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Oncolytic effect of Midkine promoter–based conditionally replicating adenoviruses expressing EGFR siRNA in head and neck squamous cancer cell line T891

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Abstract

Background: Epidermal growth factor receptor (EGFR) is overexpressed in head and neck squamous cell carcinomas (HNSCCs). Midkine expression is restricted in adult tissues but is increased in several malignant tumors, including HNSCCs.

Aim: Here, we evaluated the antitumor effect of Midkine promoter–based conditionally replicative adenovirus expressing siRNA against EGFR for targeting HNSCCs expressing Midkine.

Methods and results: A conditionally replicative adenovirus vector controlled by the Midkine promoter, Ad-MK-siEGFR, was generated by integrating gene-expressing siRNA against EGFR. Antitumor effect of Ad-MK-siEGFR was tested in vitro using established HNSCC cell line, T891 with strong Midkine expression. Expression of EGFR in T891 infected with Ad-MK-siEGFR was significantly lower than that of T891 infected with control. Cytotoxicity assays showed significant growth suppression of Ad-MK-siEGFR in T891 cells.

Conclusions: This study demonstrated the possibility of oncolytic therapy using the Midkine promoter–based conditional replication-selective adenovirus containing siRNA against EGFR in HNSCC cell line T891. Further validation of the findings in more cell lines and in vivo should be performed to clarify the potential clinical application.

KEYWORDS
conditionally replicative adenovirus, EGFR, head and neck cancer, Midkine, siRNA

INTRODUCTION

The head and neck have various roles including senses of vision, hearing, balance, smell, and taste and communication with speech, breathing, eating, and swallowing. Successful treatment of patients with head and neck cancer is required not only to eradicate the tumor and ensure against recurrence but also preserve important functions and maintain patient quality of life. Development of surgical procedures and chemoradiotherapy have improved clinical outcome. However, reported prognoses of patients with advanced head and neck...
cancer are still poor. Epidermal growth factor receptor (EGFR) has been reported to be related with growth, invasion, and metastasis of cancers including head and neck squamous cell carcinoma (HNSCC). Accordingly, antibodies against EGFR have been developed and used in combination with other chemotherapeutic agents or irradiation for advanced HNSCC. However, these antibodies can produce severe adverse events such as infusion reactions, various skin toxicities, and interstitial pneumonia. Immune checkpoint inhibitors also have been introduced for unresectable or metastatic advanced HNSCC, but previously unexperienced immuno-mediated adverse events have been reported in thyroid, lung, colon, liver, spleen, kidney, and skin. Thus, the development of effective tumor-specific therapy against advanced HNSCC is required.

Virotherapy is a tumor-specific therapy using viruses called oncolytic viruses, which selectively replicate in and kill tumor cells. Conditionally replicating adenoviruses (CRAds) are recombinant adenoviruses modified to selectively replicate in cancer cells by controlling viral genome replication such as E1A with a tumor-specific promoter. CRAds can lyse infected target cells to allow viral genome replication such as E1A with a tumor-specific promoter. CRAds controlled by Midkine promoter to increase the efficiency of oncolytic effects of CRAds can spread to a wide region of tumors compared with nonreplicating adenoviruses. Midkine is a heparin-binding growth factor that is involved in various neural development processes. Midkine expression is restricted in certain tissues in adults, but its strong expression has been detected in various malignancies including HNSCC. This tumor-specific expression of Midkine is attractive for the promoter of CRAds for the treatment of HNSCC.

EGFR is a member of the tyrosine kinase receptor ErbB family. EGFR is expressed on normal human cells, but high levels of EGFR expression have also been correlated with a variety of cancers. In addition, expression of EGFR in malignant cells is associated with poor prognosis and resistance to therapies. As described above, EGFR has already been a prime target for new anticancer therapies including antibodies, small molecular tyrosine kinase inhibitors, and antisense therapies. In the present study, we introduced small-interfering RNA (siRNA) targeting EGFR mRNA into CRAds controlled by Midkine promoter to increase the efficiencies of oncolytic adenovirus and examined the antitumor effects of Midkine promoter-controlled adenovirus carrying EGFR siRNA on Midkine expressing HNSCC cell line T891.

