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Suppressive effects of dominant negative *ras* mutant N116Y on transformed phenotypes of human bladder cancer cells

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Abstract

To investigate the suppressive effect of dominant negative H-*ras* mutant N116Y on transformed phenotypes, we established two N116Y *ras* mutant stable transfectant clones (C5, C13) of human bladder cancer cell line, UMUC-2. These N116Y *ras* mutant transfectants, especially the C5 cells, showed a dramatic change of cellular morphology and significantly reduced growth in soft agar compared to their control. Furthermore, phosphorylation of the Jun NH_2-terminal kinase (JNK) was significantly decreased in these transfectants compared to the control. These results suggest that the N116Y-induced suppression of transformed phenotypes in UMUC-2 cells is associated with inhibition of JNK phosphorylation. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Bladder cancer; Dominant negative H-*ras* mutant; Transformed phenotype; Jun NH_2-terminal kinase; Gene therapy

1. Introduction

The carcinogenesis of human bladder cancer is a multistep process. Several molecular alterations of oncogenes and tumor suppressor genes have been reported in human bladder cancers [1]. Among these, *ras* oncogenes are considered to play an important role in the pathogenesis of human bladder cancer[2]. Ras *p*21 proteins, which are encoded by *ras* oncogenes, act as an intracellular signal transducer of several extracellular stimuli that influence cellular proliferation and differentiation [3]. Oncogenic Ras constitutively activates several signaling pathways downstream of Ras. Of these, Raf-dependent activation of the extracellular signal regulated kinase (ERK) is well characterized and plays an important role [4]. Recently, Raf-independent activation of the Jun NH_2-terminal kinase (JNK) has also been reported to be crucial for cellular transformation by oncogenic Ras [5]. The importance of Ras signaling pathways in the growth and differentiation of human cancer cells has led to the development of novel cancer therapeutic modalities such as anti-Ras ribozyme that block Ras functions [6].

A dominant negative H-*ras* mutant N116Y was

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derived from the v-H-*ras* oncogene by substituting the codon for asparagine-116 with tyrosine. This mutant has been shown to suppress the transformed phenotypes of NIH/3T3 cells induced by overexpression of the H-*ras* protooncogene and several protein tyrosine kinase oncogenes [7,8]. The N116Y *ras* mutant is considered to prevent the GTP-form of the endogenous Ras proteins by inhibiting the Ras guanine-nucleotide exchange reaction [9]. The N116Y *ras* mutant also significantly inhibited the growth of a variety of human cancer cells, including human bladder cancer cell line T24 [10,11]. Furthermore, introduction of the N116Y *ras* mutant in vivo via an N116Y-containing adenoviral vector strongly suppressed in vivo growth of esophageal cancer cells [12].

Although the N116Y *ras* mutant is a potent suppressor of cellular proliferation, it has been unclear whether inhibition of the transformed phenotype in human cancer cells is brought about by this mutant or not. When the N116Y *ras* mutant was expressed under the control of the MLV retroviral promoter, we could not obtain cancer cell clones with constitutive expression of this mutant because of intensive growth suppression. The transcriptional activity of a human metallothionein (hMT) IIa promotor was reported to be more that ten times lower than that of the MLV retroviral promoter [13]. In the present study, we obtained stable clones of human bladder cancer cell line, UMUC-2 in which the N116Y *ras* mutant was constitutively expressed under the control of hMT IIa promotor. Utilizing these transfectants, we examined suppressive effects of the N116Y *ras* mutant on transformed phenotypes and signaling pathways downstream of Ras such as ERK and JNK in human bladder cancer cells, and explored the possibility of applying the N116Y *ras* mutant as a potential agent for developing gene therapy for human bladder cancer.

2. Materials and methods

2.1. Tumor cell lines

A human bladder cancer cell line, UMUC-2, was used in the present study. UMUC-2 was a kind gift from Dr B. Grossman [14]. Mutation of the H-*ras* oncogene at codon 12 was found in this cell line (unpublished data). This was maintained in Dulbecco's modified Eagle's minimum essential medium (DMEM) containing 10% fetal calf serum and 0.05% glutamine. Cells were grown in tissue-culture flasks at 37°C in an atmosphere of 95% air and 5% CO_2 and were harvested by brief treatment with trypsin/EDTA.

