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Combination of Vaccine Strain Measles Virus and Nimotuzumab in the Treatment of Laryngeal Cancer

NGUYEN LINH TOAN[1*], NGO THU HANG[1,2*], NGUYEN KIM LUU[3], CAN VAN MAO[1], NGUYEN VAN BA[3], NGUYEN THI XUAN[4], TRUONG DINH CAM[5], NAOKI YAMAMOTO[6], HOANG VAN TONG[1,7] and HO ANH SON[1,7]

[1]*Department of Pathophysiology, Vietnam Military Medical University, Hanoi, Vietnam;*
[2]*Ha Dong Medical College, Hanoi, Vietnam;*
[3]*103 Military Hospital, Vietnam Military Medical University, Hanoi, Vietnam;*
[4]*Institute of Genome Research, Vietnam Academy of Science and Technology, Hanoi, Vietnam;*
[5]*175 Military Hospital, Ho Chi Minh, Vietnam;*
[6]*Department of Microbiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore;*
[7]*Institute of Biomedicine and Pharmacy, Vietnam Military Medical University, Hanoi, Vietnam*

Abstract. *Background/Aim: This study aims to investigate whether the combination of oncolytic viruses with chemoradiotherapy or other therapies is a promising strategy for cancer treatment. Materials and Methods: The anticancer effects of measles virus (MeV) in combination with nimotuzumab in the treatment of laryngeal cancer were evaluated in vitro and in nude mice inoculated with Hep2 tumors. MTT assay and flow cytometry were used to examine cell death. Results: Laryngeal cancer cells treated with MeV+nimotuzumab combination had a significantly lower survival rate compared to those treated with MeV or nimotuzumab alone ($p<0.0001$). In an animal model bearing human laryngeal tumor, the treated group had a higher survival rate (60%) compared to a untreated group (20%) ($p<0.05$), and the survival rate of the group treated with MeV+nimotuzumab combination was higher compared to the groups received single treatment. Conclusion: The MeV+nimotuzumab combination has greater anticancer activities in both laryngeal cancer cells and an animal model.*

*These Authors share first co-authorship.

Correspondence to: A/Prof. Ho Anh Son, MD, Ph.D., Department of Pathophysiology, Vietnam Military Medical University, 160 Phung Hung Street, Ha Dong, Hanoi, Vietnam. Tel: +84 978 437229, e-mail: hoanhsonhp@gmail.com; A/Prof. Nguyen Linh Toan, MD, Ph.D., Department of Pathophysiology, Vietnam Military Medical University, 160 Phung Hung Street, Ha Dong, Hanoi, Vietnam. Tel: +84 979166868, e-mail: toannl@vmmu.edu.vn

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Head and neck cancers rank fourth among the most popular malignant diseases and are one of the most common cancers in men worldwide (1). According to statistics in various countries, laryngeal cancer accounts for 2% of all cancers (2). The disease is diagnosed in more than 10,000 men and 3,000 women in the United States annually. Most of them are at the age of 65, and laryngeal cancer ranks second after nasopharyngeal cancers among head and neck cancers and ranks tenth among all cancers, with the high incidence among men aged 40-60 years (3).

Oncolytic virus (OLV) therapy is based on the property of oncolytic viruses to selectively infect and replicate in tumor cells, and subsequently either directly kill the infected cells or stimulate immune responses against tumor cells (4, 5). Among the viruses with oncolytic activities, Measles virus (MeV) is the most systematically studied. It is a negative-strand RNA virus of the genus *Morbillivirus* that causes the infectious measles syndrome. In the urgent need for a new effective therapy for cancers, oncolytic virus-based therapy is a promising tool to complement current therapeutic strategies. The vaccine strain of MeV has been shown to have the capability to infect many cancer cell lines (6, 7). Although the exact mechanism by which MeV can enter and kill cancer cells is not fully understood, CD46, which is highly expressed on cell surface of most types of cancer cells, has been identified as one of the receptors of MeV (8). In addition, oncolytic virus has a high replication rate that can directly kill cancer cells and the vaccine strain of MeV has been demonstrated to be safe in humans (9). Therefore, MeV has a great potential to be used for cancer treatment.

The earliest *in vitro* and *in vivo* studies on attenuated MeV have demonstrated effective lysis of myeloma cell lines *in vitro,* as well as of cells of tumors implanted in a murine

xenograft model (10). Data showed complete tumor regression following intratumoural MeV treatment or intravenous virus treatment (10). Intratumoral injection of MeV resulted in regression of large established human lymphoma xenografts whereas all tumors in the control group treated with UV-inactivated virus progressed (11). The antitumor effect still occurred in the presence of anti-MeV antibody and intravenous administration of MeV also resulted in decelerating of tumor progression (12). Analysis of tumor sections confirmed replication of MeV within the tumors.

Epidermal growth factor receptor (EGFR) has been observed to be overexpressed in tumor tissues of head and neck cancer patients. EGFR plays an important role in cell growth, metastasis and proliferation of tumor cells promoting the development of head and neck cancers (13). nimotuzumab is a humanized monoclonal antibody that can bind specifically to EGFR and prevent the activation of the receptor (14). The combination of nimotuzumab with concurrent chemoradiotherapy is one of the strategies to treat several types of cancer such as esophageal cancer (15), advanced lung squamous cell carcinoma (LSCC) (16), and advanced nasopharyngeal carcinoma (17). Particularly, nimotuzumab combined with chemotherapy has been shown to have an increased effect and was suggested to be a first-line therapy for patients with LSCC (16). In addition, combination of nimotuzumab with concurrent chemoradiotherapy has been shown to have beneficial effect on advanced nasopharyngeal carcinoma (17).

Our earlier study has shown that the combination of vaccine-derived MeV and mumps virus (MuV) efficiently targeted and killed a wide range of hematopoietic cancer cell lines, exhibited greater tumor suppression and resulted in prolonged survival *in vivo* (7). A convincing evidence for the anti tumor effect of MeV+MuV combination on human solid cancers has also been demonstrated in another previous study (6). In this study, we aimed to investigate the anticancer and cytopathic effects of the combination of MeV and nimotuzumab on laryngeal cancer *in vitro* and *in vivo*.

