at MOIs ranging from 25 to 400. After 48 hr, cells were fixed with 1.2% glutaraldehyde for 5 min and stained with 0.6 mg/ml of X-gal in 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM MgCl<sub>2</sub> in PBS at 37° C, overnight. Infection efficiency equaled the number of positive cells/number of total cells  $\times$  100%.

### *Detection of N116Y mRNA expression by reverse transcriptase-polymerase chain reaction analysis*

Total cellular RNA was isolated by the RNAsol (Biotex, Houston, TX) method. Each 20  $\mu$ l cDNA synthesis reaction contained 1  $\mu$ g of total RNA, 1  $\times$  First Strand Buffer (GIBCO BRL, Grand Island, NY; 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl<sub>2</sub>), 0.5 mM each of deoxynucleotide triphosphates, 200 units of SUPERScript II (GIBCO BRL), 10 mM dithiothreitol, 0.5  $\mu$ g oligo (dT)<sub>12-18</sub> (GIBCO BRL). The reverse transcription (RT) reaction were carried out for 50 min at 42° C and

inactivated by heating at 70° C for 15 min. Multiplex polymerase chain reactions (PCR) were performed as described previously (Wong et al., 1994). Briefly, each 25 l reaction contained 2 l of RT reaction products, 1 unit of Taq DNA polymerase (Boehringer Mannheim, Germany), 1.3 PCR buffer (Boehringer Mannheim), 160 mM of each deoxynucleotide and 20 pmol each 38 and 58 primers specific for N116Y (sense, 58-GGCAAGAGCTCCTGGTTTGG-38 [37-18 bp upstream of the v-H-ras coding region]; antisense, 58CGCATGTACTGGTCCCGCAT-38). N116Y cDNA was amplified for 30 cycles; 20 pmol of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer sets (Wong et al., 1994) were added at the beginning of the 6th cycle (25 cycles remaining) by the primer- dropping method. Conditions for PCR were 94° C for 1 min, 55° C for 30 sec, then 72° C for 1 min. The PCR products were electrophoresed in a 2% agarose gel and visualized by ethidium bromide staining.

#### *Cell growth and colony formation assays*

Cells (2.3 × 10<sup>4</sup> or 5.3 × 10<sup>4</sup> per well) were plated in 6-well plates. Cells were infected with either AdCMV-N116Y or control vector and harvested every 2 days and counted; and their viability was determined by Trypan blue exclusion. For soft agar colony formation assays, infected cells were trypsinized, then 5.3 × 10<sup>3</sup> of these were mixed with 0.33% agarose and plated over a base layer of 0.5% agarose as described previously (Shichinohe et al., 1996). The colony-forming efficiency was calculated as colony-forming efficiency = (colony number in soft agar/cell number of inoculated onto soft agar) × 100 at 6 wk. Colonies that contain more than 4 cells were counted.

#### *In vivo experiments*

TE8 cells (2.3 × 10<sup>6</sup>), or 5.3 × 10<sup>6</sup> SGF3 cells in 0.1 ml medium, were injected s.c. into the back of 4- or 5-week-old male nude mice, and tumors were allowed to develop for 3 days. Intra-tumoral injections of 0.1 ml PBS containing 4.3 × 10<sup>8</sup> plaque-forming units (pfu) of AdCMV or AdCMV-N116Y were administered 3-5 and 8-10 days after cell inoculation. Tumor volume was monitored over 30 days. Tumor volumes were measured according to the formula  $V = \frac{a^3 b^2}{2}$  (a, largest superficial diameter; b, smallest superficial diameter) (Carlsson et al., 1983). The animal experiments were conducted under the Hokkaido University School of Medicine guidelines for use of experimental animals.

#### *Flow cytometry*

For detection of DNA content, 1.3 × 10<sup>6</sup> TE8 cells were infected with either AdCMV or AdCMV-N116Y at an MOI of 200; 48 hr after infection, cells were fixed in ethanol. After RNase A treatment, cells were stained with propidium iodide and then analyzed with a FACScan flow cytometer (Beckton Dickinson, San Jose, CA).