2.2. Plasmids and transfection

Plasmid pSV*neo*-116Y containing the N116Y *ras* mutant was provided by Dr T.Y. Shih (NCI, Frederick, MD). Plasmid pHS1, which has the hMT IIa promotor, was provided by Dr M. Karin (Southern California University, Los Angeles, CA). We initially inserted the 2.1-kb *Bam*HI–*Eco*RI fragment of the N116Y *ras* mutant into the multicloning site of the pHS1 vector, and constructed an N116Y expression vector, designated pHS1-116Y[15]. Thereafter, the 3.0-kb *Hind*III–*Eco*RI fragment containing both the hMT IIa promoter and the N116Y mutant gene of pHS1-116Y was inserted into the cloning site of pSV2*neo* to construct pHS1-116Y*neo*. Plasmid pSV2*neo* was used as the control.

The transfections were performed using a lipofection procedure as described in a previous study [12]. Briefly, UMUC-2 cells (1×10^5) were transfected using liposome-DNA complexes containing 10 μg of pHSI-116Y*neo* and 1 ml of gene transfer solution (Gene Transfer; Wako, Co., Tokyo, Japan) to isolate stable clones expressing the N116Y *ras* mutant. As a negative control, the cells were also transfected with pSV2*neo*. Transfected cells were incubated at 37°C for 48 h. Following incubation, the growth medium was changed to culture medium containing aminoglycoside G418 (geneticin, GIBCO BRL, Grand Island, NY) at a concentration of 500 μg/ml. After 2 weeks, selected G418-resistant colonies from the cells transfected with either pHSI-116Y*neo* or pSV2*neo* were cloned and expanded into cell lines.

2.3. Detection of N116Y mRNA expression by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total cellular RNA was isolated by Chomczynski's method [16]. The RNA was quantitated spectrophotometrically. Three micrograms of RNA were tran-

scribed in 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiotheitol, 0.5 μg of oligo $(dT)_{12–18}$ primer (GIBCO BRL, Rockville, MD), 3 units of RNase inhibitor (Boehreinger Mannheim, Heidelberg, Germany), 10 units of Moloney murine leukemia virus reverse transcriptase (GIBCO BRL), and 0.5 mM of each deoxynucleotide triphosphate (dNTP). The reaction was incubated at 42°C for 1 h and 70°C for 15 min, and then treated by 1.5 units of ribonuclease H at 37°C for 30 min. The PCR procedure was used to amplify specific cDNA sequences transcribed in the reverse transcription reaction. One microliter of each cDNA preparation was amplified by PCR using 25 pmol each of the forward and reverse primers [12]. The cycling conditions of PCR were 25 cycles of 1 min denaturation at 94°C, 2 min annealing at 55°C, and 3 min extension at 72°C. To detect the N116Y mRNA, oligonucleotide primers used in the PCR were 5′-GGTTTGGCAGCCCCTGTGAAG-3′ (37–18 bp upstream of the v-H-*ras* coding region) for the forward primer and 5′-CGCATGTAC-TGGTCCCGCAT-3′ (specific for 199–218 bp of the v-H-*ra*s coding region) for the reverse primer to generate a 242 bp fragment. To detect the endogenous β-actin mRNA, oligonucleotide primers used in the PCR were 5′-TGACGGGGTCACCCACACT-GTG-CCCATCTA-3′ for the forward primer and 5′-G-GGAGGTAGCAGGTGGCGTTTACGAAGATC-3′ for the reverse primer to generate a 661-bp fragment. Ten microliters of each sample was electrophoresed in a 1.5% agarose gel and visualized by ethidium bromide staining. Under these PCR conditions, the amount of PCR products was below the saturation point.

2.4. Assay for colony-forming ability in soft agar

For the assay of colony-forming efficiency in soft agar, 1×10^3 cells in 1.5 ml of growth medium containing 0.33% Noble agar (Difco, Detroit, MI) supplemented with 10% FCS were inoculated onto each 60-mm dish layered with 4 ml of 0.5% agar medium. The cells were incubated at 37°C for 14 days, and then the number of colonies (30 or more cells) was counted under a light microscope.

