의학박사 학위 논문

두경부암의 방사선 저항성 획득에 있어 EphA3의 역할 규명

Investigation on the role of EphA3 in the development of EMT (Epithelial Mesenchymal Transition) dependent radioresistance in HNC

울 산 대 학 교 대 학 원

의 학 과

김 송 희

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지 도 교 수　한 명 월

이 논문을 의학박사 학위 논문으로 제출함

2018년 12월

울 산 대 학 교　대 학 원

의　학　과

김 송 희

김송희의 의학박사 학위 논문을 인준함

심사위원 김 승 후 (인)

심사위원 김 상 윤 (인)

심사위원 이 종 철 (인)

심사위원 박 정 제 (인)

심사위원 한 명 월 (인)

울 산 대 학 교 대 학 원

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ABSTRACT

Radiotherapy is a well-established therapeutic modality used in the treatment of many cancers. However, radioresistance remains a serious obstacle to successful treatment. Radioresistance can cause local recurrence and distant metastases in some patients after radiation treatment. Thus, many studies have attempted to identify effective radiosensitizers.

Eph receptor functions contribute to tumor development, modulating cell-cell adhesion, invasion, neo-angiogenesis, tumor growth and metastasis. However, the role of EphA3 in radioresistance remains unclear.

In the current study, we established a stable radioresistant head and neck cancer cell line (AMC HN3R cell line) and found that EphA3 was expressed predominantly in the radioresistant head and neck cancer cell line through DNA microarray, real time PCR and Western blotting. Additionally, we found that EphA3 was overexpressed in recurrent laryngeal cancer specimens after radiation therapy. EphA3 mediated the tumor invasiveness and migration in radioresistant head and neck cancer cell lines and epithelial mesenchymal transition- related protein expression. Inhibition of EphA3 enhanced radiosensitivity in the AMC HN 3R cell line in vitro and in vivo study. In conclusion, our results suggest that EphA3 is overexpressed in radioresistant head and neck cancer and plays a crucial role in the development of radioresistance in head and neck cancers by regulating the epithelial mesenchymal transition pathway.

Keywords: Head and neck cancer, EphA3, Radioresistance, Radiosensitivity Epithelial-mesenchymal transition

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Introduction

The treatment of locoregionally advanced squamous-cell carcinoma of the head and neck has developed gradually from surgery to radiotherapy. The major reason for this gradual change in treatment modality is an increased preference for organ preservation strategies. Radiotherapy is a well-established therapeutic modality used to treat many cancers. However, radioresistance remains a serious obstacle to successful treatment. Radioresistance can cause local recurrence and distant metastases in some patients treated with radiation. Thus, the investigations of novel mechanisms that contribute to the radioresistance process are very important, particularly the discovery of effective radiosensitizers [1,2]. Accumulating evidence indicates that activated PI3K/PTEN/Akt pathway is a major predictive marker of the responsiveness of solid tumors to radiotherapy. Preclinical in vitro and in vivo studies have shown that ionizing radiation-induced upregulation of the PI3K/Akt pathway inhibits apoptosis and activates DNA double-stranded break repair machinery, thus protecting tumor cells from ionizing radiation-induced death and leading to radioresistance [3,4]. And, epthelial-to-mesenchymal transition (EMT), cancer stem cell and negative HPV status increase radioresistance [5 - 7]. Especially, radiation-mediated EMT is through PTEN-dependent pathways, highlighting adirect proinvasive effect ofradiation treatmenton tumor cells [8].

The Eph receptors constitute the largest subfamily of receptor tyrosine kinases, and interact with cell membrane bound ligands, known asephrins.Eph-ephrinmediatedcellcommunication controlsbiological functions such as cell-cell attachment and detachment, cell shape and motility and epthelial-to-mesenchymal transition (EMT), which together govern cell positioning underlying normal and oncogenic development [9 - 11]. Eph receptor functions contribute to tumor development, modulating cell-cell adhesion, invasion, neo-angiogenesis, tumor growth and metastasis [9,12].

Eph receptor overexpression occurs in a wide range of epithelial and mesenchymal tumors, and often correlates with more aggressive phenotypes and poor prognosis [13 - 15]. Similarly to other protein families involved in development, Eph receptors often re-emerge in cancer. And EphA3, like many Eph receptors, was initially identified in tumor cell lines [16]. EphA3 is frequently overexpressed in glioblastoma and, in particular, in the most aggressive mesenchymal subtype [17]. Recent EphA3 targeting studies in pre-clinical models of glioblastoma have been very encouraging and may provide an avenue to treat these highly refractory aggressive tumors [18]. A recent study demonstrated that EphA3 was overexpressed within the microenvironment of a range of human cancers and the potential of using EphA3 agonists for anticancer therapy [19]. However, the role of EphA3 in radioresistance remains unclear.

Here, we found that EphA3 was overexpressed in a radioresistant head and neck cancer cell line through microarray analysis. We investigated the role of EphA3 in radioresistance and whether EphA3 inhibition can regulate radiation sensitivity. We found that EphA3 is associated with radiation resistance in head and neck cancer and the inhibition of EphA3 enhances the efficiency of radiotherapy significantly in vitro and in vivo through the PTEN/Akt/EMT pathway.

Materials and methods

1. Cell culture and establishment of radioresistant head and neck cancer cell line

Various head and neck cancer cell lines (AMC HN3, AMC HN8, UMSCC1, UDSCC2) were used in this study. Cancer cell lines were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Invitrogen) and 100μg/mL of penicillin/streptomycin and incubated at 37°C with 5% CO^2 in a humidfied incubator. Head and neck cancer cells were grown to approximately 50% cofluence in vented 75-cm^2 culture flasks and irradiated using 6-MV photon beam generated by a linear accelerator (CLINAC 600; Varian, Palo Alto, CA, USA) at a dose rate of 2Gy or 4Gy. Cell lines were kept in continuous culture for < 5 passages and tested by PCR or FACS before evaluating the phenotype and expression of relevant proteins. By using the previously established human laryngeal SCC cell line AMC HN3 (11), clinically relevant fractionated radiation doses (2Gy) at 2day intervals were successively delivered. After receiving a cumulative dose of 70Gy, the isogenic model of successively irradiated AMC HN3R cell line was considered to be established. This model was originally designed for investigating radioresistance, which uses cells of the same origin that differ only in terms of radiosensitivity [20 - 22]. We prepared various additional radioresistant head and neck cancer cell lines (AMC HN8R cell line, UMSCC1R cell line, UDSCC2R cell line) using the same procedure.

2. RNA preparation and gene expression profiling

Total RNA was isolated using the PureLink™ RNA mini kit (Life Technologies, Carlsbad, CA, USA). RNA quality was assessed by an Agilent

2100 bioanalyzer using the RNA 6000 Nano chip Nano Chip (Agilent Technologies, Santa Clara, CA, USA), and quality was determined using a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Briefly, 300ng of total RNA from each sample was converted to double-strand cDNA using a random hexamer incorporating a T7 promoter; amplfiied RNA was generated from the Affymetrix sample cleanup module. cDNA was regenerated through a random-primed reverse transcription using a dNTP mix containing dUTP. The cDNA was then fragmented by the UDG and APE1 restriction endonucleases and end-labeled in a terminal transferase reaction incorporating a biotinylateddideoxynucleotide. Fragmented end-labeled cDNA was hybridized to the GeneChip® Human Gene 1.0 ST arrays as described in the Gene Chip Whole Transcript(WT)Sense Target Labeling Assay Manual(Affymetrix, Santa Clara, CA, USA). Next, the chips were stained and washed in a GeneChip Fluidics Station 450 (Affymetrix) and scanned using a GeneChip Array scanner 3000 7G (Affymetrix).