Materials and Methods

Cell lines. Vero cell line (kidney, African green monkey) and human laryngeal squamous cell carcinoma Hep 2 cell lines (ATCC CCL-23, laryngeal SCC) were purchased from the American Type Cell Culture (ATCC, Manassas, VA, USA). Vero cells were cultured in M199 medium (Biowest, Maine-et-Loire, France) supplemented with 10% Foetal Bovine Serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (ATCC). Hep2 cells were cultured in Eagle's Minimum Essential Medium (EMEM) (ATCC-formulated F-12K) (Catalog No.30-2004) medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in a 75 cm^2 culture flash. The cells were maintained at 37˚C in a humidified incubator with 5% CO_2. The cells were used at 80% confluence. The cells were harvested by using Trypsin EDTA, and centrifuged to remove the culture medium. The density of cancer cells was determined using Neubauer counters and optical microscopes. A standard density of 10^7 cells/ml was used in the experiments.

Propagation of measles viruses. MeV was plaque purified from Priorix (GlaxoSmithKline, UK) containing the attenuated measles virus (Schwarz MeV strain), mumps virus (RIT 4385 strain) and rubella virus (Wistar RA 27/3 RV strain) and was maintained in Vero cells. The procedure to prepare MeV for further experiments has been described in detail in our previous study (6). We also confirmed the presence of only MeV in each viral clone by using RT-PCR with specific primer pairs (6).

Death cell evaluation by MTT assay. The MTT assay was used to evaluate cell viability. This method is based on the principle that in living cells mitochondrial succinate dehydrogenase metabolizes 3-(4,5- Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) (yellow) into formazan which is violet. When the cells are dead, the mitochondria are no longer able to convert MTT into a colorant formazan, which can be quantified by measuring optical density. The optical density measured by the MTT assay is proportional to the number of living cells (18). Cancer cells were harvested from cultured plates, divided equally in culture medium, adjusted at a density of 10^4 cells/ml, and transferred to 96-well plates. Each well contained 200 μl of culture medium and 2,000 cells. After 24 h of incubation, when the cells adhered to the bottom of the wells, the old culture medium was removed and replaced with 200 μl of new culture medium in the MeV 1MOI control wells, 200 μl new culture medium supplemented with MeV in the MeV group, 200 μl new culture medium supplemented with nimotuzumab at a dose of 100 μg/ml in the nimotuzumab group. The MTT assay was performed following 72 h and 96 h incubation. The medium in the culture wells was completely removed and replaced with 90 μl of fresh culture medium containing 10 μl of MTT solution. The cells were incubated for 4 h to allow mitochondria to convert the yellow MTT into purple formazan crystals. Then, the culture medium was removed again and 100 μl of MTT crystalline solution was added. Optical density (OD) was measured at 570 nm and the background OD was measured at 690 nm.

Phosphatidylserine translocation and 7AAD incorporation. To discriminate necrotic/late apoptotic from early apoptotic cells by flow cytometry using the Annexin V/7AAD kit (Biolegend, CA, USA) according to the manufacturer's instructions. The presence of phosphatidylserine (PS) on the outer surface of the apoptotic cells was assessed from FITC-conjugated annexin V binding to PS at the cell surface and necrosis/late apoptosis was assessed from the amount of 7AAD-positive cells. In brief, approximately 10^6 cells (human laryngeal squamous cell carcinoma Hep2 cell line) were seeded in each 60 mm cell culture dish with EMEM, 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin for 24 h before treatment. The cells were then treated with control medium, MeV, nimotuzumab or MeV+nimotuzumab combination. Apoptosis was assayed at 48, 72 and 96 h after treatment.

Quantification of gene expression by RT-PCR. Human laryngeal squamous cell carcinoma Hep 2 cell lines cultured in EMEM medium were harvested in trypsin EDTA bottles by centrifuging to remove the culture medium. Cell density was determined using Neubauer counters and a microscope. 10^6 laryngeal tumor cells were used for total RNA isolation by using total RNA isolation kit

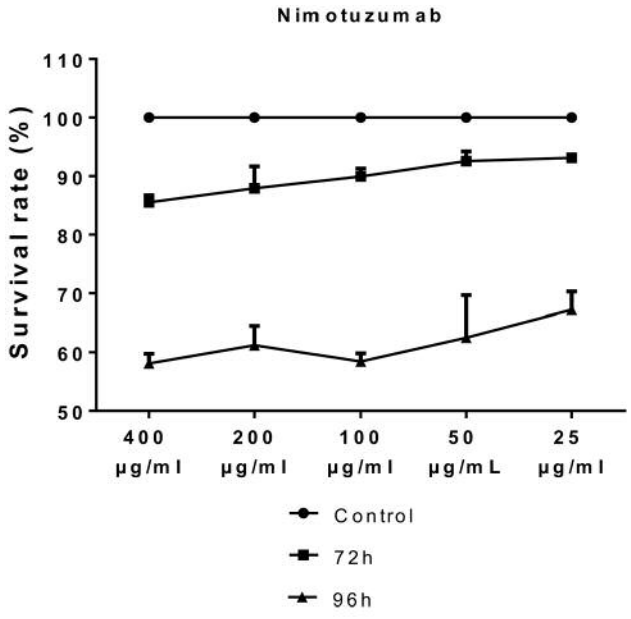

Figure 1. *Anticancer effect of nimotuzumab at different concentrations. A total of 10^6 laryngeal cancer cells (Hep2) were cultured and treated with different concentrations of nimotuzumab (400, 200, 100, 50 and 25 µg/ml). MTT assay was used to evaluate the survival rate of Hep2 cells at 72 and 96 h.*