2 | MATERIALS AND METHODS

2.1 | Cells and cell culture

The tongue SCC cell line T891 was provided by Dr Mamoru Tsukuda (Department of Otolaryngology, Yokohama City University Medical Center, Yokohama, Japan). The lung cancer cell line A549 and transformed human embryonic kidney cell line HEK293 were obtained from the American Type Culture Collection (ATCC). T891 and A549 cells were cultured in RPMI-1640 with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin. HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS. All cell lines were maintained at 37°C in a 5% CO2 humidified incubator. Cells were passaged three times a week into fresh growth medium. Authentication of the cell lines was done by short tandem repeat (STR) profiling by ATCC.

2.2 | Quantitative real-time polymerase chain reaction

Total RNA was extracted from T891 and A549 cells by TRIzol (Invitrogen, Eugene, OR, USA). Expression of Midkine and EGFR mRNA was analyzed by real-time polymerase chain reaction (RT-PCR) according to the manufacturer’s protocol. The first strand cDNA was generated from 600 ng of total RNA in a final volume of 20 μL using Rever Tra Ace qPCR RT master mix (TOYOBO, Osaka, Japan). The PCR reaction contained 2× Power SYBR Green PCR Mix (Applied Biosystems, CA, USA), 5 μM of each primer, and 1 μL (out of 20 μL) of synthesized cDNA. Reactions were performed in triplicate for each sample. RT-PCR conditions were as follows: initial denaturation at 95°C for 10 minutes, followed by 40 cycles at 95°C for 30 seconds, 55°C for 1 minute, and 72°C for 1 minute. Beta-actin and GAPDH mRNAs were used as internal controls. The primer sequences to amplify each gene were as follows: EGFR: forward primer CAGGGCTACC TTGTCAATCA -3′; reverse primer TGGACTCAGAGCTCAGAGA -3′. Midkine: forward primer AGGGTGAGGAGGAGGAAGC -3′; reverse primer CTTTCCCTCCCTTCTTG -3′. Beta-actin: forward primer GGACTTCCGCAAGAGATGG -3′; reverse primer AGCATCTGTT TGGCGTACAG -3′. GAPDH: TaqMan GAPDH Control Reagents (Applied Biosystems; Cat# 402869).

2.3 | Construction of plasmids and transfection

EGFR siRNA was cloned into pBAsi-hU6 vector (Takara Bio, Kusatsu, Japan) stepwise according to the manufacturer’s instructions and the resultant vector, named pBAsi-hU6-EGFR. The sequence of inserted nucleotide the EGFR-siRNA is as follows: 5′- GATCC G aggaattaagagaagcaacat TTCAAGACA aagttgcttcAaactct TTTTT A-3′ (underline: position2480-2500 nucleotide in EGFR mRNA and complementary sequences of each other; double underline: hairpin region). Scrambled siRNA sequence was used as negative control, and the control construct containing the scrambled sequence was named as psi-Scr. The scrambled siRNA sequence was submitted to a BLAST search against the human and murine genome sequence to insure that no gene of the human genome was targeted. For siRNA transfection, 2×10^5 per well were seeded into a six-well culture plate and grown overnight until they were 60% to 80% confluent. pBAsi-hU6-EGFR and psi-Scr were transfected into T891 cells using Xfect transfection reagent (Takara) according to the manufacturer’s instructions. Mock transfection was used a plasmid that did not contain siRNA. After 48 hours, the total RNA was extracted as described above.
The expression cassette of siRNA of EGFR gene containing hU6 promoter (hU6-siEGFR) was digested with EcoRV from pBAsi-hU6-EGFR.

2.4 | Construction of adenovirus vectors

The sequence including adenoviral E1A-E1B genes controlled by the Midkine promoter (MK-E1A + E1B) was digested with I-CeuI and PI-SceI from the plasmid pMK using a previous study (a 600-bp Midkine promoter and adenoviral E1a-E1B gene fragment were inserted into pshuttle2PL). Expression cassette of hU6-siEGFR was ligated into the pMK vector at the BamHI restriction site, which was blunt ended with Klenow Fragment (TAKARA Bio). After confirmation by DNA sequencing, the MK-E1A + E1B and hU6-siEGFR cassettes were digested from vectors with PI-SceI and I-CeuI enzyme, respectively; then, the target DNA was isolated from an agarosegel.

The MK-E1A + E1B cassette was subcloned into the adenoviral cosmid pAxCawtit2 (Takara Bio) in which E1A, E1B, and E3 genes were deleted to generate the control cosmid pAd-MK (Figure 1A). The MK-E1A + E1B and hU6-siEGFR cassettes, which were blunt ended with Klenow Fragment, were subcloned into pAxCawtit2 vector at the SmiI restriction site to generate pAd-MK-siEGFR (Figure 1B). Ad-LacZ containing LacZ gene (encoding beta-galactosidase) was constructed as previously described, which is through replication-defective vectors.