2.5. MTT survival assay

Cells (2000 cells in 100 μl of culture medium/well) were seeded into 96-well flat bottom plates. At indicated time points, the medium was removed, and the cells were assayed by the method of Mosmann [17]. Briefly, medium was aspirated, and fresh medium with 10% MTT (3-4,5 dimethylthiazol-2, 5 diphenyl tetrazolium bromide) (Sigma, St. Louis, MO) was added to each well and incubated for 2 h at 37°C, in 5% CO_2, in a humidified incubator. The cells were solubilized by the addition of 100 μl of dimethyl sulfoxide (Sigma, St. Louis, MO) and analyzed on an ELISA plate reader (Bio Rad, Hercules, CA) at 540 nm.

2.6. Expression and activation of ERK and JNK

Cells in the subconfluent state were serum-starved for 48 h followed by either no stimulation or stimulation with 10% FCS for 15 min at 37°C. Stimulated and unstimulated cells were lysed in ice-cold NP-40 lysis buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 100 μg/ml PMSF, 1 μg/ml aprotinin, 1% NP-40. Then 20 μg of cell lysate was separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto a polyvinylidene difluoride microporous membrane (Fluorotrans, Pall BioSupport, Port Washington, NY) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) with a semidry transfer apparatus (BIO RAD).

For the determination of endogenous ERK or JNK expression, blots were incubated with an anti-ERK or anti-JNK primary antibody (p42/44 MAP Kinase Antibody or SAPK/JNK antibody) (New England Biolabs, Beverly, MA) that recognized phosphorylated (active) and unphosphorylated (inactive) forms of ERK and JNK proteins, respectively. Furthermore, to assess the phosphorylation state of ERK and JNK proteins, antibodies specific for the phosphorylated forms of the ERK proteins (phospho-p42/44 MAP kinase Antibody) (New England Biolabs, Beverly, MA) or specific for the phosphorylated forms of the JNK proteins (phospho-SAPK/JNK Antibody) (New England Biolabs, Beverly, MA) were utilized. The primary antibodies were detected using a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Biosource International, Camarillo, CA). Immunoreactive bands were detected using enhanced chemiluminescence reagents (ECL; Amersham,

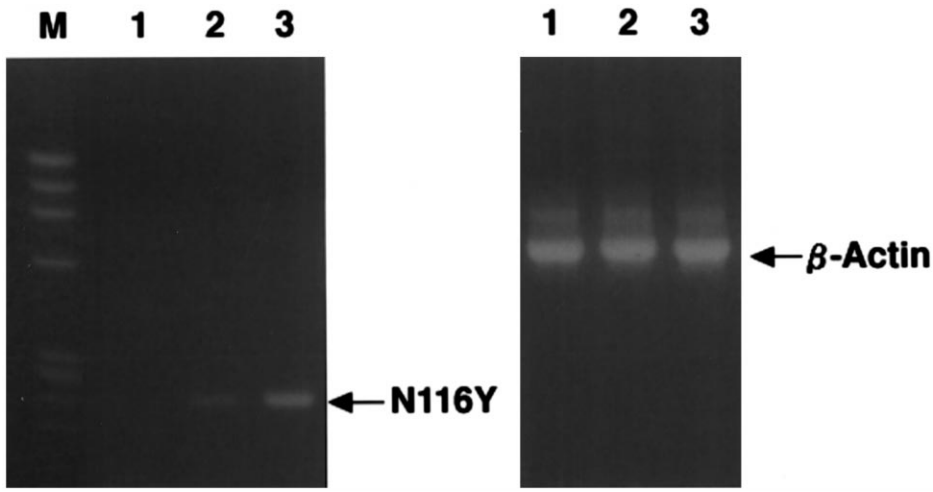

Fig. 1. Detection of N116Y mRNA in the N116Y *ras* mutant transfectant of UMUC-2 cells. The expression of N116Y mRNA (left) and β-actin mRNA (right). Lane 1, total RNA from the control clone N1; Lane 2, from the clone C13; Lane 3, from the clone C5; Lane M, standard DNA marker Φx174/*Hae*III digests.

Buckinghamshire, UK) according to manufacturer's instructions.