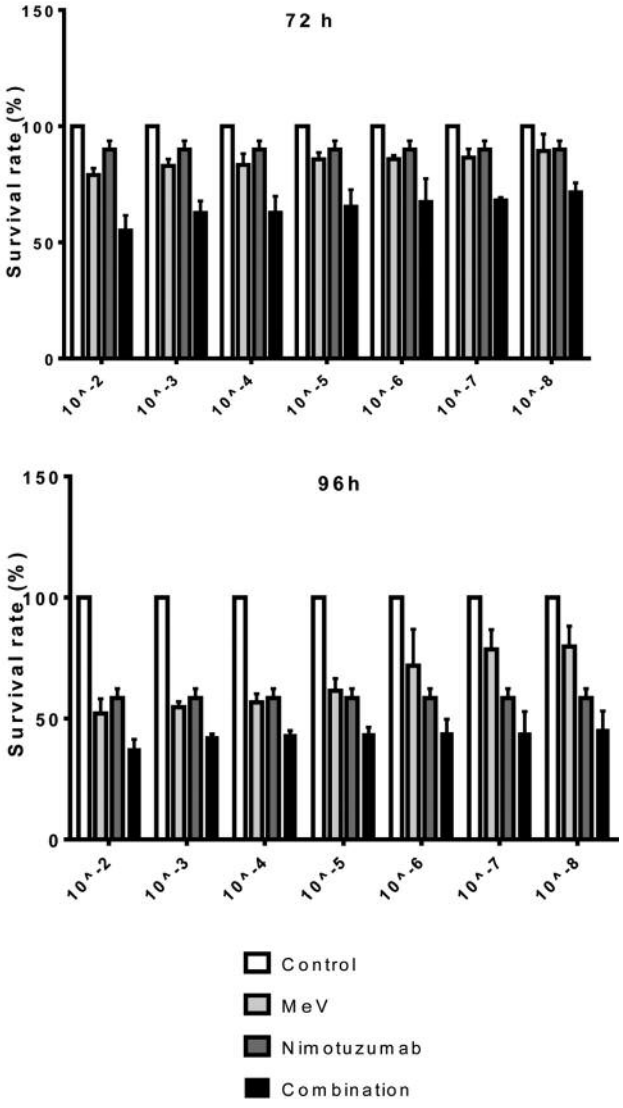

Figure 2. *Anticancer effects of MeV and nimotuzumab in vitro. A total of 10^6 laryngeal cancer cells (Hep2) were cultured and treated with MeV, nimotuzumab alone, or with MeV+nimotuzumab combination. Non-treated cells were used as control. MTT assay was employed to evaluate the survival rate of Hep2 cells at 72 and 96 h.*

(Qiagen, Hilden, Germany) following the instructions of the manufacturer. RNA was reversely transcribed into cDNA by using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, MA, USA). Quantification of *STAT3* and *ISG15* genes was performed by qRT-PCR, and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) was used as a reference gene. The reaction contained: 10 µl of 2× SYBR Green PCR master mix (Enzynomics, Daejeon, South Korea), 0.4 µM of forward and reverse primers for the reference and target genes, 50 ng of cDNA and 6 µl and RNase-free water (Qiagen, Hilden, Germany) in a final volume of 20 µl. All reactions were performed in duplicate and were repeated twice. Primer sequences used were: STAT3 (sense): 5'-GGA GGA GTT GCA GCA AAA AG-3'; STAT3 (antisense): 5'-TGT GTT TGT GCC CAG AAT GT-3' (19); ISG15 (sense): 5'-GAG AGG CAG CGA ACT CAT CT-3'; ISG15 antisense: 5'-CTT CAG CTC TGA CAC CGA CA-3'. GAPDH (sense): 5'-TTG GTA TCG TGG AAG GAC TCA-3'; GAPDH (antisense): 5'-TGT CAT CAT ATT TGG CAG GTT-3'. (20) Thermal cycling conditions were: 2 min at 95˚C followed by 45 cycles of denaturation for 5 sec at 95˚C, annealing for 20 sec at 52˚C for *STAT3*, 58˚C for *ISG15* and 50˚C for *GAPDH* and extension for 20 sec at 72˚C. Reaction specificity was confirmed by melting curve analyses starting from 50˚C to 85˚C. The fold change of *STAT3* and *ISG15* was normalized using the ΔCt method against the expression of *GAPDH*.

Animal experiments. Six to eight-week old male BALB/c nude mice were purchased from BioLASCO (Taipei City, Taiwan) and were kept under pathogen-free conditions in accordance with Animal Center Guidelines. The procedures were approved by the Vietnam Military Medical University, Vietnam (072/13). To evaluate the effect of MeV, nimotuzumab or MeV+nimotuzumab combination on tumor growth and survival time of nude mice bearing Hep2 tumors, the mice were inoculated with 10^6 Hep2 cells in 100 µl FBS on the right rear flanks of male nude mice. One week after inoculation, the formation of tumor in mice was checked twice per week, when the tumor reached the size of 7-10 cm in diameter, the mice were divided into 4 groups, and each group consisted of 10 mice. The mice were treated with PBS as control, MeV, nimotuzumab or MeV+nimotuzumab combination. They received a single dose (10^6 CFU/mouse/time) or multiple doses (10^7 CFU/ mouse/time, twice a week for 3 weeks) by intratumor injection. Tumor volume and survival time were observed and recorded. Tumor volume was calculated from caliper measurements