#### *Western blot analysis*

For analysis of Erk2 phosphorylation, cells (5.3 × 10<sup>5</sup>) were infected with either AdCMV-N116Y or Ad CMV; 48 hr later, cells were starved for 24 hr and then stimulated by epidermal growth factor (EGF) (20 ng/ml) for 10 min. Cell lysates were prepared in RIPA buffer containing 1% NP-40, 0.1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 10 mM NaF, 4 mM EDTA, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 0.2 U/ml aprotinin, 1 mM PMSF; 30 g of total proteins were electrophoresed in 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The

mouse anti-human Erk2 monoclonal antibody (Transduction Laboratory, Lexington, KY) was used as the primary antibody (1:5,000). Peroxidase-conjugated goat F(ab')<sub>2</sub> anti-mouse IgG+M (Jackson ImmunoResearch, West Grove, PA) was used as secondary antibody (1:5,000). Detection of the bound antibodies was performed using the ECL system (Amersham, Aylesbury, UK).

### *Statistical analysis*

The results are presented as mean  $\pm$  6 standard error of the mean. Statistical analysis of the differences between the mean was calculated using Student's t-test, and a p value  $\leq$  0.05 was taken to indicate statistical significance.

## **RESULTS**

### *Cell line characterization*

Before evaluating the effect of the dominant negative H-ras mutant N116Y on esophageal cancer cell proliferation, we performed PCR-SSCP and sequence analyses to examine the K-ras and H-ras status of 4 human esophageal cancer cell lines. TE8, SGF3 and SGF7 exhibited wt K-ras and H-ras sequences at codons 12, 13 and 61. HEC46 had a K-ras mutation at codon 12 (GGT/GTT; Gly/Val). To examine the infection efficiency and transgene expression of the adenovirus in esophageal cancer cells, we infected recombinant adenovirus AdCMV-LacZ carrying the Escherichia coli  $\beta$ -galactosidase gene at different MOI. AdCMV-LacZ-mediated expression of  $\beta$ -galactosidase activity was detected by X-gal staining. All cell lines exhibited 75–85% positive staining at 200 MOI. Based on these results, an MOI of 200 or 400 was used in this study.

### *Expression of N116Y mRNA in AdCMV-N116Y- infected cells*

To confirm the expression of AdCMV-N116Y at the mRNA level, cells were infected with AdCMV control virus or AdCMV-N116Y at an MOI of 200. After 48 hr, total RNA was isolated and RT-PCR was performed. A 254 bp N116Y fragment was detected only in cells infected with AdCMV-N116Y, but was not detected in cells infected with AdCMV control vector (Fig. 1a). These results indicated that the exogenous N116Y mRNA was successfully expressed in AdCMV-N116Y- infected esophageal cancer cells.

### *Effect of AdCMV-N116Y on cell growth in vitro*

Cells infected with the control AdCMV vector had growth rates similar to those of the uninfected cells in vitro (Fig. 1b). HEC46 cells, which have a mutated K-ras gene, exhibited approximately 50% growth inhibition when infected at an MOI of 400. TE8 and SGF3 cells that have wt K-ras and H-ras exhibited greater growth inhibition when infected with 200 MOI of AdCMV-N116Y, and SGF7 cells with wt K-ras and H-ras also exhibited a remarkable growth inhibition following infection with AdCMV-N116Y at an MOI of 400. We also investigated the effect of AdCMV-N116Y on the anchorage dependency of esophageal cancer cells (Fig. 2a). HEC46, SGF7 and TE8 cells infected with either AdCMV-N116Y or AdCMV (400 MOI) were grown in the agar medium in triplicate. There were no significant differences between AdCMV (19.0  $\pm$  2.8%) and AdCMV-N116Y (13.9  $\pm$  1.2%) in HEC46 cells. In contrast, in TE8 and SGF3 cells, the colony forming efficiency of AdCMV-N116Y- infected cells (TE8: 0.8  $\pm$  0.6

0.6%; SGF7: 1.2  $\pm$  0.3%) was significantly ( $p$ , 0.01) reduced in comparison with that of AdCMV-infected cells (TE8: 9.6  $\pm$  1.0%; SGF7: 13.5  $\pm$  1.9%).