3. Microarray expression data analysis

Expression data were generated by Affymetrix expression console software version 1.1. For normalization, the Robust Multi-Average algorithm implemented in Affymetrix Expression Console software was used. To determine whether genes were differentially expressed between the two groups, one-way analysis of variance was performed on the Robust Nulti-Average expression values (2-fold change, $p < 0.05$). In order to classify the co-expression gene group showing similar expression patterns, we performed hierarchical clustering and K-mean clustering in Multi Experiment Viewer software 4.4 (www.tm4.org). The web-based tool Database for Annotation, Visualization, and Integrated Discovery was used to perform the biological interpretation of differentially expressed genes. Next,

the genes were classfied based on the information of gene function in Gene Ontology, KEGG pathway database (http://david.abcc.ncifcrf.gov/home.jsp).

4. RNA interference

At 24h before transfection, AMC HN3R cells wereplated into 6-well plates (1×10^5 cells per well). Cells were transfected with 30nM of EphA3 siRNA (sc-39934) or control siRNA (sc-37007) using siRNA Transfection reagent from Santa Cruz Biotechnology (Santa Cruz, CA, USA). EphA3 shRNA and control construct was the mission lentivirus SCHLNV, Clone ID TRCN0000196830 (Sigma). shRNA sequences are listed in previous article [17]. The cells were grown for 72h prior to western blot analysis.

5. RNA extraction and quantitative real-time PCR assay

Total RNA isolated using the PureLink RNA Mini Kit (ambion by life technologies™) and cDNA was synthesized from 1μg RNA with Maxime RT-PCR PreMix Kit (iNtRON Biotechnology). SYBR Green (Bio rad) PCR performed in triplicate using the CFX96 Touch™ Real-Time PCR Detection System. All samples were normalized to the signal generated from β-actin. Primer sequences of EphA3 and EMT associated genes were presented in Supplementary Table 1. Data was shown as fold change ($2-\Delta\Delta Ct$) and analyzed initially using Bio-rad CFX Software. All experiments were carried out>3times. The level mRNA expression was presented as the mean of three experiments and the standard error was indicated.

6. Western blot analysis

Total protein was extracted using PRO-PREP protein extraction solution (Intron Biotechnology). Protein concentrations were determined using the

PierceTM BCA Protein Assay Kit (Rockford, IL, USA). Equal amounts of protein were separated by SDS-PAGE and blotted to nitrocellulose transfer membranes (Whatman, Maidstone, UK). This was followed by incubation with primary antibodies to EphA3 (sc-920), fibronectin (sc-18825), snail (sc-28199), E-cadherin (BD610182), Akt (#4691), phsopho-Akt Ser473 (#4060), phospho-PTEN (#9551) andβactin (sc-47778), which were purchased from Cell Signaling Technology (Danvers, MA, USA) and horseradish peroxidase-conjugated secondary antibodies, followed by detection with a SuperSignal West Pico Trial kit (Thermo Scientfic) according to the manufacturer's instructions (Cayman chemical, Ann Arbor, MI). All western blot analysis was performed at least 3 times and the representative figures were presented.

7. Clonogenic assay

Parental and R cells were plated on a 6-cm dish at 100, 500, 1000, and 4000 cells per dish. Brifly, cells were treated with different doses of irradiation (0, 2, 4, and 8Gy, respectively) in the presence of siRNA. Irradiated cells were plated in duplicate in a 6-cm dish. After 14 days, the colonies were fxed with methanol and stained with crystal violet. Colonies containing>50 cells were counted under the microscope. Not every cell seeded will form a colony, even in the absence of irradiation, due to factors such as errors in counting, stress of manipulation, suboptimal growth medium, etc. The plating efficiency (PE) is defined as the number of colonies observed/the number of cells plated

$$\mathrm{PE} = \frac{colonies\ \ observed}{number\ of\ cells\ plated}$$

Parallel dishes are seeded with cells that have been exposed to increasing

doses of radiation. The number of cells plated is increased so that a countable number of colonies results. Surviving fraction (SF) is the colonies counted divided by the number of colonies plated with a correction for the plating efficiency

$$SF = \frac{colonies\ \ counted}{cells\ \ seeded\ x\,(PE\ /100)}$$

8. Invasion and migration assay

Migration and invasion were examined in a Transwell assay using a CytoSelectTM 24-Well kit (Cell Biolabs, Inc., San Diego, CA, USA), according to the manufacturer's instructions. For the migration assay, briefly, the inner chambers of the Transwells containing polycarbonate membrane inserts were seeded with cells (1×10^5 cells/well) that had been transfected with EphA3 siRNA or control siRNA. Media containing 10% fetal bovine serum was added to the lower well of the migration plate. The migrated cells were stained with a cell staining solution and extracted with an extraction solution (both Cell Biolabs, San Diego, CA, USA) according to the manufacturer's instructions. The optical density of the extracted solution was measured at 560nm using an Emax Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). For the invasion assay, the kit required a 24-well plate containing polycarbonate membrane inserts; the upper surface of the insert membrane was coated with a uniform layer of dried basement membrane matrix solution. This basement membrane layer served as a barrier to discriminate invasive from non-invasive cells. The invasion assay was performed simultaneously using an identical protocol to that used for the migration assay, but with a different insert.

9. Xenograft model

To determine anti-tumoral activities in combination of EphA inhibition and irradiation, xenografts were established in 5-to 6-week-old male athymic nude mice (nu/nu; Harlan-Sprague-Dawley) by subcutaneous injection of 5×10^6 AMC HN3R cells. When tumor volume reached $50mm^3$ mice were treated into four treatment groups (each group with 4 mice): (a) AMC HN3R control, vehicle: (b) AMC HN3R with radiation only: (c) AMC HN3R with EphA3 shRNA treatment: and (d) AMC HN3R with EphA3 shRNA+radiation. All mice were treated for 1week with 4 mice per group and sacrificed after approximately 4weeks of treatment. Radiation was delivered with 4Gy, using a 6-MV therapeutic linear accelerator (CLINAC EX: Varian, Palo Alto, CA) at a dose rate of 2Gy/min: 1.0-cm bolus was used for radiation dose buildup. Tumor volumes were calculated ($V=(length\times Width^2)/2$).

10. Immunohistochemical analysis

For immunohistochemical evaluation, this study included 104 tissue specimens of 100 patients with laryngeal cancer. Regions of each primary tumor were chosen under microscopy and arranged pair-wise in tissue microarray blocks. The invasive front of each tumor was represented by two validated tissue cores on a tissue microarray. Fiftynine cases of primary total laryngectomy or laryngomicrosopic biopsy specimens and 45 cases of salvage total laryngectomy specimens for recurred cancer after radiotherapy or concurrent chemoradiotherapy were examined. After deparaffinization and rehydration, 4-μm thick sections were subjected to heat-induced antigen retrieval using a 0.01M citrate buffer (pH6.0) for 1h. Sections were incubated in aqueous 3% H_2O_2 for 15min to quench endogenous peroxidase activity and then washed with 1×PBS. Slides were loaded into a humid chamber and blocked for 30min with 1× Universal Blocking Agent (10× Power Block™:

BioGenex, San Ramon, CA, USA) before incubation overnight at 4°C with primary antibodies against EphA3 (1:100; Sigma-Aldrich, Inc., St. Louis, MO, USA). The next day, slides were incubated for 1 h at room temperature and then treated with Envision Reagent (Dako REALTM EnVisionTM, Glostrup, Denmark) for 30min. Slides were washed with PBS and treated with thechromogen DAB for 15min to allow formation of the brown reaction product. The slides were counterstained in Mayer's hematoxylin, dehydrated in graded alcohol, cleared in xylene, and mounted. The slides were independently interpreted by two reviewers with no knowledge of the clinical data. Immunostaining was graded by the reactivity score (IRS), which reflects staining intensity (SI),assessed to be negative(=0), weak(=1), moderate(=2) or strong(=3). For further analysis, the specimens were divided into two groups: negative with score 0 and positive with score 1 - 3 (Supplementary Fig. 1).

