Role of Histone Deacetylase Inhibitor in Adenovirus-mediated 
\textit{p53} Gene Therapy in Esophageal Cancer

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\textbf{Abstract.} \textit{Background:} Recently, the use of histone deacetylase inhibitors (HDACIs) with gene therapy has been shown to improve the effect of this therapy. The effectiveness of one of the novel HDACIs, FK228, was examined in adenovirus-mediated \textit{p53} gene therapy of esophageal squamous cell carcinoma (ESCC). \textit{Materials and Methods:} The expression levels of coxsackie and adenovirus receptor (CAR) in ESCC patients were examined immunohistochemically. CAR induction by FK228 in ESCC cells was analyzed by real-time PCR and Western blotting. The efficiencies of adenoviral transduction treated with FK228 were determined using AV1.0CMV-\textit{gal}. The acetylation of \textit{p53} protein was detected by Western blotting. \textit{Results:} CAR expression was reduced in some tumor specimens compared to that in normal specimens. CAR expression was increased by FK228 in both in vitro and in vivo experiments. FK228 improved the efficiency of adenovirus infection. Acetylated \textit{p53} protein was increased in some tumor specimens compared to that in normal specimens. CAR expression was increased by FK228 in both in vitro and in vivo experiments. FK228 improved the efficiency of adenovirus infection. Acetylation of \textit{p53} protein was increased in a dose- and a time-dependent manner. \textit{Conclusion:} Our findings suggest that FK228 has a potent ability to augment the effect of adenovirus-mediated \textit{p53} gene therapy in ESCC cells.

Esophageal squamous cell carcinoma (ESCC) is known as one of the most aggressive types of malignant cancer due to its high mortality rate. Recently, improvement of diagnostic apparatuses and development of treatment by a combination therapy of surgery, radiation, and chemotherapy have improved the prognosis of this cancer. However, the number of advanced cancer cases still remains high and, even if the tumor is radically removed, the 5-year survival rate is still around 60\% (1, 2).

Currently, the regimen of chemotherapy for ESCC patients has most frequently involved using a combination of cisplatin and 5-fluorouracil (5-FU) (3). This regimen is effective to some extent, but the outcome is unsatisfactory as a radical therapy and because of the lack of specificity for any certain type of cancer, the side-effects of these anticancer drugs cannot be ignored.

In our department, we have conducted a phase I/II trial of \textit{p53} gene therapy in ESCC patients and have examined the effect of histone deacetylase inhibitor (HDACI) on ESCC (4, 5). Ten patients received a total of 26 cycles of gene administration mediated by adenovirus in our department. Six of the ten patients survived for more than 1 year and one of these six stayed alive for more than 3 years. In addition, the administration of \textit{p53} gene mediated by adenovirus was carried out safely without any side-effects.

The role of histone deacetylase in chromatin remodelling is to repress transcription by the deacetylation of lysine residues (6, 7). Presently, the occurrence of cancers is mostly thought to be related to an abnormality in gene expression and numerous reports have shown decreases in tumor suppressor expression in cancer tissue compared to that in normal tissue (8). Acetylation of lysine residues can release this restraint and histone deacetylase inhibitor (HDACI) is a novel drug that has been investigated vigorously in recent years to induce acetylation of lysine residues (9, 10). One of the HDACIs, FK228 (depsipeptide), is a promising anticancer agent and has been used in a phase II clinical study in some patients with hematopoietic and solid malignancies (11). We also reported that FK228 induces growth inhibition and apoptosis, and investigated those effects associated with the modulation of various gene expressions by acetylation of chromatin (5, 12). Furthermore, it is known that HDACIs induce the expression of adenovirus receptor, coxsackie and adenovirus receptor (CAR) (13, 14), and exert an effect on \textit{p53} protein by acetylating its lysine residues in the regulatory domain (15, 16).

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The p53 gene therapy that we carried out was to assess the efficiency of that therapy alone. The outcome of the phase I/II trial showed its safety and moderate effect, and henceforth we are considering applying it as one of the finest tools to eradicate ESCC. In this study, we investigated the potential of FK228 as a new partner in p53 gene therapy in ESCC patients.