3. Results

3.1. Isolation of N116Y ras mutant transfectants of human bladder cancer cell line UMUC-2

By the transfection of an N116Y expression vector, pHSI-116Y*neo*, we obtained 2 N116Y *ras* mutant transfectant clones (C5 and C13) expressing N116Y mRNA. One clone, C5, expressed a larger amount of N116Y mRNA than the other, C13 (Fig. 1, left). All neomycin-alone transfectant clones expressed no N116Y mRNA, as shown in the N1 cells. The amount of β-actin mRNA for the internal control was the same for the N116Y *ras* mutant transfectants and the neomycin-alone transfectant clones (Fig. 1, right).

3.2. Cellular morphology of the N116Y *ras* mutant transfectants of UMUC-2

We examined the cellular morphology of the N116Y *ras* mutant transfectants (Fig. 2). The C5

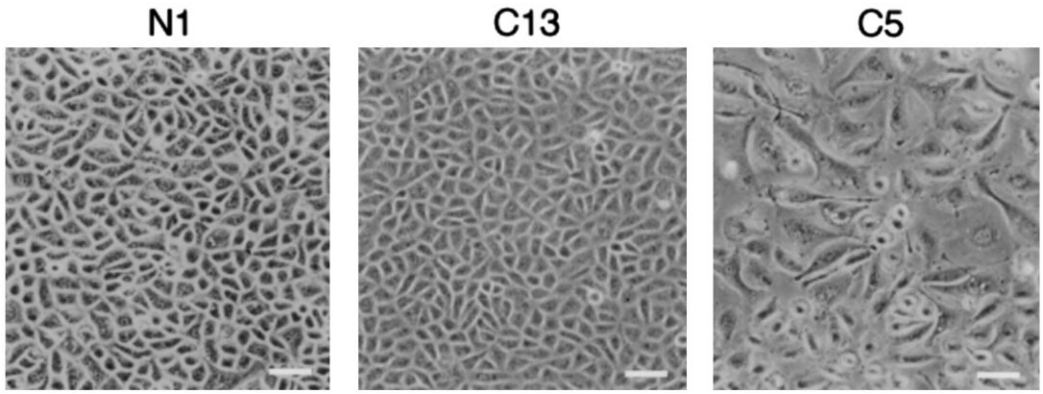

Fig. 2. Morphology of the N116Y *ras* mutant transfectant clones. Phase-contrast micrograph. Bar, 40 μm.

cells showed distinctive morphologies; polynuclei, flatter and enlarged cytoplasm, and obscure cell borders, compared with the N1 cells. The C13 cells showed slightly flatter cytoplasm and obscure cell borders compared with the N1 cells. These results indicated that the cellular morphology was changed in proportion to the amount of N116Y mRNA.

3.3. In vitro growth characteristics of the N116Y ras mutant transfectants of UMUC-2.

There was no significant difference among the in vitro growth rates of all clones examined: C5, C13, and N1 exhibited doubling times of 25.5, 21.3, and 20.3 h, respectively. On the other hand, the N116Y *ras* mutant transfectant clones (C5 and C13) demonstrated significantly reduced growth in soft agar compared to the neomycin-alone transfectant clone (Fig. 3). Furthermore, the colony-forming efficacy of the C5 cells was significantly decreased in comparison with that of the C13 cells. These results indicate that the transformed phenotype of UMUC-2 cells was suppressed in proportion to the amount of N116Y mRNA.

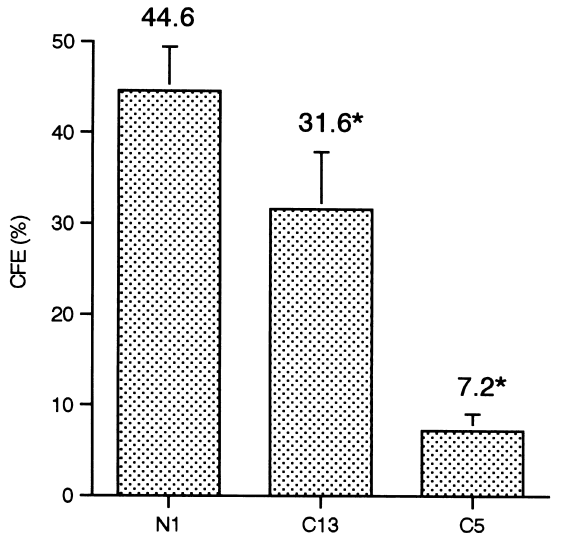

Fig. 3. Colony forming efficiencies of the N116Y expressing clones. CFE, percentage of input cells (1×10^3) grown as soft agar colonies. The * indicates that CFE (C5 or C13) is significantly different than CFE (N1) with $P < 0.05$. Average of tripricate experiments are presented.