11. Immunofluroscence microscopy

The cells were plated on 18-mm coverslips and fixed with 3.7% paraformaldehyde, permeabilized in 0.1% Triton X-100 in PBS, and blocked in PBS/5% BSA. EphA3 was detected using anti-EphA3 (Santa Cruz) at 4°C overnight followed by incubation with fluorescent-conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA). After washing, the cells were mounted on glass slides and examined under a DP40 (Olympus, Tokyo, Japan).

12. Statistical analysis

Statistical analyses were conducted using one way or two way ANOVA to identify statistically. To determine statistically significant relationships between the distribution of categorical values the Chisquared test. Differences with p-values < 0.05 were considered statistically significant and the p-value

of tests is provided in the figure legends.

Results

1. EphA3 is expressed highly in radioresistant head and neck cancer cell line

Analysis of the microarray data revealed that the expression of nine genes (PLXDC2, Robo1, SNORD77, EphA3, LMO3, LPHN3, SULT1B1, SULT1E1 and TPTE2) was significantly increased in the AMC HN3R cell line comparing with the expression in the AMC HN3 cell lines. Robo1 [23], EphA3 [11,17,24], LMO3 [25] and TPTE2 [26] were linked cell death and Robo1 [27] and EphA3 [15] could regulate EMT. We had conducted studies with emphasis on EphA3 among several genes (Expression difference: EphA3 AMC HN3R/AMC HN3 = 4.762). To shown between AMC HN3 and AMC HN3R ($p < 0.01$, Fig. 1A). Western blotting analysis of EphA3 protein expression revealed EphA3 overexpression in all R cell lines (Fig. 1B).

Additionally, we investigated the effect of radiation dose on EphA3 levels. EphA3 mRNA and protein levels in the AMC HN3R-70 Gy cell line were higher than in AMC HN3 and AMC HN3R-40 Gy (Fig. 1C, D). Immunofluorescence staining of EphA3 in the three cell lines revealed that EphA3 was abundantly expressed in AMC HN3R-70 Gy (Fig. 1E).

2. EphA3 is overexpressed in recurrent laryngeal cancer specimen

For immunohistochemical evaluation, this study included 104 tissue specimens of 100 patients with laryngeal cancer. The staining score for EphA3 expression in 45 salvage surgical specimens after radiation failure was significantly higher than that in 59 surgical specimens without radiation treatment according to univariate analysis ($p = 0.016$, Table 1). Interestingly, among 5 cases in which we could evaluate cancer specimens before and after

radiation, 3 specimens overexpressed in radioresistant head and neck cancer and may play a crucial role in the development radioresistance in head and neck cancers.

3. EphA3 mediates tumor invasiveness and migration in radioresistant head and neck cancer cell lines

At first, we confirmed the effective knockdown of EphA3 for AMC HN3R cell line (Fig. 2A). We next performed cell migration and invasion Transwell assays to examine the effect on the cell migration and invasion capabilities of EphA3. The results showed that the AMC HN3R cell line significantly enhanced the capabilities of migration and invasion of tumor cells compared with their corresponding AMC HN3 cell line (Fig. 2B). In contrast, silencing of EphA3 (EphA3 siRNA) in the AMC HN3R cell line inhibited cell migration and invasion (Fig. 2C). Taken together, these results support that EphA3 plays an important role in migration and invasion of radioresistant head and neck cancer cell.

4. The expression of EphA3 correlates the expression of EMT related protein.

EMT is one of the key mechanisms by which cancer cells acquire the ability to undergo metastasis and invasion and an EMT-like phenotype was associated with radioresistance [5,28]. To evaluate whether EphA3 influences EMT-related proteins in radioresistant head and neck cancer cell lines, we carried out real-time PCR and western blotting to detect the expression of Snail, fibronectin, vimentin, E-cadherin, and N-cadherin. The results showed that in the AMC HN3R cancer cell line, the mRNA expression of Snail, fibronectin, and vimentin was significantly increased with overexpression of EphA3 and the expression of N-cadherin was increased although there is no

statistical significance while the expression of E-cadherin was decreased compared to that in the AMC HN3 cancer cell line (Fig. 2D). Furthermore, the difference in the protein levels of Snail, fibronectin, vimentin, E-cadherin, and N-cadherin were confirmed using western blotting (Fig. 2E).

To evaluate whether EMT-related proteins increased in other radioresistant head and neck cancer cell lines, we carried out real-time PCR, western blotting, and immunofluorescent staining in AMC HN8, AMC HN8R, UDSCC2, and UDSCC2R cells. We obtained the same results for the AMC HN3R cell line; the expression of Snail, fibronectin, vimentin and N-cadherin was increased, while E-cadherin was decreased (Supplementary Fig. 2).

5. EphA3 regulates expression of EMT related protein.

To determine the effects of EphA3 increased on EMT, we investigated the expression of EMT related protein after treatment of siRNA to inhibit expression of EphA3 (Fig. 3). And we carried out realtime PCR, western blotting, and immunofluorescence to detect the expression of Snail, fibronectin, vimentin, E-cadherin, and N-cadherin.

Real-time PCR and western blotting showed that in AMC HN3R + EphA3 siRNA cells, expression of the mesenchymal differentiation markers, Snail, fibronectin, vimentin and N-cadherin was decreased compared to that in the control cells and N-cadherin and while E-cadherin was increased (Fig. 3A and B). Additionally, we determined these results by immunofluorescence staining, which showed that Snail and fibronectin expression decreased after blocking of EphA3 by treatment with EphA3 siRNA in the AMC HN3R cell line (Fig. 3C).

It is known that EPH receptors can regulate of Akt via PTEN [16,29 - 31] and Akt activation mediate cellular migration and invasion through EMT [32]. In order to investigate the effects of EphA3 knockdown on the activity of the PTEN/Akt/EMT signaling pathway in AMC HN3R cells, the protein

expression levels of PTEN, Akt and P-Akt were measured using western blotting. We identified the Akt activation with decreased PTEN activation in AMC HN3R comparing with AMC HN3 cell line. When EphA3 was inhibited with siRNA, Akt activation was decreased with increased PTEN expression compared to that in the control cells (Supplementary Fig. 3). These results indicate that EphA3 may regulate the EMT through PTEN/Akt signaling pathway.

6. EphA3 inhibition increase radiosensitivity in radioresistant cell line.

Next, we assessed the effect of inhibition of EphA3 on the response to irradiation in cancer cells. The regulatory effects of the EphA3 inhibitor (siRNA of EphA3) were assessed by cell survival. We observed the sensitivity of AMC HN3R cells to radiation after silencing of EphA3.

AMC HN3, AMC HN3R, and AMC HN3R cells transfected with siRNA of EphA3 were exposed to various doses of irradiation (0, 2, 4, and 8 Gy) and then the clonogenic assay was performed at 11 days after irradiation. The surviving fraction of EphA3 siRNA-treated AMC HN3R cells was significantly lower compared to untreated AMC HN3R cells (Fig. 4). Similar results were shown in AMC HN3 cell line. Taken together, these findings suggest that inhibition of EphA3 enhances radiosensitivity in radioresistant cancer cells and that EphA3 can mediate radioresistance in head and neck cancer.