**Materials and Methods**

**Immunohistochemistry.** A total of 40 primary esophageal squamous cancer samples were obtained at the Department of Frontier Surgery, Chiba University Hospital, Chiba, Japan. All of these patients providing samples were undergoing surgical operations without any preoperative radiotherapy or chemotherapy. The histological diagnosis revealed that all of the patients had squamous cell carcinoma. The specimens were immersed in 10% formaldehyde immediately after removal and then were embedded in a paraffin block. Blocks containing both carcinoma and the adjacent normal epithelium were chosen and sections were stained with anti-human CAR antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). In brief, the sections were deparaffinized and the endogenous peroxidase activity was inactivated in 100% methanol containing 3% hydrogen peroxide. After the blocking of non-specific binding by treating the slides with 5% skim milk at 37°C for 60 min, the slides were incubated at room temperature with the primary antibody at 1:200 dilution. The sections were then washed and incubated with the ENVISION+ kit (DAKO, Copenhagen, Denmark) for 60 min. 3, Diaminobenzidine was used as a chromogen to reveal the antigen and the sections were then counterstained with Harris’ hematoxylin.

**Cell culture and chemicals.** All cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS). T.Tn cells were obtained from the Japanese Cancer Research Resources Bank. TE2 cell were kindly provided by Dr. T. Nishihira (Tohoku University, Sendai, Japan). FK228 was provided by Fujisawa Pharmaceutical Company (Tokyo, Japan). In in vitro studies, FK228 was dissolved in 100% ethanol and diluted with each experimental medium. In in vitro studies, FK228 was dissolved and diluted with 10% polyoxyethylated (60 mol) hydrogenated castor oil (HCO60) in saline.

**Western blotting analysis.** T.Tn and TE2 cells were incubated with 1 ng/ml of FK228 and harvested at 0, 6, 12, 24, and 48 hours or incubated with 0, 0.1, 1.0, 10, 100, or 1000 ng/ml of FK228 and harvested at 24 hours. Whole cell pellets were washed three times in phosphate-buffered saline (PBS), resuspended in lysis buffer [20 mM Tris-HCl (pH 7.5), 5% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 50 μM leupeptin, 50 μM antipain, 50 μM pepstatin, 50 μM N-acetyl-leucyl-leucyl-norleucinal], Protein extracts (40 μg) were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked in PBS containing 5% skim milk and 0.1% TWEEN 20. Anti-human CAR antibody (1:1000; Santa Cruz Biotechnology), anti-P53 (Ab-6) (1:1000; Oncogene Research Products, San Diego, CA, USA), anti-acetylated P53 L373, 382 antibody (1:1000; Upstate Biotechnology, Lake Placid, NY, USA) were used. After incubation with a primary antibody for 1 hour at 37°C, the membranes were incubated for 45 min in the second antibody in PBS and visualized using ECL Western Blotting Detection Reagents (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA).

**Real-time quantitative reverse transcription-PCR.** Cells were seeded into a 225 cm² flask and incubated with 1 ng/ml of FK228 and harvested at 0, 6, 12, 24, and 48 hours or incubated with 0, 0.1, 1.0, 10, 100, or 1000 ng/ml of FK228 and harvested at 24 hours. Cells were washed with PBS and processed for RNA extraction with RNeasy Kit; (Qiagen, Inc., Chatsworth, CA, USA) following the manufacturer’s protocol. The cDNA templates for real-time PCR were synthesized from 1 μg of total RNA using SuperScript II reverse transcriptase and an oligo-dT primer.

The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene served as an internal control. The PCR reaction mixture consisted of DNA Master SYBR Green I mix (LightCycler-FastStart DNA Master SYBR Green I kit; Roche Diagnostics, Basel, Switzerland; containing Taq DNA polymerase, dNTP, 3 mM MgCl₂, and SYBR Green dye), 0.5 μM of each primer and cDNA. The PCR processes were as follows: initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 57°C for 10 s and elongation at 72°C for 8-18 s. The Fit Points method provided by the LightCycler software was used to estimate the concentration of each sample. Primers were chosen using Primer3 (available at: http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The following primer sequences were used: CAR: 5’-GCCCT CAGGTGCGAGAGTGTAC-3’ and 5’-CTTAAGTCGAACTGGGTGCCAAG-3’. GAPDH: 5’-ACCACAGTCTACGCCCATC-3’ and 5’-TCACACCACCTGTGCTGTA-3’.