#### *Inhibition of tumor growth in vivo*

To explore the potential for N116Y gene therapy, TE8 or SGF3 were injected s.c. into the back of nude mice at day 0, then AdCMV-N116Y or Ad CMV were injected directly into tumors at days 3-5 and 8-10 (Fig. 2b). At day 3, tumor volumes were 79.1  $\pm$  8.1 mm<sup>3</sup> (TE8) and 114  $\pm$  17.1 mm<sup>3</sup> (SGF3). Compared with AdCMV, AdCMV-N116Y injections suppressed the tumor growth of both TE8 and SGF3. Tumor volume following treatment with AdCMV-N116Y (TE8: 359  $\pm$  47.6 mm<sup>3</sup>; SGF3: 535  $\pm$  52.8 mm<sup>3</sup>) was significantly ( $p$ , 0.01) smaller than following AdCMV treatment (TE8: 1278.5  $\pm$  61.3 mm<sup>3</sup>; SGF3: 1660.3  $\pm$  198 mm<sup>3</sup>) at the end of a 30-day period after inoculation. These data demonstrate that adenovirus-mediated N116Y gene transfer can suppress the growth of human esophageal cancers in vivo.

#### *Effect of AdCMV-N116Y on the cell cycle*

To examine the mechanism of significant cell growth reduction, we investigated the effect of AdCMV-N116Y infection on the cell cycle by flow cytometry analysis. TE8 cells were infected with AdCMV-N116Y at an MOI of 200, and the DNA profiles were analyzed 2 days later. In AdCMV-infected cells, 30% of cells were in G0-G1 phase, 50% were in S phase and 20% were in G2-M (Fig. 3). These results were similar to those of uninfected cells. In contrast, in AdCMV-N116Y- infected cells, progression into S phase was blocked (78% G0-G1 phase, 11% S phase, 11% G2-M phase).

#### *Effect of AdCMV-N116Y on phosphorylation of Erk2*

To examine the inhibitory effect of N116Y on the phosphorylation of Erk2, which is downstream of Ras, cells infected with AdCMV or AdCMV-N116Y were starved for 24 hr, then stimulated by 20 ng/ml of EGF for 10 min. In TE8, SGF3, SGF7 and HEC46 cells infected with AdCMV, the phosphorylated forms of Erk2 (pp42) were detected (Fig. 4). Whereas infection of AdCMV-N116Y completely suppressed the phosphorylation of Erk2 in TE8, SGF3 and SGF7 cells, in ras-mutated HEC46 cells, pp42 was detected. These results demonstrate that growth inhibitory effects of N116Y correlate with inhibition of Erk activation.

## **DISCUSSION**

Cancer is the end result of an acquisition of genetic alterations in key regulatory molecules, resulting in unregulated cell growth. In the case of esophageal cancers, overexpression of EGFR and cyclin D1 and mutation of p53 are the most common of these genetic events (Montesano et al., 1996). Cyclin D1, in conjunction with their catalytic partners cyclin-dependent kinases (Cdk)4 and Cdk6, appears to regulate the initial phases of G1 progression (Quelle et al., 1993). In normal untransformed cells, the growth factor- dependent accumulation of cyclin D1 has been shown to be required to allow cells to pass the G1 restriction point (Quelle et al., 1993). Evidence has accumulated that Ras regulates the expression of cyclin D1. Transformation of NIH3T3 cells by constitutive overexpression of v-H-Ras protein has been associated with increased levels of cyclin D and shortened G1 phase (Liu et al., 1995). Inactivation of Ras caused a decline in cyclin D1 protein levels; accumulation of the

hypophosphorylated, growth-suppressive form of retinoblastoma tumor-suppressor protein; and G1 arrest (Peeper et al., 1997). These reports suggest that the inactivation of Ras function might be useful in gene therapy for human esophageal cancer.