7. EphA3 inhibition increase radiosensitivity in AMC HN3R xenografts model.

Having established the in vitro effects of combined EphA3 inhibition on sensitivity of radioresistant cancer cells, the biologic efficacy of combined treatment was tested in tumor growth delay using AMC HN3R xenografts

established in nude mice. Tumor mice (each group with 4 mice) were treated with AMC 3R cell line vehicle, AMC HN3R cell line with 4 Gy radiation, AMC HN3R cell line with EphA3 shRNA treatment, and AMC HN3R cell line with EphA3 shRNA treatment and radiation combined. EphA3 inhibition enhanced the efficacy of radiation: combination therapy of EphA3 shRNA and radiation resulted in a noticeable tumor growth delay at 27 days, compared with irradiation alone (Fig. 5). Mouse body weight monitoring suggested that all treatments were relatively well tolerated. These data suggest that EphA3 inhibition can enhance the radiosensitivity in irradiated AMC HN3R xenografts.

Table 1
EphA3 expression in laryngeal cancer specimen in tissue microarray ($N = 104$ specimens).

	Negative	Positive	*P* value
Primary surgery specimen ($N = 59$)	28	31	*0.016*
Recurred cancer specimen ($N = 45$)	11	34	

Table 2
Comparison of EphA3 expression with preRT specimen and postRT specimen after recurrence in same patients.

	PreRT EphA3 expression	PostRT EphA3 expression
Case 1	0	1
Case 2	0	2
Case 3	0	1
Case 4	1	1
Case 5	0	0

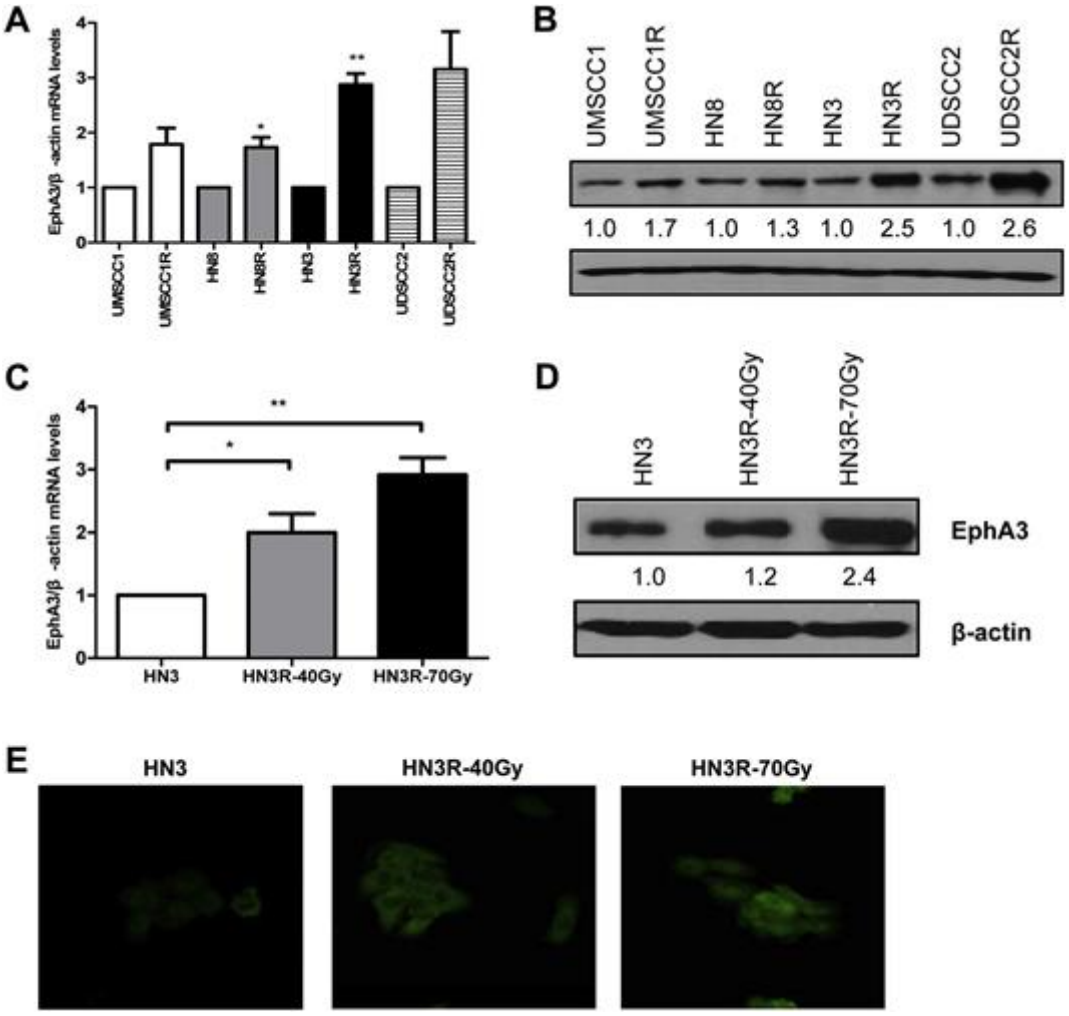


Fig. 1. EphA3 is expressed highly in radioresistant head and neck cancer cell line.

(A) EphA3 mRNA expression level was assessed using real time PCR in various mother cell line and radioresistant head and cancer cell line. (B) EphA3 protein expression level was assessed using Western blotting in various mother cell line and radioresistant head and cancer cell line. EphA3 expression level was investigated in radiation dose dependent manner in AMC HN 3R cell line using (C) Real time PCR, (D) Western blot and (E) Immunofluorescent staining.*,$P < 0.01$; **, $P < 0.005$; ***, $P < 0.0001$. All experiments were carried out > 3 times. The level mRNA expression was presented as the mean of three experiments and the standard error was

indicated. The expression of the protein was quantified as compared to the beta-actin in the Western blot. AMC was omitted in AMC HN cell lines for simplicity in figures.