The expression value was calculated as follows: expression level of each mRNA/expression level of GAPDH. Experiments were performed in duplicate.

**Xenograft model of real-time quantitative PCR for CAR gene.** T.Tn (2.5x10⁶ cells) and TE2 (2.5x10⁶ cells) were injected into the backs of 7-week-old female BALB/C nu/nu mice (around 30g). Twenty five mice were injected for each cell line. When the tumor weight reached about 200 mg, animals were treated i.v. once with 3.0 mg/kg of FK228, and RNA was extracted from solid tumors at 0, 6, 12, 24, 48 hours post injection using RNeasy Mini Kits (Qiagen, Chatsworth, CA, USA). Five mice were sacrificed at each time point. RNA was reverse-transcribed and PCR analysis for CAR and GAPDH was performed as described above. Tumor weight was calculated from the following formula: tumor weight (mg) = length x width² / 2.

**The augmentation of adenoviral in vitro transduction efficiency by FK228 treatment.** T.Tn and TE2 cells were plated into 24-well plates at a density of 2x10⁴ per well with the medium containing 10% FBS. At 48 hours after seeding, cells were exposed to 0, 1, or 10 ng/ml of FK228 and incubated for a further 48 hours. Cells were then infected with the AV1.0CMV-βgal (17) at multiplicities of infection (MOI) of 100 at 37°C for 60 min and were gently shaken every 15 min. The medium was changed to fresh DMEM and the cells incubated for a further 24 hours. Infected cells were determined by βgal Staining Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s protocol and blue color cells were counted as positive cells.
Results

Immunohistochemical analysis of CAR expression in ESCC. Immunohistochemical staining of CAR expression in tumors from 40 ESCC patients who were not administrated any preoperative radiotherapy or chemotherapy. CAR expression was observed in all of the specimens examined and the expression in tumor was lower in 9 cases compared to that in the corresponding normal epithelium (Figure 1). In all other cases, no observable difference was detected between the tumor and normal epithelium expression.

FK228 activates CAR expression. To determine whether CAR expression levels were activated by FK228, real-time reverse transcription-PCR and Western blotting analysis were performed. CAR expression levels increased both in a time and dose-dependent manner, the maximum levels reached were at least three times higher than the control levels in both cell lines (Figure 2A and B). Furthermore, similar results were observed in the Western blotting analysis (Figure 3A and B).

Effect of FK228 on the expression of CAR in T.Tn and TE2 xenografts. In in vivo experiments, CAR expression changes by FK228 were examined using real-time reverse transcription-PCR and remarkable induction of CAR expression was found (Figure 4). At 6 or 12 hours after FK228 injection, the expression levels of CAR mRNA peaked, with maximum levels at two and a half times that of the control.

Enhancement of adenovirus gene transfer by FK228. The efficiencies of adenoviral transduction in T.Tn and TE2 cells were determined using AV1.0CMV-βgal (Figure 5A). The transduction efficiencies of these cell lines were similar and there appeared to be a linear relationship between the transduction efficiency and MOI ranging from 10 to 3,000. Although the cells were inoculated with a dose of AV1.0CMV-βgal at MOI 3,000, the transduction efficiencies did not reach 100%. On the contrary, the dose of AV1.0CMV-βgal at MOI 100 with 10 ng/ml of FK228 induced more than 90% efficiency in both cell lines (Figure 5B). In comparison, inoculation with a dose of AV1.0CMV-βgal at 100 alone showed less than 20% transduction efficiency.

p53 acetylation by FK228. We confirmed whether FK228 can induce the acetylation of the lysine residues of p53 protein by Western blotting analysis using the specific antibody to recognize the acetylated p53 373, 382 lysine residues. The total amount of p53 protein was reduced in T.Tn cells and relatively unchanged in TE2 cells. However, the amount of acetylated p53 protein increased in both cell lines in a dose-dependent manner (Figure 6A). In addition, the increase was also time-dependent (Figure 6B).