Recombinant cosmids were digested by BspT104I to generate linear recombinant adenoviral genomics and then transfected into HEK293 cells. These recombinant adenoviruses were verified and propagated in HEK293 cells. After large-scale preparation, adenoviruses were concentrated using ViralBind Adenovirus Purification Kit (Cell Biolabs, San Diego, CA, USA), dialyzed in phosphate-buffered saline (PBS), and stored in aliquots at −80°C until use. The titer of each virus stock was determined by a standard plaque-forming assay on 293 cells. This study was approved by the Committee for Safe Handling of Living Modified Organisms at Kobe University and carried out according to the committee guidelines.

2.5 | Cytotoxic assay using crystal violet staining

Cells were seeded at a density of 2 × 10^5 per well in a six-well culture plate. After 24 hours, cells were infected with Ad-MK-siEGFR, Ad-MK, or Ad-LacZ at multiplicity of infections (MOIs) of 10 and 1. Four days after infection, the cells were stained with 2% crystal violet in 20% methanol for 15 minutes, washed with distilled water three times, and photographed with a digital camera (OLYMPUS STYLUS XZ-2).

2.6 | Cytotoxic assay using AlamarBlue

Cells were seeded at a density of 250 per well in a 96-well culture plate. After 24 hours, cells were infected with Ad-MK-siEGFR, Ad-MK, or Ad-LacZ, respectively, at MOIs of 20 and 10. Cell proliferation was detected at 0, 24, 48, and 96 hours after infection. AlamarBlue Cell Viability Reagent (Invitrogen, Carlsbad, CA, USA) was added to each well, and cells were further incubated at 37°C for 1 hour. The absorbance at wave-length 590 nm was measured with a Synergy HTX (Biotec, Bunkyo-ku Tokyo, Japan). The ratios of absorbance of treated cells relative to those untreated cells were determined.

2.7 | Western blotting

Cells were seeded at a density of 2 × 10^5 per well in a six-well culture plate. After 24 hours, cells were infected with Ad-MK-siEGFR or Ad-MK, at MOIs of 10. At indicated time points after infection, cells were harvested, and protein extracts were prepared using M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of total proteins were resolved on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel under reducing conditions. The proteins were then electrotransferred onto a polyvinylidene difluoride membranes Immobilon-P (Millipore, Billerica, MA, USA). After blocking, antigen detection was performed using anti-EGFR rabbit antibody (1:1000) (Santa Cruz Biotechnology: EGFR [1005] sc-03) for overnight at 4°C. Blots were then incubated with a horseradish peroxidase-conjugated IgG secondary antibody, Anti-Rabbit IgG, HRP-Linked Whole Ab Donkey (NA934-100UL, GE Health Care). After several washes with PBS, the bands were visualized using the Chemi-Lumi One L (Nacalai tesque, Kyoto, Japan). Blots were then stripped and re-probed with anti-GAPDH mouse antibody (1:1000) (GAPDH [0411] sc-47 724, Santa Cruz Biotechnology) and then incubated.
with HRP-conjugated goat anti-mouse IgG secondary antibody, Anti-Mouse IgG, HRP-Linked Whole Ab Sheep (NA931-100UL, GE Health care) and developed as above. Densitometry was performed on scanned western blot images using the ImageJ gel analysis tool (Rasband, W.S., ImageJ, US National Institutes of Health, Bethesda, MD, USA. https://imagej.nih.gov/ij/).15

### 2.8 Statistical analyses

Statistical significance was determined using a t test for direct two-group comparisons (Figure 2A,B) and analysis of variance (ANOVA) for multiple-group comparisons. A P value of <.05 was considered statistically significant. These analyses were performed using R version 3.5.2 (R Foundation for Statistical Computing, Vienna, Austria).16

### 3 RESULTS

#### 3.1 Ad-MK-siEGFR inhibited EGFR mRNA and protein expressions in T891 cells

First, we evaluated the expression of Midkine and EGFR mRNA in T891 cells using A549 for comparison, which were strong Midkine expression as previously described.11 As shown in Figure 2A,B, T891 cells showed significantly higher expression of Midkine mRNA and EGFR mRNA compared with A549 cells. Then, to knockdown EGFR expression in T891 cells, we first used siRNA targeting EGFR. EGFR expression was significantly suppressed in cells transfected with pBAsi-hU6-EGFR compared with the control and psi-Scr groups (P = .0001 and P = .0003, respectively) (Figure 2C). To evaluate the efficiency of siRNA-mediated knockdown of EGFR by adenovirus, we infected T891 cells with Ad-LacZ, Ad-MK, and Ad-MK-siEGFR at 0.1 and 1 MOI and performed RT-PCR at 40 hours after infection. Cells without infection were used as negative control.