Based on this hypothesis, we used the dominant negative H-ras mutant, N116Y, to abolish cellular Ras function. This mutant, N116Y, was derived from the v-H-ras oncogene by substituting asparagine with tyrosine at codon 116 (Clanton et al., 1986). In our previous study, N116Y suppressed the transformed phenotype of NIH/3T3 cells transformed by overexpression of the c-H-ras protooncogene and by the viral oncogenes encoding tyrosine kinase proteins (Ogiso et al., 1994). Moreover, N116Y-expressing clones of a human pancreatic cancer cell line (PCI 35) became less spread and lost their anchorage-independent growth ability and tumorigenicity in vivo (Shichinohe et al., 1996). Using an adenovirus vector system, we demonstrated that AdCMV-N116Y infection effectively reduced the growth of 4 human squamous cell carcinoma of esophagus cell lines in vitro. Our in vivo results also showed that direct injection of AdCMV-N116Y into the TE8 cell or SGF3 cell-induced tumors in nude mice suppressed their growth compared with the control group. This in vivo study confirmed the in vitro effects of AdCMV-N116Y on human esophageal cancer cells, suggesting that N116Y had a therapeutic tumor-suppressing effect.

A number of effectors for Ras proteins have been identified that bind preferentially to Ras in the GTP-bound states. These include Raf1, the p110 PI3 kinase catalytic subunit, PKC  $\zeta$ , RaIGDS, Rin1 and MEKK1. Several of these have been implicated in tumorigenesis. Among these, the Ras/Raf/Erk MAPK pathway has emerged as one of the most important membrane-nucleus signaling pathways (Hunter, 1997). Erk is a serine/threonine kinase that is rapidly activated in cells stimulated with various extracellular signals by dual phosphorylation of tyrosine and threonine residues. Previous studies have shown that sustained activation of Erk is required for fibroblasts to pass the G1 restriction point and enter S-phase, while inhibition of the Erk "cascade" inhibits expression of endogenous cyclin D1 protein, DNA synthesis and cell proliferation (Pages et al., 1993; Lavoie et al., 1996). In the present study, we showed that activation of Erk2 was strongly inhibited in three human esophageal cancer cell lines after infection with AdCMV-N116Y, and that the growth inhibitory effects of N116Y on esophageal cancer cells correlated with inhibition of Erk activation. We therefore conclude that the H-ras mutant N116Y blocked the Erk signaling pathway, resulting in reduction of cell proliferation in esophageal cancer by imposing G1 arrest.

Although results using this ras mutant are very promising, there are some potential problems in the application of N116Y in gene therapy for human cancers. One of these is the possibility of reactivation or reversion of N116Y, which originated from the v-H-ras oncogene. This does not seem to be significant, because infection by adenovirus containing N116Y cDNA does not result in the integration of N116Y cDNA into the host genome. However, we are now investigating the effect of deletion of the carboxyl-terminus of N116Y on esophageal cancer cells, because Ras proteins contain a carboxyl-terminal that has been shown to be essential for mutant Ras proteins to transform cells in culture (Lowy and Willumsen, 1993). Another problem are the potential side effects of N116Y on normal cells. In gene therapy for cancer, transcriptional elements that drive expression of proteins unique to or overexpressed in malignant cells, such as carcinoembryonic antigen in colorectal cancer or  $\alpha$ -fetoprotein in hepatoma, are used to reduce toxicity to normal cells (Miller and

Whelan, 1997). We believe that the promoters of cyclin D1 or EGFR may be applicable in esophageal cancer gene therapy, because these gene products are most commonly overexpressed in esophageal cancers.

In conclusion, our results demonstrate that a dominant negative H-ras mutant (N116Y) inhibits the activation of Erk2 in esophageal cancer cells stimulated by EGF, and that expression of this mutant using the adenoviral vectors significantly reduces the growth of human squamous cell carcinoma of esophagus cells in vitro and in vivo.

## **ACKNOWLEDGMENTS**

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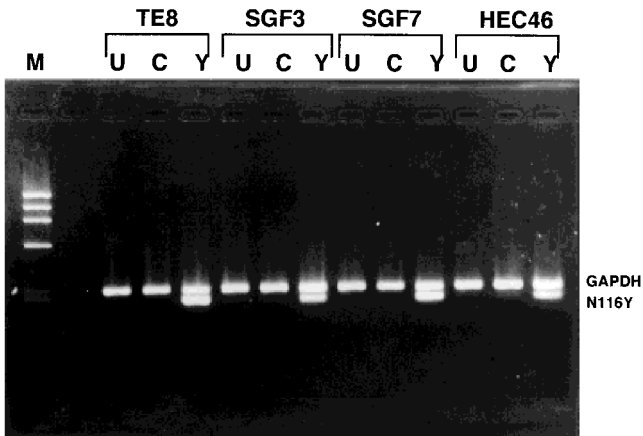
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A