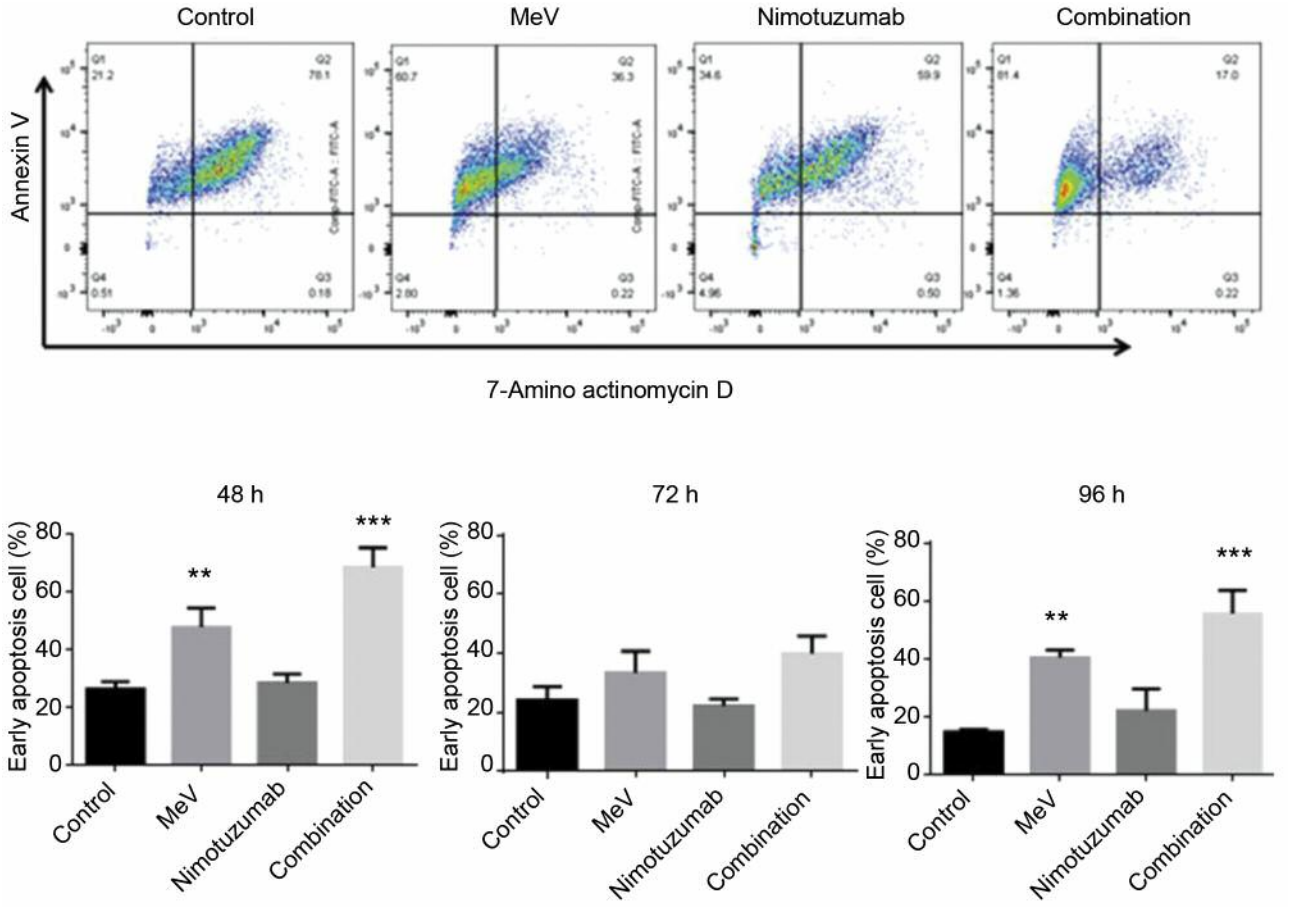

Figure 3. *Apoptosis induction by MeV and nimotuzumab treatment of laryngeal cancer cells. A total of 10^6 laryngeal cancer cells (Hep2) were cultured and treated with MeV, nimotuzumab alone, or MeV+nimotuzumab combination. Non-treated cells were used as control. Apoptotic cells were detected after 48, 72 and 96 h after treatment. Original dot plots of early-stage apoptotic cells (Annexin $V^+/7AAD^-$ laryngeal cancer cells, in upper left quadrant) detected 48 h after treatment by flow cytometry (upper panels). Arithmetic means±SD of the percentage of Annexin $V^+/7AAD^-$ cells in untreated cells (control) or cells treated with MeV, nimotuzumab alone, or MeV+nimotuzumab combination for 48 h (1st lower panel), 72 h (2nd lower panel) and 96 h (3rd lower panel); (**), p<0.01 and (***), p<0.0001 compared with other groups.*

of length and width of masses (volume (mm^3)=length × ½ × $width^2$). Relative tumor volume was calculated as the volume at a given time divided by the volume on the indicated time points after initiation of treatment.

Analysis of tumor cell structure by Transmission electron microscopy. Biopsy samples of Hep2 tumors were washed twice with cacodylate buffer solution pH 7.3 then fixed with glutaraldehyde 2% in a cacodylate buffer (pH 7.3) with a ratio of sample volume and solution volume of 1:10 for 2 to 3 days. The samples were divided into small pieces with size of 1×1×2 mm, washed 2-3 times in 1 h with cacodylate buffer, fixed with 1% osmic acid in cacodylate buffer (pH 7.3) for 1 h, and washed again 2-3 times with cacodylate buffer (pH 7.3). The samples were then dehydrated by consecutively transferring them for 15 min to different alcohol solutions of 50%, 60%, 70%, 80%, 90%, and 100%. After dehydration, the samples were transferred for 10 min to a solution of propylene+ethylene (1:1 ratio in volume), then passed to propylene solution for 10 min. The samples were moved to a mixture of propylene+epon 812 with a volume ratio of 1:1 for 15 min followed by a propylene+epon 812 mixture of 1: 2 in volume for 30 min. Finally, the samples were moved to epon 812 for 30 min, blocked and maintained at 37˚C for 24 h and polymerized at 60˚C for 48 h. Subsequently, the sample blocks were thinly cut, stained with toluidine blue and were cut by a microtome (Walldorf, Germany) at thickness of 50 nm. The slides were put in a 200-hole copper net, stained with 2% uranyl acetate for 5 min, washed twice with distilled water and stained with 5% lead citrate for 5 min, and washed twice with distilled water. The sections were observed under the transmission electron microscopy (TEM) JEM 1400 (JEOL, Japan).

Statistical analysis. Data were analyzed with the GraphPad Prism 5.0 software (GraphPad Software, CA, USA) and SPSS v.20 (SPSS Statistics, IBM, Armonk, NY, USA). Student's *t*-test, Mann-Whitney *U*-test or Fisher's exact test was applied to compare groups. The Kaplan–Meier method and the log-rank test were used to compare the survival time of nude mice between groups. Statistical significance was defined as p-value≤0.05.