3.4. Suppression of JNK activation in the N116Y ras mutant transfectants of UMUC-2

As shown in Fig. 4a, the expression of phosphorylated-JNKs in the serum-starved condition was significantly decreased in the N116Y *ras* mutant transfectants (C5 and C13) compared to the neomycin-alone transfectant clone (N1), whereas a transient increase in the expression of phosphorylated-JNKs was seen in these cells when they were treated with 10% FCS. Endogenous JNKs were equally expressed in all transfectants examined. On the other hand, endogenous ERKs and phosphorylated-ERKs in serum-starved and serum-stimulated conditions and were equally expressed in all transfectants (Fig. 4b). These results indicated that the N116Y *ra*s mutant suppressed JNK activation but did not affect ERK activation in UMUC-2 cells.

4. Discussion

It has been shown that the N116Y *ras* mutant is a potential candidate gene for gene therapy of a variety of human cancers [10,11,18]. However, when we choose a target gene for cancer gene therapy, the expression level of the target gene must be taken into consideration. Because the efficacy of in vivo gene transduction is relatively faint, it is important that the target gene be effective even though its expression level is low in view of the clinical application. We, therefore, investigated the effects of the N116Y *ras* mutant on human bladder cancer cell line, UMUC-2 when it was weakly expressed under the control of promotor hMT IIa. The present study offered evidence that, although the N116Y *ras* mutant could not significantly suppress cellular growth under such conditions, constitutive expression of dominant negative *ras* mutant N116Y caused a dramatic change of cellular morphology and a corresponding significant impairment in its ability to grow in soft agar. Ogiso et al. showed that the N116Y *ras* mutant significantly suppressed the transformed phenotypes of 18A cells, NIH3T3 cells transformed by an LTR-linked rat c-H-*ras* protooncogene [7]. These results suggest that the N116Y *ras* mutant suppresses tumorigenic transformation of human bladder cancer cells as shown in rodent fibroblast cells.