B

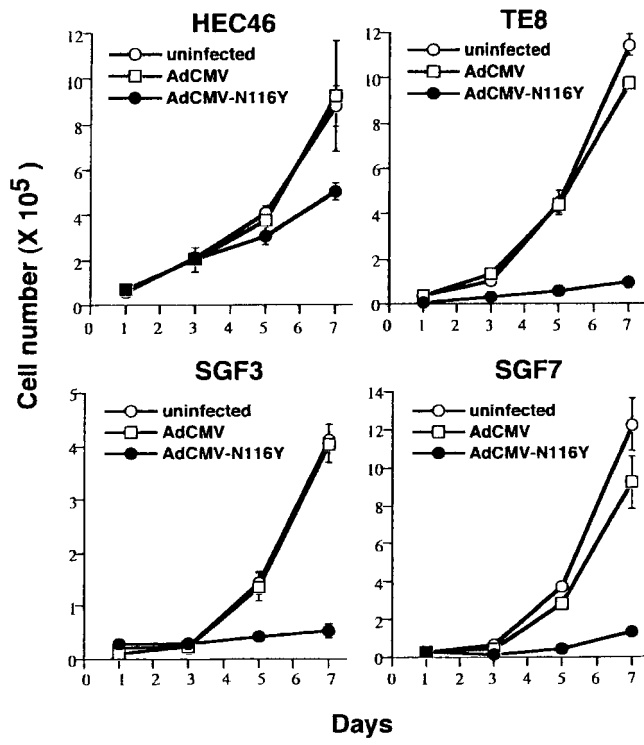
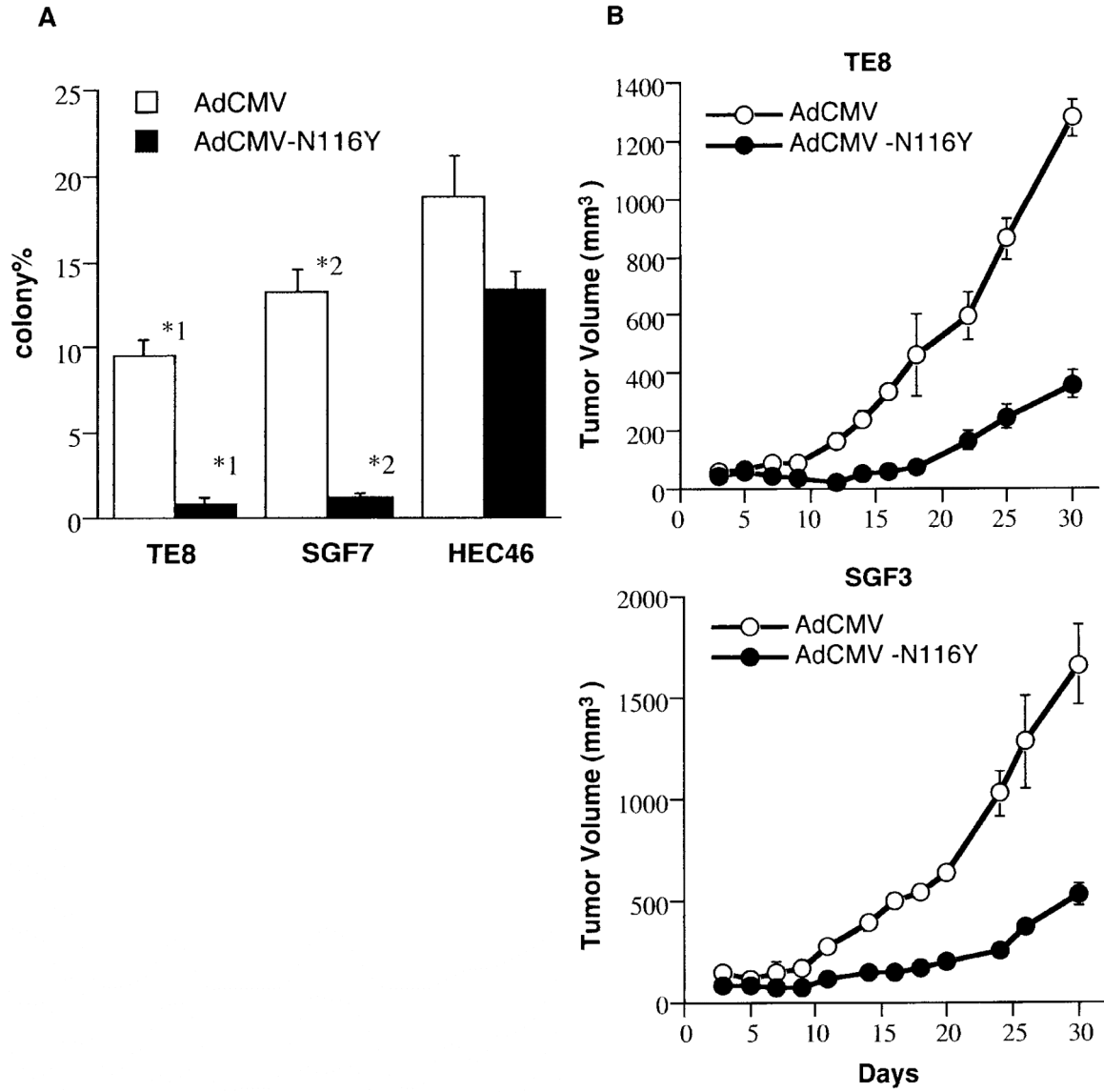