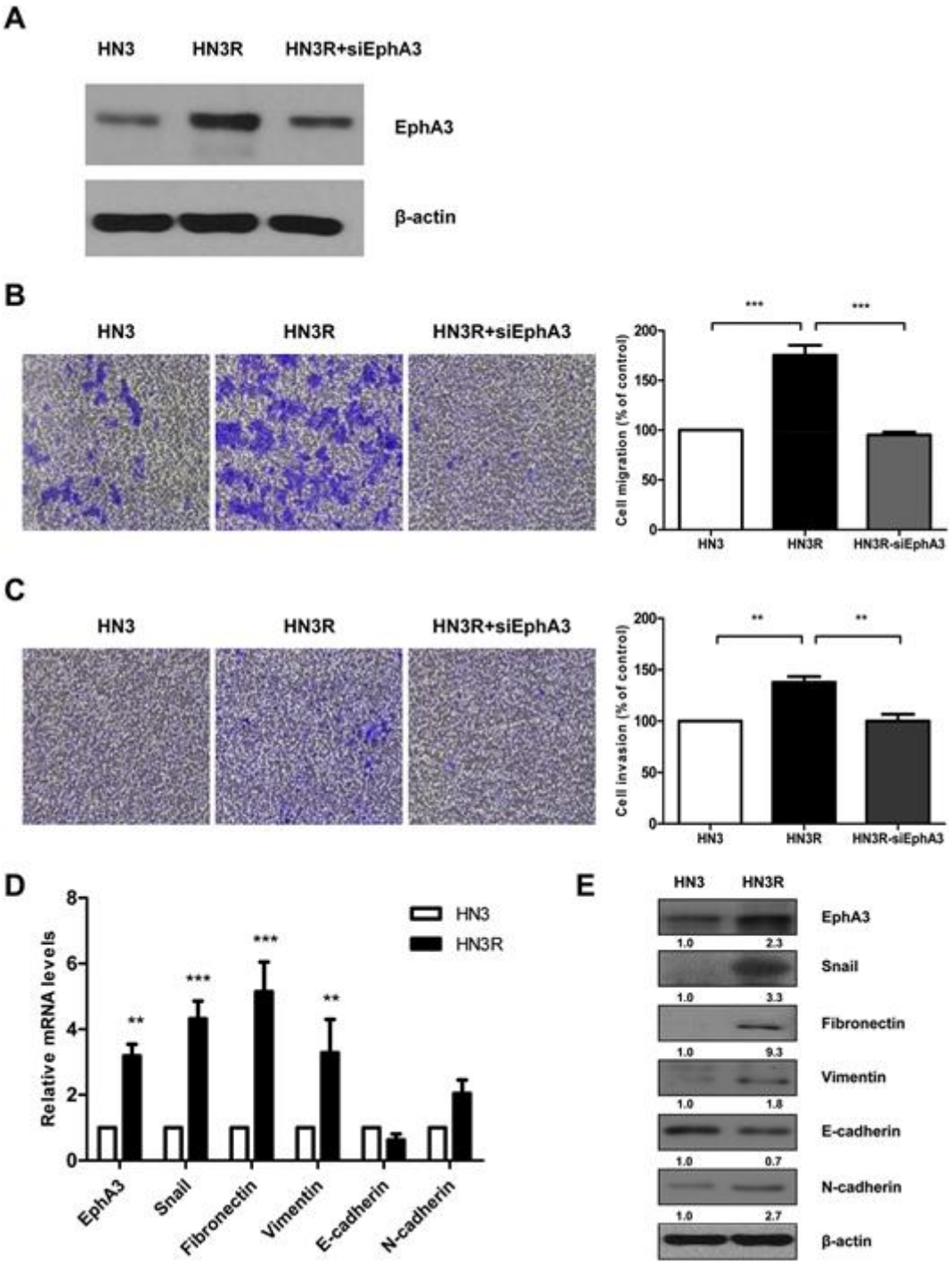


Fig. 2. EphA3 mediates tumor invasiveness and migration in radioresistant head and neck cancer cell lines through EMT pathway.

(A) The effective knockdown of EphA3 for AMC HN3R cell line been shown using western blotting. (B) Migration and (C) invasion assay were examined in a Transwell assay using a CytoSelectTM 24Well kit in AMC HN3, AMC HN3R and AMC HN3R + siRNA of EphA3 cell line. (D) The mRNA expression of Snail, fibronection, vimentin, E-cadherin and N-cadherin was assessed using Real time PCR in the AMC HN3 and AMC HN3R cell line. (E) The difference in the protein levels of Snail, fibronection, vimentin, E-cadherin and N-cadherin were confirmed via western blotting in AMC HN3 and AMC HN3R cell line.*, $P < 0.01$; **, $P < 0.005$; ***, $P < 0.0001$. All experiments were carried out > 3 times. The level mRNA expression was presented as the mean of three experiments and the standard error was indicated. The expression of the protein was quantified as compared to the beta-actin in the Western blot. AMC was omitted in AMC HN cell lines for simplicity in figures

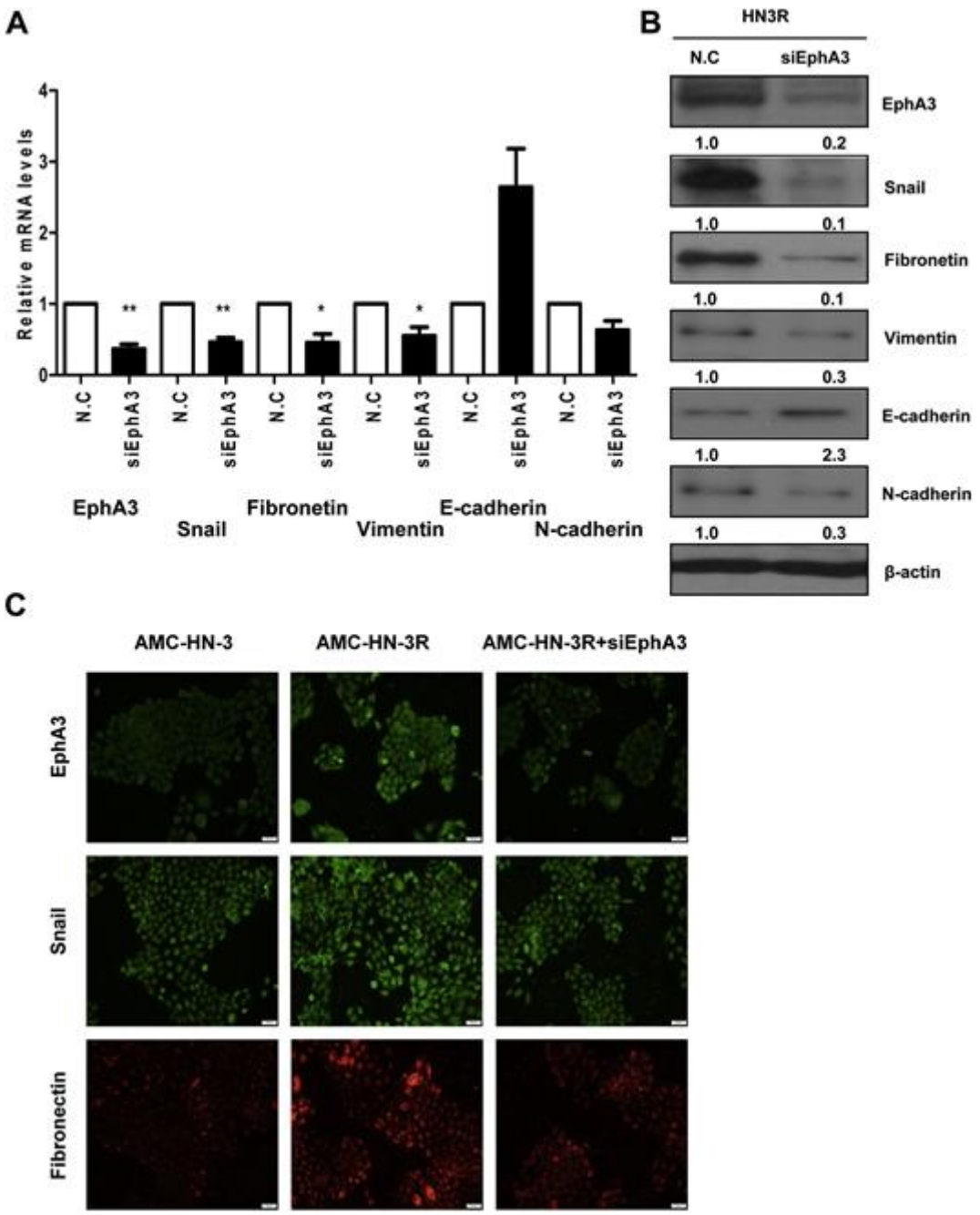


Fig. 3. The expression of EphA3 correlates the expression of EMT related protein.