Discussion

P53 gene therapy mediated by adenovirus conducted in our department has shown its promising efficacy in ESCC (4). However, the effectiveness of p53 gene therapy alone was thought to be inadequate to eradicate this highly malignant disease. Therefore, a new strategy to improve the efficiency of p53 gene therapy is a very promising treatment for ESCC patients. In addition, we have shown the effectiveness of a novel histone deacetylase inhibitor, FK228, in ESCC cells and the results have suggested that FK228 has a potent tumor suppressive function derived from its distinctive character to affect gene transcription (5). In this study, we demonstrated the role of FK228 in adenovirus-mediated p53 gene therapy in ESCC cells.

Various reports have shown that HDACIs induced CAR expression in several kinds of cancer cell line (13, 14).
CAR is the receptor of adenovirus and the level of CAR expression should affect the efficiency of the infection with this virus (18). In our study, we determined the expression levels in ESCC and normal esophageal epithelium. However, the expression level of CAR in ESCC was actually reduced in 9 cases (out of a total of 40 cases) compared to that in normal epithelium. This result suggested to us the possibility that it may be necessary to use highly concentrated adenovirus in adenovirus-mediated gene therapy, thus raising the threat of side-effects due to adenovirus infection of the normal epithelium (19).

We showed that CAR expression was increased by FK228 at both the transcriptional and protein levels. In addition, we examined the expression of CAR mRNA in T.Tn and TE2 xenografts after treatment with FK228 and the
Figure 4. Expression of CAR mRNA in T.Tn and TE2 xenografts after FK228 administration. Relative expression levels of CAR were analyzed by real-time quantitative PCR analysis at various time points after 3.0 mg/kg of FK228 injection. Bars, SD.

Figure 5. Transduction efficiency of adenovirus vector in T.Tn and TE2 cells after FK228 treatment. A, AV1.0CMVβgal was used to infect the cells at different multiplicities of infection (MOI). The percentages of positive cells were obtained from triplicate wells. B, Expression of AV1.0CMVβgal in control and FK228-treated cell lines. Upper panels show the data of positive cells obtained from triplicate wells. Cells were grown in 24 wells and incubated with or without FK228 prior to infection with AdCMVβgal (MOI, 100). Bars, SD. Lower panels show the photographs of βgal-staining cells corresponding to these shown in the upper panel.
expression significantly increased. Moreover, FK228 was able to improve the efficacy of adenovirus infection dramatically and this result indicates that FK228 should be useful for adenovirus-mediated gene therapy in ESCC. However, the change of transduction efficacy was too immense to be explained by the change of CAR expression alone, therefore we need to consider other possibilities. One potential molecule to consider is $\alpha_v\beta_3$ integrin, which is thought to be one of the important molecules involved in the infection of mammalian cells with adenovirus (20). Kitazono et al. (13) reported that FK228 induced the expression of $\alpha_v\beta_3$ integrin in some cell lines similar to CAR. In addition, since the vector used in this study contained a CMV promoter, FK228 could possibly have induced the acetylation of the CMV promoter, thus augmenting its transcriptional function.

The acetylation of P53 lysine residues 320, 373 and 382 has been known as a critical factor in the regulation of p53-specific gene regulation (15, 16). In this study, FK228 therapy did not increase the expression of endogenous p53 in ESCC cells, although we did find an increase in acetylation of p53 lysine residues. These data indicate the combinatory therapy of p53 gene therapy with FK228 as a useful method for cancer treatment.

This study suggests FK228 improved the infection efficiency of adenovirus by inducing the CAR expression in ESCC cells. We also confirm that the lysine residues of p53 protein were acetylated by this HDACI. In the near future, HDACIs may be useful for malignant diseases in the clinical setting and the combination of p53 gene therapy and HDACIs could become a novel molecular targeting therapy for cancer.

References


Figure 6. Acetylation of p53 protein by FK228. A, T.Tn and TE2 cells were incubated with different amounts of FK228 for 2 hours and then analyzed for total p53 protein expression and p53 acetylated lysine residues by Western blotting. B, CAR protein expression levels were analyzed by Western blotting at various time points after administration of 1 ng/ml FK228.


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