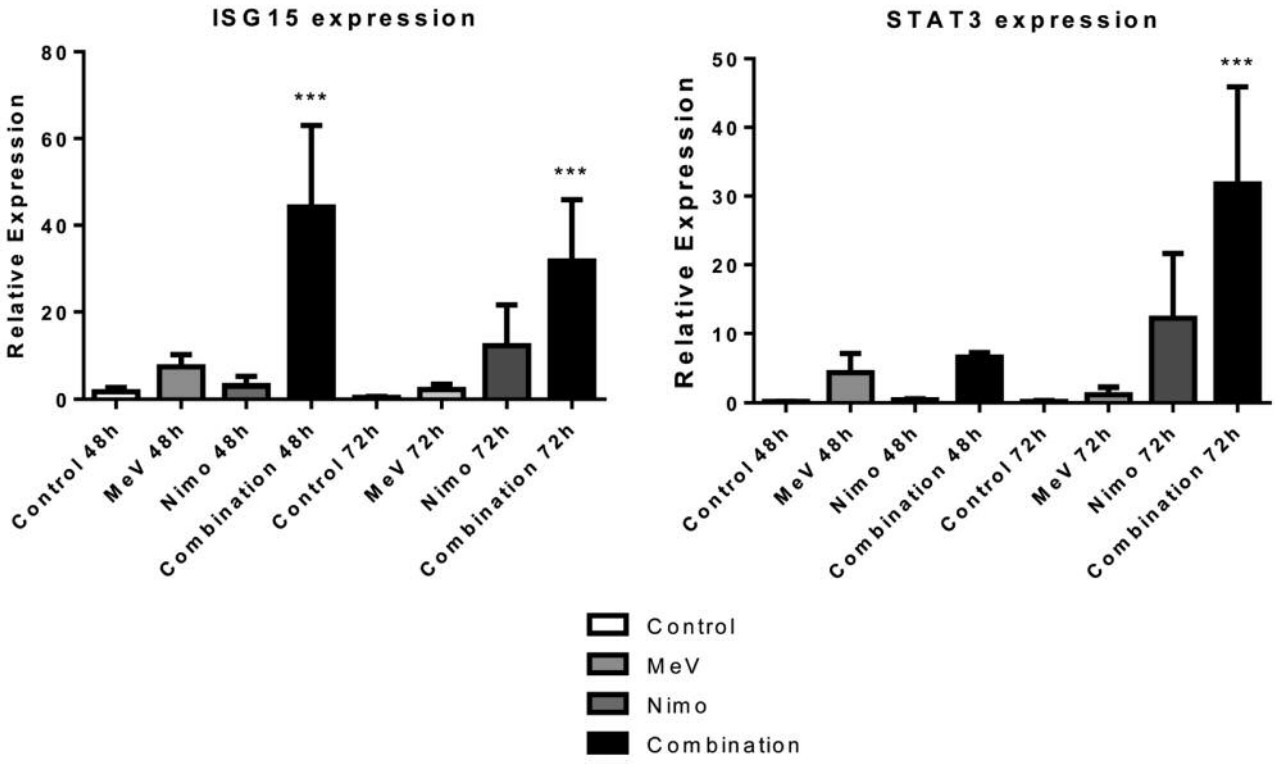

Figure 4. *Expression of ISG15 and STAT3 genes in laryngeal cancer cells. Laryngeal cancer cells were treated for 48 h and 72 h with PBS, MeV, nimotuzumab and MeV+nimotuzumab combination. Relative expression of ISG15 and STAT3 was quantified by real-time PCR and calculated by the ΔCt method normalized with GAPDH as a reference gene. The levels of relative expression were compared between groups and p-value was calculated by the ANOVA test, (***), p<0.0001 compared to other groups.*

Results

Anti tumor effects of MeV+nimotuzumab treatment against laryngeal cancer in vitro. The anticancer effects of different concentrations (400 μg/ml, 200 μg/ml, 100 μg/ml, 50 μg/ml and 25 μg/ml) of the monoclonal antibody nimotuzumab on the laryngeal cancer cell line (Hep2) were examined. The result showed that nimotuzumab treatment resulted in a concentration-depended increase in laryngeal cancer cell death (Figure 1). In addition, the IC_{50} of nimotuzumab were 2146 μg/ml and 876.9 μg/ml when determined at 72 h and 96 h post-infection, respectively.

Subsequently the effect of MeV, nimotuzumab and MeV+nimotuzumab combination on the viability of Hep2 laryngeal cancer cells) was examined at 100 μg/ml nimotuzumab. Hep2 cells were infected and cell death was measured at 72 and 96 h post-infection. The results showed that MeV also had a strong killing effect on Hep2 cells compared to control. Remarkably, treatment with MeV and nimotuzumab (MeV+nimotuzumab) combination sh owed a significantly stronger anticancer activitiy compared to treatment with MeV or nimotuzumab alone as well as compared to control at both 72 h and 96 h post-infection (Figure 2).

MeV+nimotuzumab treatment induces apoptosis. Next, the mechanism by which MeV and nimotuzumab treatment results in cell death was examined. Laryngeal cancer cells were infected with MeV, nimotuzumab or MeV+nimotuzumab combination, and apoptosis was assayed by flow cytometry using staining with Annexin-V. The results showed that the percentage of apoptotic cells (Annexin V^+/PI^-) was significantly higher in the group treated with MeV+nimotuzumab combination compared to the groups treated with MeV or nimotuzumab alone at 48, 72 and 96 h (Figure 3, upper panel). However, higher rate of early apoptotic cells was observed in the group treated with MeV+nimotuzumab combination at only 48 h post-infection (Figure 3, middle panel), and higher rate of late apoptotic cells was observed in the group treated with MeV+nimotuzumab combination at only 96 h post-infection (Figure 3, lower panel). These results indicated that the enhancement of the cytotoxic effect by MeV+nimotuzumab combination is due to increased induction of apoptosis.