A) The mRNA expression of Snail, fibronection, vimentin, E-cadherin and Ncadherin was investigated after silencing of EphA3 in AMC HN3R cell line using Real-time PCR. B) The protein expression level of Snail, fibronection,

vimentin, Ecadherin and N-cadherin was assessed after silencing of EphA3 in AMC HN3R cell line using western blotting, and (C) The difference in the expression levels of Snail and fibronectin were confirmed via immunofluorescence in AMC HN3, AMC HN3R and AMC HN3R+ siRNA of EphA3 cell line. *, $P < 0.01$; **, $P < 0.005$; ***, $P < 0.0001$. All experiments were carried out >3 times. The level mRNA expression was presented as the mean of three experiments and the standard error was indicated. The expression of the protein was quantified as compared to the beta-actin in the Western blot. AMC was omitted in AMC HN cell lines for simplicity in figures

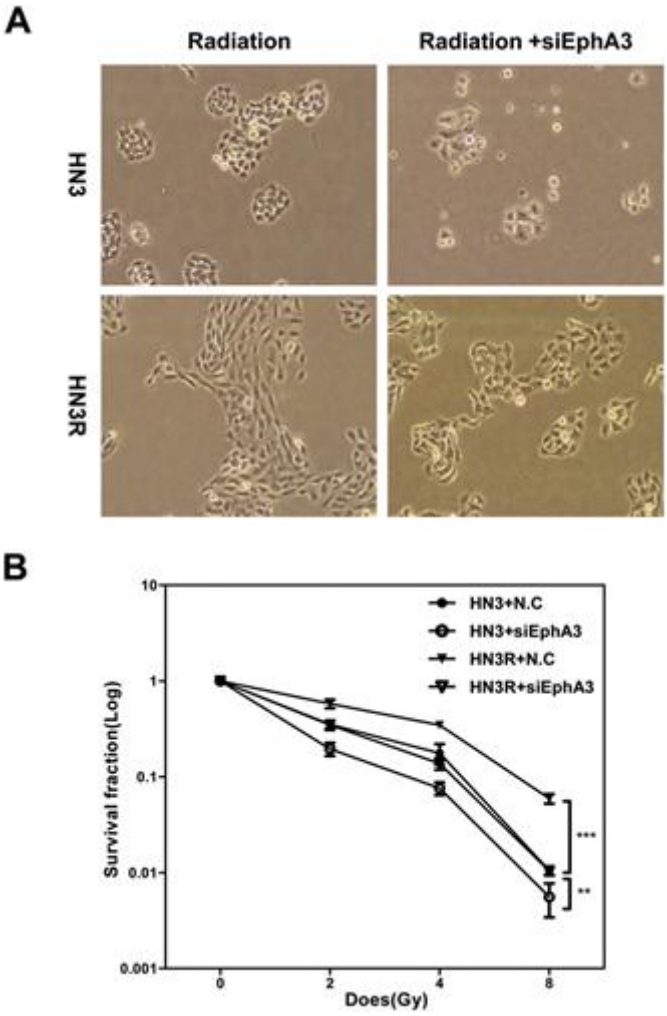


Fig. 4. EphA3 inhibition increase radiosensitivity in radioresistant cell line.

(A) Cell growth was assessed after radiation (4 Gy) and siRNA of EphA3 treatment in AMC HN3 and HN3R cell line. (B) AMC HN3, AMC HN3 cells transfected with siRNA of EphA3, AMC HN3R, and AMC HN3R cells transfected with siRNA of EphA3 were exposed to various doses of irradiation (0, 2, 4, and 8 Gy) and then the clonogenic assay was performed at 11 days after irradiation. *, $P < 0.01$; **, $P < 0.005$; ***, $P < 0.0001$. All experiments were carried out > 3 times. The survival fraction was presented as the mean of three experiments and the standard error was indicated. AMC was omitted in AMC HN cell lines for simplicity in figures

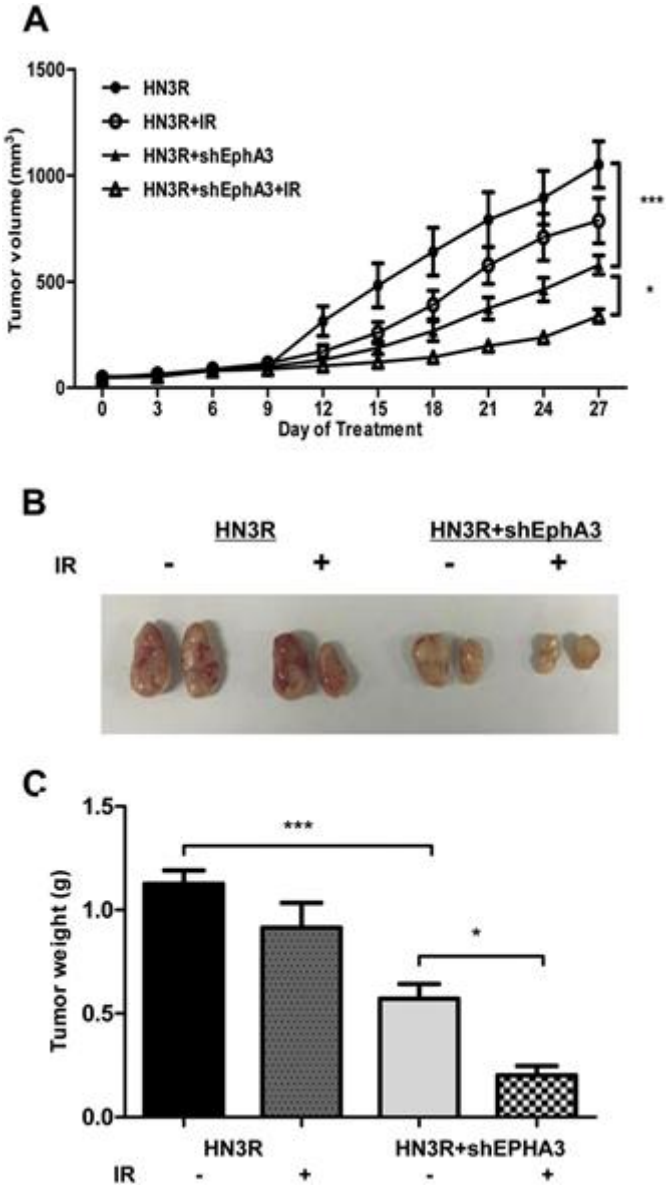
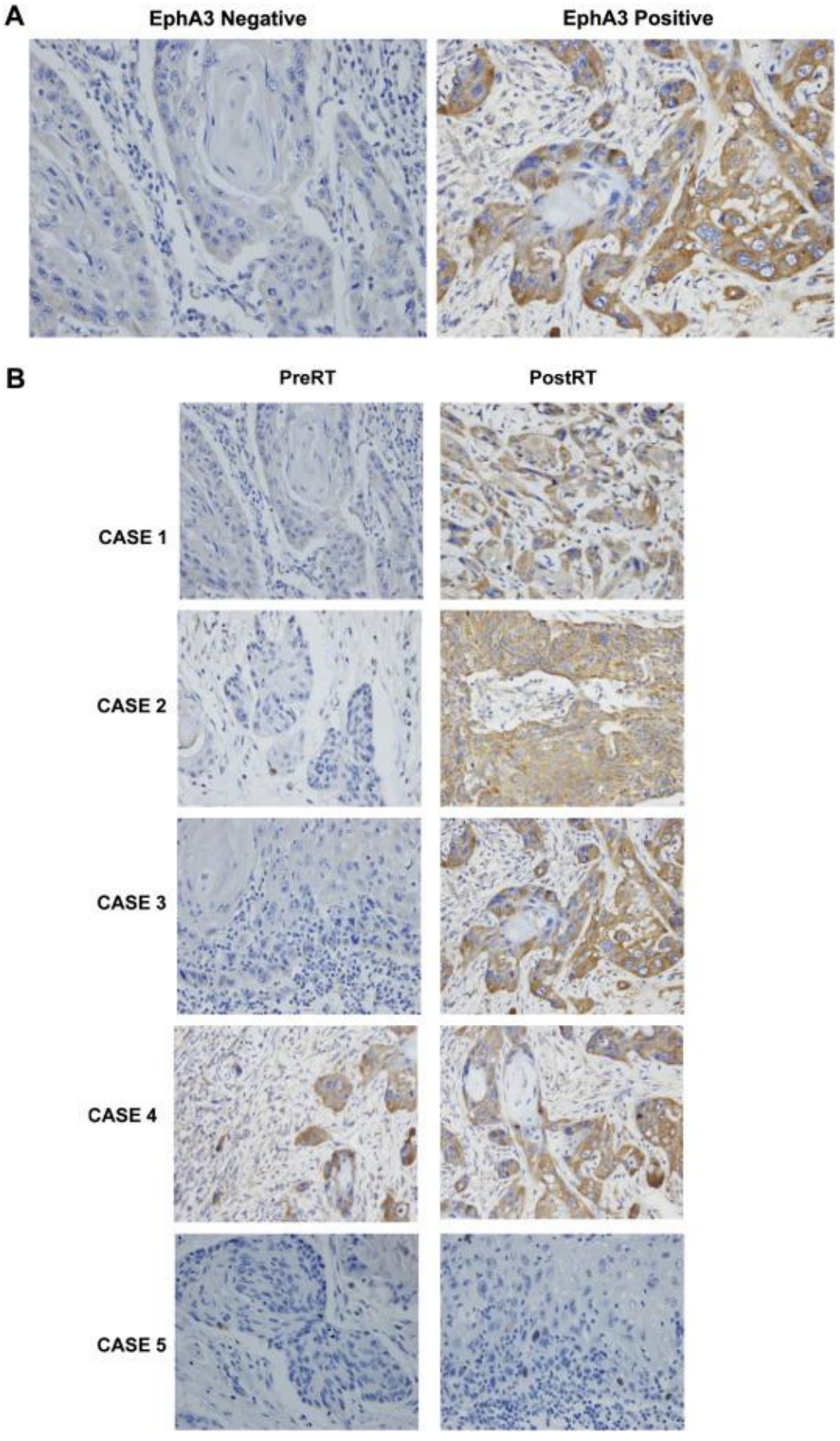