We observed a significant reduction of EGFR mRNA expression between the groups treated with Ad-MK and Ad-MK-siEGFR compared with Ad-LacZ at 1 MOI. In addition, expression of EGFR mRNA of T891 infected with Ad-MK-siEGFR was significantly lower than that of T891 infected with Ad-MK at 0.1 MOI (Figure 3, P = .00011).

Next, we investigated the expression of EGFR protein in T891 cells infected with Ad-MK or Ad-MK-siEGFR at 10 MOI. At day 1 after infection, there was no significant difference in EGFR protein levels among the adenoviral infected cells and control cells (Figure 4A,B). At day 4, the expression of EGFR protein was significantly decreased in cells infected with Ad-MK and Ad-MK-siEGFR compared with untreated cells, while expression of EGFR protein was increased in untreated T891 cells at day 4 compared with levels at day 1.

![Figure 2](image-url)

**Figure 2** Expression of (A) Midkine and (B) epidermal growth factor receptor (EGFR) mRNA in A549 and T891 cells. Control was untreated cells.

![Figure 3](image-url)

**Figure 3** Expression of EGFR mRNA in T891 cells infected with Ad-LacZ, Ad-MK, and Ad-MK-siEGFR. Control was untreated cells.

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*P < 0.05, **P < 0.01 (analysis of variance), Ad-LacZ vs Ad-MK-siEGFR (1 MOI), P = .0045, Ad-LacZ vs Ad-MK (1 MOI), P = .034, Ad-MK vs Ad-MK-siEGFR (0.1 MOI), P = .00011. Data are given as the mean values ± SD (n = 3). The graphs shown are representative of three independent experiments. EGFR, epidermal growth factor receptor; MOI, multiplicity of infection.
3.2 | Ad-MK-siEGFR showed oncolytic effects in T891 cells

To examine the oncolytic activity of Ad-MK-siEGFR, we conducted an in vitro cytotoxicity assay in T891 cells using crystal violet staining. Cells were infected with Ad-MK, Ad-MK-siEGFR, or Ad-LacZ as control at MOIs of 10 and 1. Four days later, cells were stained with crystal violet. As shown in Figure 5, 10 MOI of Ad-MK and Ad-MK-siEGFR markedly killed T891 cells. We also observed partial cell killing in cells infected with Ad-MK-siEGFR at only MOI of 1. Ad-LacZ had no lethal effect on T891 cells even at an MOI of 10.

3.3 | Ad-MK-siEGFR inhibited the cell proliferation of T891 cells

Tumor-suppressing ability of Ad-MK-siEGFR was also assessed in vitro using AlamarBlue assay. T891 cells were infected with Ad-MK, Ad-MK-siEGFR, or Ad-lacZ at MOIs of 20 and 10. Cell proliferation was evaluated at 0, 24, 48, and 96 hours after infection. As shown in Figure 6, cell proliferation increased in T891 cells infected with Ad-LacZ and untreated T891 cells at day 4 compared with that at day 0. At 20 MOI, proliferation was significantly inhibited in T891 cell infected with Ad-MK-siEGFR compared with Ad-MK at day 2 (P = .0325), while it was markedly reduced at day 4 (P = .0637).

4 | DISCUSSION

Midkine, a product of retinoic acid-responsive gene during embryogenesis, has been reported to play important roles in tumor progression and is highly expressed in various malignant tumors. While Midkine shows high expression during embryogenesis and in advanced tumors, it is not detectable in healthy adults. Because of its biological significance in carcinogenesis and its cancer-specific expression, Midkine has been considered as a candidate molecular target for therapy against human carcinoma. Taking advantage of this tumor-specific expression of Midkine, we generated the Midkine promoter-based replication selective adenoviral vector Ad-MK and demonstrated its antitumor effect against Midkine expressing bladder cancer cells. Notably, we previously demonstrated nontoxic effects in low levels of MK expression. Several prior studies also showed that Midkine expression was significantly greater in cancer cell lines than in noncancerous tissues, and the promoter activity of Midkine in Midkine-positive cells was greater than that in Midkine-negative cells. Consequently, Midkine promoter-based CRAd showed specific cytotoxicity in Midkine-positive cells.