Fig. 1



**Fig. 2**

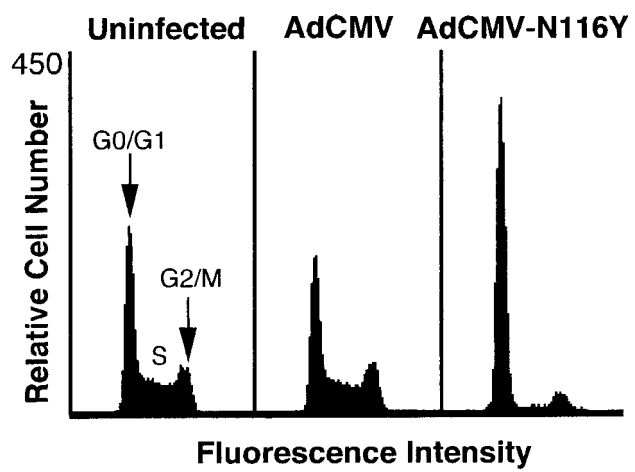


Fig. 3

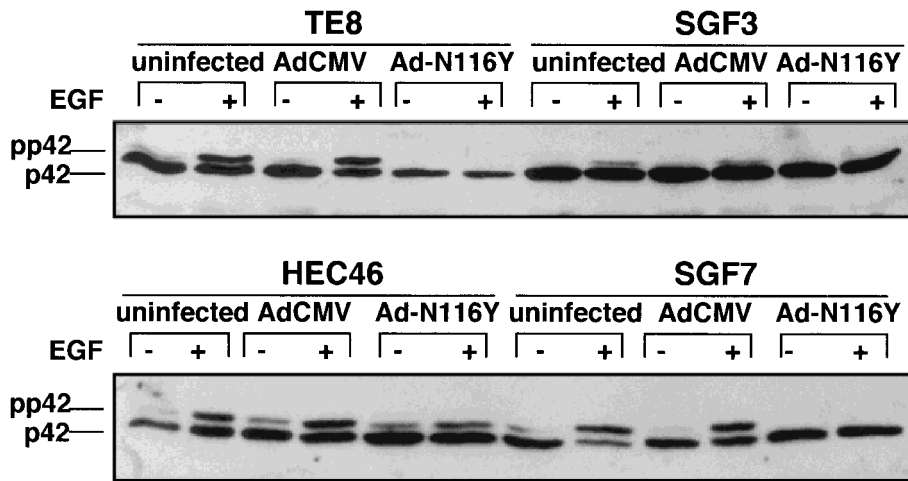


Fig. 4