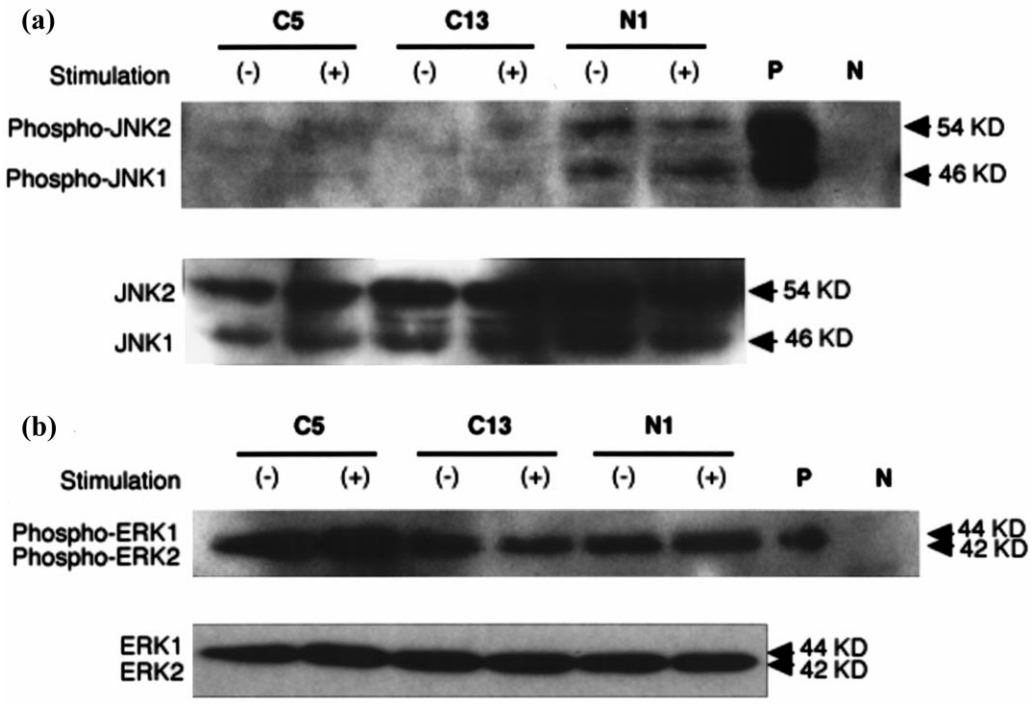

Fig. 4. (a) JNK phosphorylation in the N116Y *ras* mutant transfectant. Each clone was serum starved for 48 h prior to serum stimulation (10% FCS). (Upper panel) Phosphorylated JNKs detected by anti-phospho-JNK antibody. Lane P, UV-treated 293 cells as a positive control; Lane N, UV-untreated 293 cells as a negative control. (Lower panel) Expression of JNKs detected by anti-JNK antibody. (b) ERK phosphorylation in the N116Y *ras* mutant transfectant. Each clone was serum starved for 48 h prior to serum stimulation (10% FCS). (Upper panel) Phosphorylated ERKs detected by anti-phospho-ERK antibody. Lane P, phosphorylated ERK2 protein as a positive control; Lane N, non-phosphorylated ERK2 protein as a negative control. (Lower panel) Expression of ERKs detected by anti-ERK antibody.

However, it has remained unclear how suppression of the transformed phenotype is brought about by the N116Y *ras* mutant. Several studies showed that the mutant might suppress the Ras guanine–nucleotide exchange reaction and prevent the production of the GTP-form of endogenous Ras proteins [8,9]. Since Ras mediates its action through interaction with downstream effector targets preferentially associated with Ras-GTP [19], the N116Y ras mutant could cause suppression of several signaling pathways downstream of Ras. Of these, the Raf-dependent pathway plays an important role in Ras-mediated transformation and cellular proliferation. Activated Ras promotes the activation of Raf serine/threonine kinases. It then causes activation of the mitogen-activated protein kinase (MAPK) kinases (MEKs), which in turn activate the ERKs [4]. Ras also causes activation of a Raf-independent pathway that leads to activation of the JNKs [5]. Clark et al. reported that inhibition of JNK activation inhibited Ras transformation of NIH3T3 cells [20]. Furthermore, Ras suppressor Rsu-1 suppressed Ras transformation by inhibition of JNK activation even though the Raf/MEK/ERK pathway was enhanced [21]. These results suggest that JNK activation via a Raf-independent pathway is also associated with Ras-mediated transformation. We therefore compared the phosphorylation of ERKs and JNKs between the N116Y *ras* mutant transfectant clones of UMUC-2 and their control clone in order to clarify the mechanism by which the N116Y *ras* mutant caused suppression of the transformed phenotype. In the present study, the N116Y *ras* mutant did not suppress the ERK activation of UMUC-2 cells that had an H-*ras* mutation. On the other hand, we found

that phosphorylation of JNKs was significantly decreased in the N116Y *ras* mutant transfectant clones. Thus, the N116Y *ras* mutant could suppress the phosphorylation of JNKs in UMUC-2 cells when it was weakly expressed under the control of the hMT IIa promotor. Considering that JNK activation is crucial for cellular transformation by oncogenic Ras [20,22], suppression of transformed phenotypes in UMUC-2 cells appears to be significantly associated with the inhibition of JNK phosphorylation by the N116Y *ras* mutant.

The results described in this study indicate that the N116Y *ras* mutant is capable of suppressing the transformed phenotype of the UMUC-2 bladder cancer cell line, even when its expression is relatively low. In general, the goal of gene therapy for human cancer is to suppress cellular proliferation and to promote programmed cell death [23]. High expression of the N116Y *ras* mutant could significantly suppress the growth of human cancer cells in vitro and in vivo. However, we have to take into consideration that the efficacy of in vivo gene transduction is relatively low. Since the N116Y *ras* mutant could suppress some aspects of tumorigenicity of UMUC-2 cells at its low expression level, this mutant might be applicable for gene therapy of human bladder cancer.

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