As previously reported, insertion of antitumor gene into the adenovirus vector can be expected to enforce the ability of an oncolytic adenovirus to kill tumor cells. EGFR is associated with tumor progression, early metastatic spread, and poor prognosis of HNSCC, and EGFR is highly expressed in most HNSCC. Recently, monoclonal...
antibodies against EGFR were approved for the treatment of advanced HNSCC. However, various side effects have been reported as described above. Taken together, we decided to select EGFR as the target gene for antitumor therapies and developed the novel gene therapy using a tumor-specific Midkine promoter-based CRAd expressing siRNA against EGFR for head and neck cancer, which has antitumor effects of oncolytic virus and of siRNA suppressing expression of the carcinogenic gene, EGFR.

As we expected, expression of EGFR mRNA was significantly inhibited in tumor cells treated with Ad-MK-siEGFR in comparison with control. Moreover, expression of EGFR mRNA was suppressed in tumor cells treated with Ad-MK-siEGFR in comparison with Ad-MK at 0.1 MOI. At a strong titer, the expression of EGFR was also strongly decreased by Ad-MK alone. Oncolytic adenovirus induces autophagic cell death in human cancer cells through induction of miR-7 upregulation via enhancement of E2F1 expression and through suppression of oncogenic EGFR expression. Thus, decreased expression of EGFR by Ad-MK may reflect the strong oncolytic effect of Ad-MK but not the specific and direct effect against EGFR. Cytotoxic assay using crystal violet showed that tumor cells infected with both Ad-MK and Ad-MK-siEGFR showed poor cancer cell survival at 10 MOI. At MOI of 1, an antitumor effect was observed in the tumor cells infected with Ad-MK-siEGFR but not in those with Ad-MK or Ad-LacZ. In addition, Ad-MK-siEGFR showed antitumor effect from the earlier period at high concentration in the AlamarBlue assay. These results demonstrated that Ad-MK-siEGFR has dual antitumor effects of oncolytic virus and siRNA suppressing expression of the carcinogenic gene, EGFR, as we intended.

From a clinical point of view, Ad-MK-siEGFR shows an advantage for the treatment of unresectable and/or metastatic HNSCC, because head and neck cancer is easy to access for direct intratumoral injection of Ad-MK-siEGFR. Injected Ad-MK-siEGFR can proliferate and lyse infected target cells and spread to the neighboring cells and undergo further replications. The oncolytic effects of Ad-MK-siEGFR could thus spread to wide regions, in lung, liver, and bones, even to metastatic lesions, and exhibit dual antitumor effects. Furthermore, direct intratumoral injection of Ad-MK-siEGFR is expected to result in reduced systemic adverse effects compared with currently used EGFR-targeted therapeutic agents because Ad-MK-siEGFR selectively replicates in tumor cells and exerts antitumor effect in tumor cells, but it is not expected in normal cells. While Midkine promoter-based CRAd showed specific cytotoxicity for Midkine-positive cells in vivo as well as in vitro in previous studies, replicative viruses have shown low-level viral production and/or systemic toxicity in clinical trials. In this study, we demonstrated the synergic effect of Midkine promoter-based CRAd and siRNA against EGFR in tongue cancer cell line with high Midkine expression. We are currently planning to examine the effects of Ad-MK-siEGFR on a variety of head and neck cancer cell lines originating from other locations with various expression levels of EGFR and MK to see whether Ad-MK-siEGFR is effective at lower titer in comparison with Ad-MK in vivo. Precise requirements for selective targeting to prevent damage to normal tissues in vivo should be clarified for clinical application.

5 | CONCLUSIONS

The present results demonstrated the possibility of oncolytic therapy using Midkine promoter–based CRAd containing siRNA against EGFR in HNSCC cell line T891 with strong Midkine expression.
these results were obtained from a single cell line. Further in vitro studies with more cell lines and in vivo studies should be performed to clarify the potential clinical application.

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CONFLICT OF INTEREST
The authors have no conflict of interest to be disclosed related to this manuscript.

AUTHORS’ CONTRIBUTIONS
All authors had full access to the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Conceptualization, K.N.; Methodology, N.U., K.N., T.S.; Investigation, N.U., M.K., J.K., Y.K.; Formal analysis, N.U., M.T., H.S.; Resources, K.N., N.O., T.S.; Writing—original draft, N.U.; Writing—review and editing, N.U., N.O., K.N.; Visualization, N.U., M.K.; Supervision, N.U., N.O., K.N.; Project Administration, K.N.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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