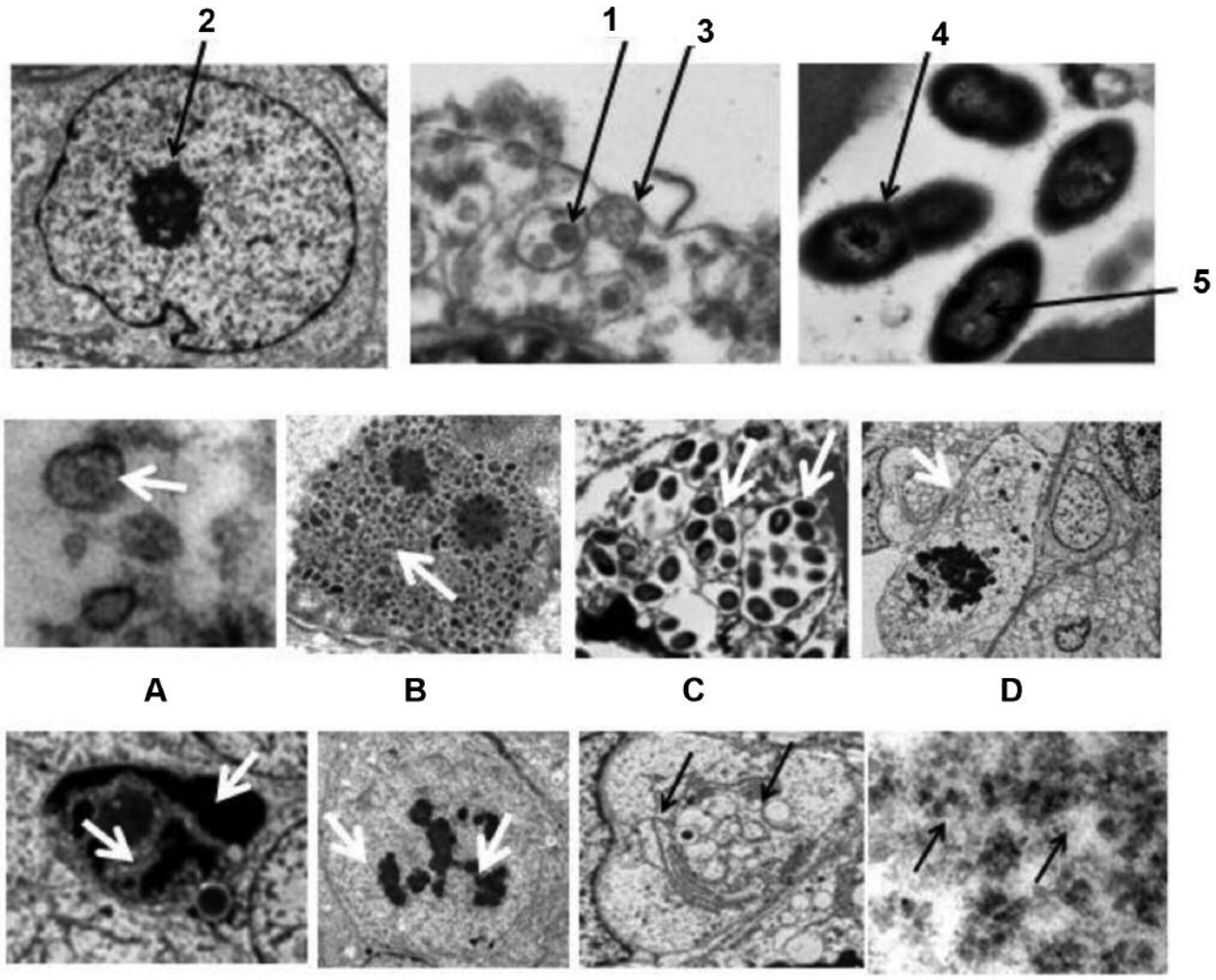

Figure 5. *Analysis of the structure of laryngeal tumor cells before and after treatment by electron microscope. Upper panel: Normal structure of laryngeal tumor cells before treatment with MeV, nimotuzumab or MeV+nimotuzumab combination. (1), normal structure of laryngeal tumor cells; (2), nucleus; (3), mucous membrane; (4, 5), dividing cells. Middle panel: Structure of laryngeal tumor cells after treatment with MeV, nimotuzumab or MeV+nimotuzumab combination. (A), Untreated cells; (B), laryngeal tumor Hep2 cells infected with MeV; (C, D), syncytia of cells that result from cell fusions. Lower panel: (E), Hep2 laryngeal tumor cells with chromosome condensation; (F), Hep2 laryngeal tumor cells with chromosome fragmentation; (G), Hep2 laryngeal tumor cells with many vacuoles; (H), necrotizing cells.*

STAT3 and ISG15 are proteins that play an important role in cancer development and apoptosis (21, 22). Therefore, the expression of *STAT3* and *ISG15* genes was examined in the laryngeal cancer cell line 48 h and 72 h post-infection. The results showed that the relative expression of *STAT3* and *ISG15* genes was highest in the groups treated with MeV+nimotuzumab combination compared to the groups treated with MeV or nimotuzumab alone at both 48 h and 72 h post-infection (Figure 4). This result suggested that MeV and nimotuzumab might induce apoptosis through STAT3 and ISG15 signaling pathways.

Then assessed the morphological changes in laryngeal cancer cells undergoing apoptosis. Laryngeal cancer cells (Hep2) were transplanted into nude mice to form tumors. After treatment with MeV and nimotuzumab, the tumors were biopsied to observe the structure of tumor cells by electron microscopy. Untreated Hep2 cells had round nuclei, dark color, clear boundaries, intact organs, and capillaries were observed at the outer surface of the cells (Figure 5, upper panel). Whereas, in the treated tumors, MeV infected cells were observed and the Hep2 tumor cells formed large, multi-nucleated, dark-colored syncytia resulting from cell

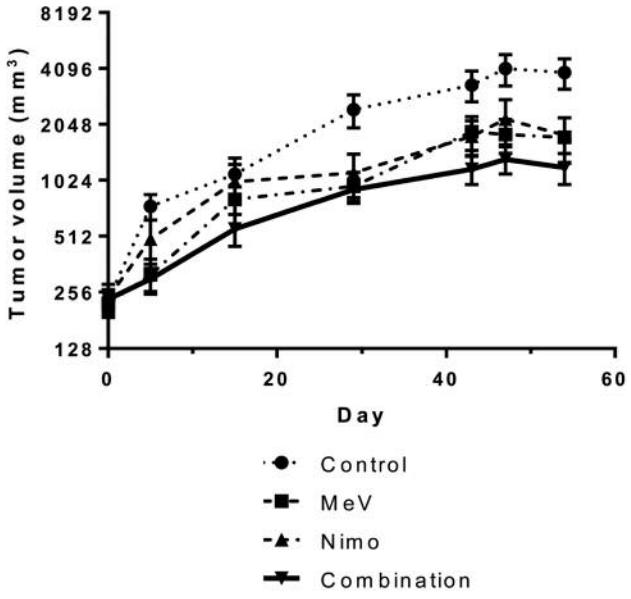

Figure 6. *Anti tumor effect of MeV, nimotuzumab or MeV+nimotuzumab combination in a laryngeal cancer xenograft tumor model. Mice bearing laryngeal tumors were infected intratumorally with multiple doses of PBS, MeV, nimotuzumab or MeV+nimotuzumab combination (twice per week for 3 weeks). Tumor volume and survival time were recorded after treatment and during a 60 days of follow-up.*

fusions (Figure 5, middle panel). We also observed Hep2 tumor cells in the process of apoptosis; rounded cells, microvascular lesions, nuclear fragmentation, chromosome condensation, lipid droplets, and appearance of multiple cytoplasmic vacuoles, as well as necrotizing cells (Figure 5, lower panel). The results indicated that treatment with MeV and nimotuzumab targets tumor cells and induces the apoptotic process.