Fig. 5. EphA3 inhibition increase radiosensitivity in AMC HN3Rxenografts model.

(A) Tumors were measured regularly and the relative tumor volume was tracked for each animal. (B) The photographs show representative tumors from each mouse of approximate 4 weeks (each group: 4 mice). (C) Tumor weight was examined in each treatment groups at 28 days. *, $P < 0.01$; **, $P < 0.005$; ***, $P < 0.0001$

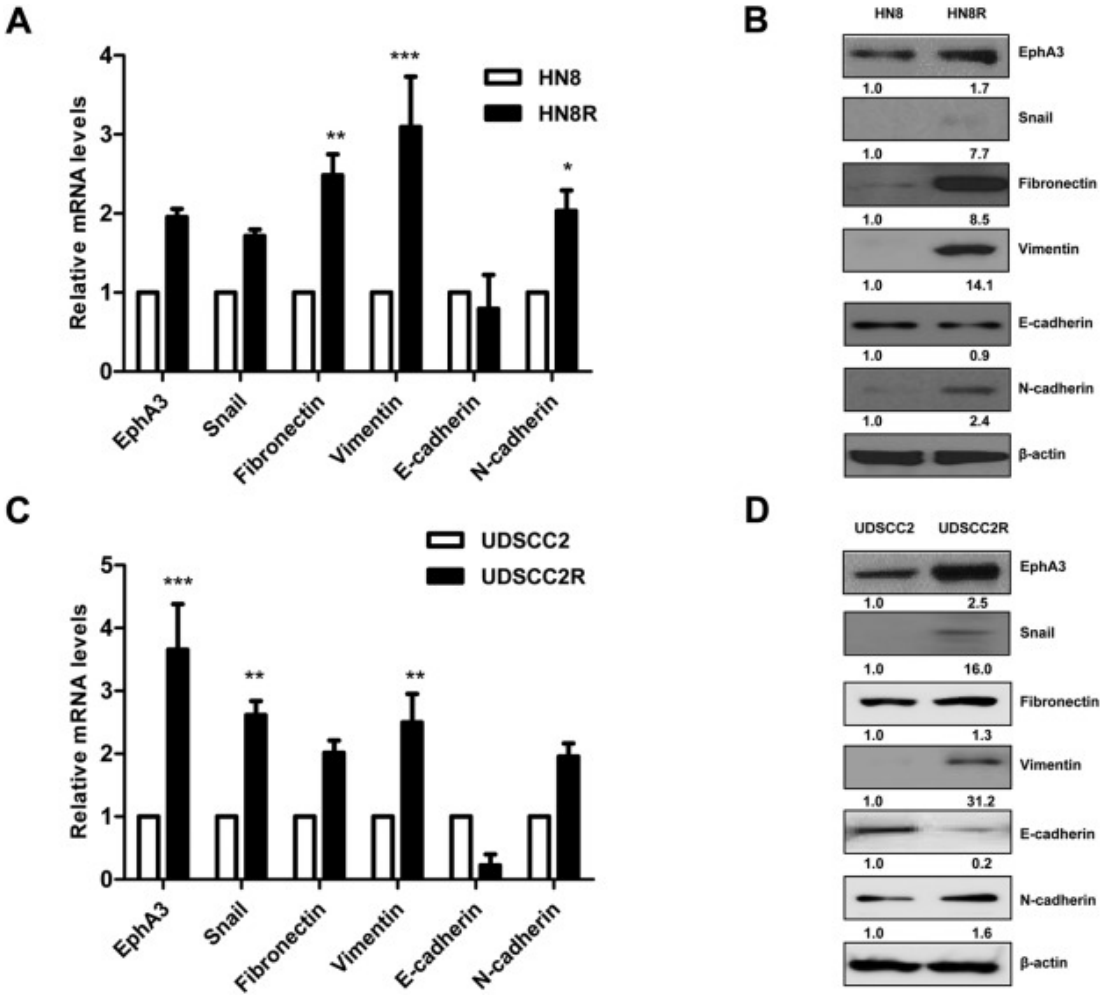
Supplementary Table 1. PCR primer sequence

EphA3	FW: GATGTTGGTGCTTGTGTTGC RV: GTGTCTGGAAACATAGCCAGATT
Snail	FW: CCCTCAAGATGCACATCCGAA RV: GACTCTTGGTGCTTGTGGAGCA
Fibronectin	FW: CTGGAACCGGGAACCGAATATA RV: TTCTTGTCCTACATTCGGCGG
Vimentin	FW: GGTGGACCAGCTAACCAACGA RV: TCAAGGTCAAGACGTGCCAGA
E-cadherin	FW: GAGTGCCAACTGGACCATTCAGTA RV: AGTCACCCACCTCTAAGGCCATC
N-cadherin	FW: TGCGGTACAGTGTAACTGGG RV: GAAACCGGGCTATCTGCTCG
β-actin	FW: CACACTGTGCCCATCTACGA RV: GTGGTGGTGAAGCTGTAGCC


A
EphA3 Negative
EphA3 Positive
B
PreRT
PostRT
CASE 1
CASE 2
CASE 3
CASE 4
CASE 5

Supplementary Fig. 1. EphA3 expression was evaluated in laryngeal cancer.