Anti tumor effects of MeV+nimotuzumab treatment in mouse model with laryngeal cancer. The tumor suppressive effect of MeV, nimotuzumab or MeV+nimotuzumab combination was examined in human laryngeal cancer xenograft tumor model. Mice bearing laryngeal tumor were injected intratumorally with multiple doses of MeV, nimotuzumab or MeV+nimotuzumab combination (twice per week for 3 weeks). Tumor size and survival time were examined at different time points (on days 5, 15, 29, 40, 43, 47, 54 and day 60) after treatment. The results showed that the tumor volume was increasing after transplantation of laryngeal cancer cells into the mouse and after treated with MeV, nimotuzumab and MeV+nimotuzumab combination. However, the tumor volume in the treatment groups was increasing at a slower rate compared to the control group at all time points examined (Figure 6). On day 43, 47 and 54, the tumor volume in the treatment groups was significantly smaller compared to control ($p<0.0001$), and tumor volume in the group treated with MeV+nimotuzumab combination was also significantly smaller compared to groups treated with MeV or nimotuzumab alone (Figure 7).

During follow-up, eight mice in the control group died on day 12,15, 33, 36, 40, 43, and day 57; four mice in the group treated with MeV died on day 22, 29, 47, and day 54; six mice in the group treated with nimotuzumab died on day 15, 22, 40, 47, and day 54; while only two mice in the group treated with MeV+nimotuzumab combination died on day 54 and day 57. After 60 days of follow-up, the survival rate in the treated group was 60% (18/30), which was significantly higher compared to the untreated group (2/10, 20%) ($p<0.05$) (Figure 8). This result indicated that treatment with MeV and nimotuzumab increased the survival rate in the human laryngeal cancer xenograft tumor model.

Discussion

Cancer therapy based on oncolytic virus using vaccine strains is new strategy due to the great advantages such as safety and capability to target a wide range of cancer cell types. Previously, we have shown that the combination of vaccine strains MeV and MuV efficiently targeted and killed a number of hematopoietic cancer cells and cells of human solid malignancies *in vitro* and *in vivo* (6, 7). In this study, MeV in combination with nimotuzumab was found to have a greater anti cancer effect against laryngeal cancer cells compared to treatment with MeV or nimotuzumab alone. Treatment with MeV+nimotuzumab combination increased cytopathic activities and promoted apoptosis by stimulating cellular signaling pathways such as EGFR, ISG15 and STAT3 pathways. Treatment with MeV+nimotuzumab combination had anti cancer effect in a mouse model of head and neck cancer as was indicated by the reduction of tumor sizes and the increase in survival rate.

Several studies have shown that MeV exhibits a great antitumor effect in bone marrow (23) and neurological cancers (24). Galanis *et al*. have used measles viruses to treat 21 patients with ovarian cancer who had been previously treated with Taxol and Platinum (25). The results indicated that patients who were treated with measles viruses had twice longer survival time (12,5 months) compared to the untreated group (6 months) without side effects (25). In this study, we also observed a strong oncolytic activity of MeV in laryngeal cancer cells and cytotoxicity was mediated by virus replication and apoptosis induction. This observation is in line with several previous studies demonstrating that MeV can induce cancer cell apoptosis (26-28).

Oncolytic virus therapy is based on the property of oncolytic viruses to selectively enter tumor cells through the CD46 receptor, which is abundantly expressed on the surface of tumor cells, including laryngeal cancer (8). Once oncolytic

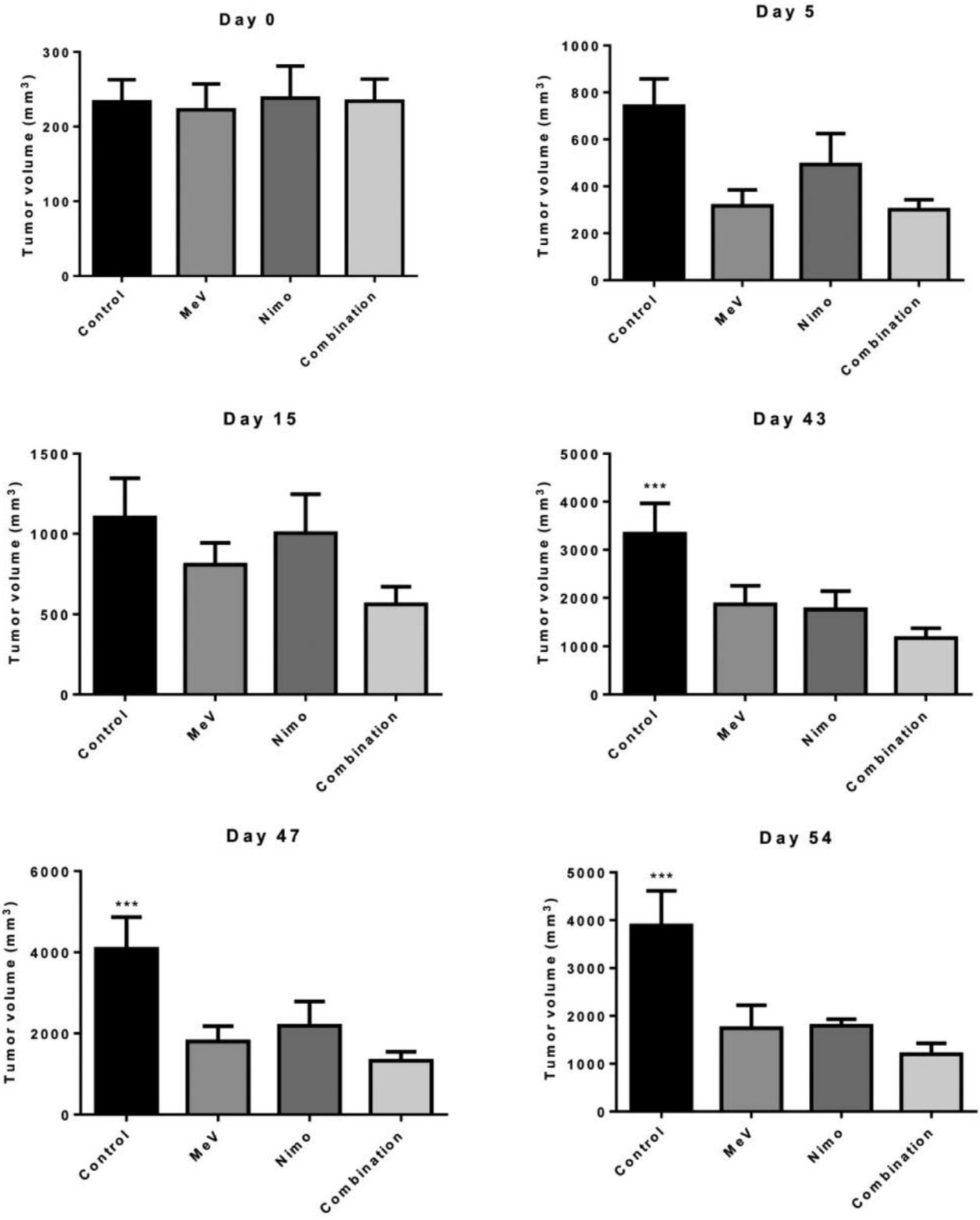

Figure 7. *Comparison of anti tumor effect between single and combined treatment in a laryngeal cancer xenograft tumor model. Mice bearing laryngeal tumors were infected intratumorally with multiple doses of PBS, MeV, nimotuzumab or MeV+nimotuzumab combination (twice per week for 3 weeks). Tumor volume and survival time were recorded and compared between groups after treatment at day 0, day 5, day 15, day 43, day 47 and day 54. Data are mean±SD of measurements obtained from 5 mice per group and (***), p<0.0001 compared to other groups.*

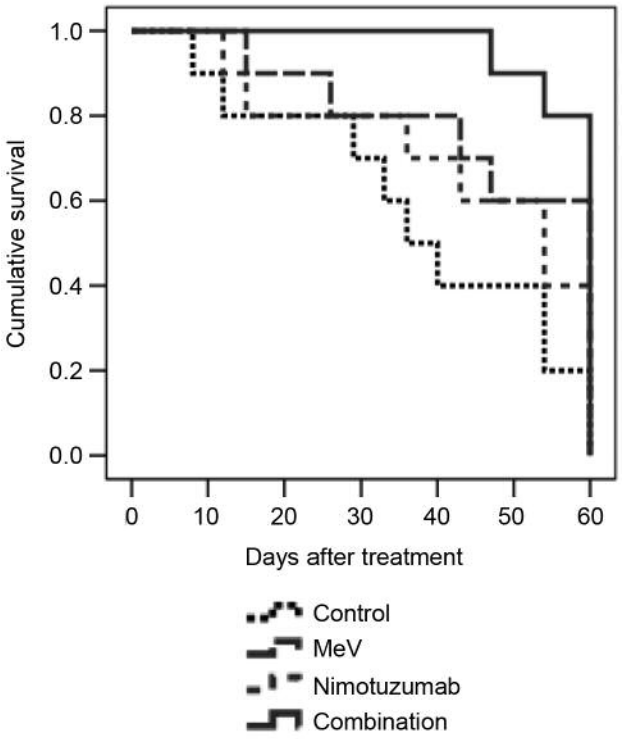

Figure 8. *Survival time of nude mice bearing laryngeal tumor during 60 days follow-up. Mice bearing laryngeal tumor were intratumorally treated with PBS, MeV, nimotuzumab or MeV+nimotuzumab combination (twice per week for 3 weeks). Survival time was recorded after treatment during 60 days follow-up.*

viruses infect tumor cells, its high replication rate results in the lysis of tumor cells. Subsequently, the newly produced oncolytic viruses continuingly infect other cancer cells (29). In the current study, MeV infected laryngeal tumor cells were observed in accordance with our previous studies detecting RNA of MeV and MuV in a number of cancer cell lines (6, 7). Oncolytic viruses may have anti cancer effects by stimulating an immune response against tumor cells, that is mediated by a number of unspecific innate immune cells such as NK, NKT cells and neutrophils (4, 5). Particularly, it has been shown that MeV induces expression of HSP72 and activates the immune system by stimulating NK cell activity and enhancing tumor-antigen presentation to professional antigen presenting cells (30). A study has showed that vaccine strain MeV infects and replicates within neutrophils and also stimulates neutrophils to secrete several specific antitumor cytokines (IL-8, TNF-α, MCP-1, and IFN-α), suggesting a neutrophil-mediated mechanism for the attenuated MeV vaccine antitumor activities (31). Our previous studies have also demonstrated that treatment with MeV, MuV and MeV+MuV combination significantly enhanced spleen-infiltrating lymphocyte populations such as macrophages, NK and dendritic cells (6).

EGFR plays a crucial role in the pathogenesis of head and neck squamous cell carcinoma (HNSCC). Specifically, overexpression of EGFR and its ligand TGFα has frequently been observed in HNSCC, and the presence of high levels of *EGFR* and *TGFα* mRNA has also been observed in tumor tissues as 92% and 87%, respectively (13). nimotuzumab is able to bind specifically to EGFR and prevent the activation of the receptor (14). nimotuzumab recognizes the extracellular domain of EGFR, competently binds to EGFR and prevents binding of EGF ligand and activation of the receptor. As a result, the tumor cells reduce secretion of vascular hypertrophy factors, decreasing blood vessel formation and increasing the number of dead cells through apoptosis (32). This is in accordance with our result indicating that treatment with MeV and nimotuzumab increased cytotoxicity and initialized apoptosis signaling. Moreover, nimotuzumab also plays a role in promoting other mechanisms of the immune response such as T and NK cells and contributes to cytotoxicity dependent effects (33).

Therapeutic regimens using the monoclonal antibodies have greatly changed the paradigm for treatment of cancer. Recently, the combination of nimotuzumab with other cancer therapies such as chemoradiotherapy has been used for the treatment of several cancers including esophageal cancer (15), LSCC (16), and advanced nasopharyngeal carcinoma (17). Another study have shown radiation-enhancing effects when using the combined treatment of nimotuzumab and celecoxib in human nasopharyngeal carcinoma cells, suggesting that this could be a promising approach for the treatment of poorly differentiated nasopharyngeal carcinoma (12). Accordingly, we showed here that the combination of MeV with nimotuzumab significantly increased cytotoxic activity in laryngeal cancer *in vitro* and *in vivo*, signifying a great potential of the combined use of oncolytic virus with monoclonal antibodies such as nimotuzumab in the treatment of various cancers.

In conclusion, these results showed a strong anticancer effect of MeV in combination with nimotuzumab in the treatment of laryngeal cancers *in vitro* and *in vivo* compared to a single therapy. Combination of oncolytic viruses and monoclonal antibodies should be considered for improving the efficiency of cancer treatment.

Conflicts of Interest

All Authors have no conflicts of interest to declare.

Authors' Contributions

NLT and HAS supervised the study and contributed to the materials and reagents. NLT, NTH, NY, HVT and HAS participated in the study design. NTH, NTX performed the experimental procedures. HVT, NLT and NTH analyzed data, interpreted results, and wrote

the manuscript. NKL, CVM, NVB, NTX and TDC contributed to the materials and revision of the manuscript. All authors agreed with both the results and conclusions.

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