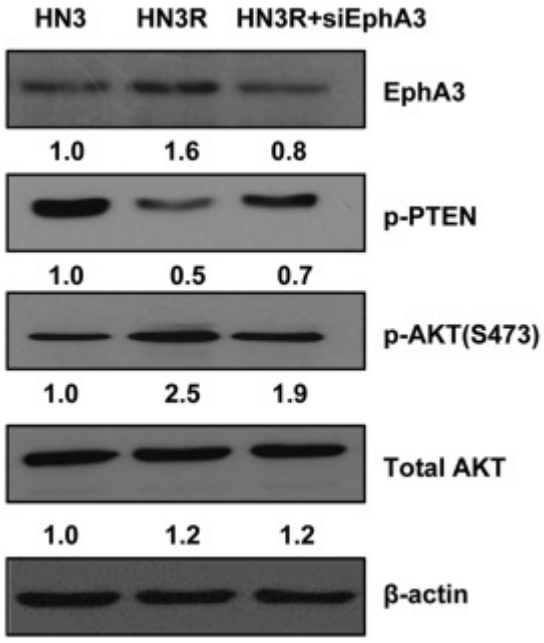
(A) EphA3 expression was evaluated in laryngeal cancer specimen using tissue microarray (×400). (B) The pretreatment specimens and the specimens of recurred tumors after radiation were shown in five patients



Supplementary Fig. 2. EphA3 regulates EMT pathway protein in various radioresistant head and neck cancer cell lines.

(A) The mRNA expression of Snail, fibronection, vimentin, E-cadherin and N-cadherin was assessed using Real time PCR in the AMC HN8 and AMC HN 8R cell line. (B) The difference in the protein levels of Snail, fibronection, vimentin, E-cadherin and N-cadherin with EphA3 expression were confirmed via western blotting in AMC HN8 and AMC HN8R cell line. (C) The mRNA expression of Snail, fibronection, vimentin, E-cadherin and N-cadherin was assessed using Real time PCR in the UDSCC2 and UDSCC2R cell line. (D) The difference in the protein levels of Snail, fibronection, vimentin, E-cadherin and N-cadherin with EphA3 expression were confirmed via

western blotting in UDSCC2 and UDSCC2R cell line. *, $P < 0.01$; **, $P < 0.005$; ***, $P < 0.0001$. All experiments were carried out >3 times. The survival fraction was presented as the mean of three experiments and the standard error was indicated. AMC was omitted in AMC HN cell lines for simplicity in figures.



Supplementary Fig. 3. EphA3 can regulate the EMT through PTEN signaling pathway.

The protein expression level of phospho-PTEN, phospho-Akt, total Akt was assessed after silencing of EphA3 in AMC HN3R cell line using western blotting.

Discussion

Radiation exposure induces numerous cellular signaling pathways, which can lead to cellular responses including apoptosis, cellular senescence, and cell cycle checkpoint activation/DNA repair [33]. Among the radiation-induced pro-survival signaling pathways, some are involved in inducing cell cycle arrest, induction of EMT, and promoting DNA repair, while others are engaged in suppressing apoptosis induction [6,33 - 39]. These pathways act synergistically to protect cancer cells from the cytotoxic effects of radiation, ultimately leading to the development of radioresistance. To overcome these problems, determining the underlying molecular mechanisms associated with radiation-induced EMT would be helpful for predicting solid cancer relapse and would greatly improve the therapeutic approaches for this disease [6,37 - 39]. Recent studies suggested that EMT plays a crucial role in the development of cancer radioresistance.

In the present study, we found that the EphA3 receptor could be an attractive molecular target for radioresistant head and neck cancer. Our findings indicate that EphA3 generates the EMT-like phenotype observed in the progeny of irradiation survivor head and neck cancer cells and has the potential to make the remaining cells in head and neck cancer patients more malignant after radiation therapy. The acquisition of such a phenotype may contribute to therapeutic failure after radiotherapy in head and neck cancer patients, the metastatic lesions.

Based on these findings, we hypothesized that inhibition of this pathway would overcome the radioresistance phenotype of EphA3 overexpressing cells. Thus, we investigated whether EphA3 inhibition can modulate radiation sensitivity. We found that inhibition of EphA3 significantly enhanced the efficiency of radiotherapy in radioresistant head and neck cancer cell by inhibiting the EMT pathway. This is the first study to associate EphA3 with

the radioresponse; translating these findings into the clinical setting may lead to novel treatment strategies. Studies focusing on the development of EphA3 inhibitors such as those conducted by Ferluga et al. [40] and Vail et al. [19] can be important for testing the effect of EphA3 blockage in the clinical setting.

Recent articles have indicated that EPH receptors crosstalk with EMT-related signal transduction pathways such as those induced by nuclear factor-κB and phosphoinositide 3-kinase (PI3K) [41]. EphA3 is involved in regulating the multidrug resistance of small cell lung cancer via PI3K/BMX/STAT3 signaling [24]. And EphA7 have an important role in the pathogenesis of NSCLC by regulating PTEN expression via the PTEN/AKT pathway [31]. However, the role of EphA3 and its relationship with PETN/Akt/EMT pathway in radioresistance remains unclear. We investigated the effects of EphA3 knockdown on the activity of the PTEN/AkT/EMT signaling pathway in AMC HN3R cells. When EphA3 was inhibited with siRNA, Akt activation was decreased with increased PTEN expression in radioresistant cell line. These results indicate that EphA3 may regulate the EMT through PTEN/Akt signaling pathway but, further studies are needed to analyze the exact association in the signaling pathway, additionally.

Conclusions

In conclusion, our results suggest that EphA3 is overexpressed in radioresistant head and neck cancer and plays a crucial role in the development of radioresistance in head and neck cancers by regulating the epithelial mesenchymal transition pathway. A greater understanding of the resistance mechanisms determined in this study willIn conclusion, our results suggest that EphA3 is over expressed in enable the rational design of combination regimens and sequential treatment algorithms for improving clinical outcomes. Further studies will help to fully clarify the potential role of EphA3 in cancer treatment. Moreover, identification of accurate biomarkers in patients who are unlikely to respond to radiation therapy may promote the development of rational drug combinations that will overcome this problem.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2018.04.001.

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국문요약

두경부암은 조기 발견 시 완치률이 매우 높지만, 일반적으로 발견이 늦고 수술이나 방사선치료로 인한 기능장애를 유발 할 수 있기 때문에 예후가 좋지 않은 질환 중 하나이다. 두경부암의 치료 방법에는 암의 종류와 발생 위치, 병기에 따라 수술적 방법, 항암화학요법, 방사선치료가 있는데 그 중 방사선치료는 암환자를 대상으로 기관보존 (organ preservation)과 기능 보존을 위하여 현재 부각 되고 있는 중요한 치료법이다.

방사선 치료 후 일부 환자에서 재발 및 전이를 일으킬 수 있다. 방사선치료로 발생하는 환자의 방사선 저항성 획득은 치료 실패 및 재발암의 주요 원인으로 부각 될 수 있다. 방사선 저항은 암치료의 가장 중요한 실패요인이고, 이러한 방사선 저항성은 극복해야 할 과제이고, 효과적인 방사선 감수제 (radiosensitizer)의 개발이 활발히 진행되고 있다.

Eph 수용체는 thyrosine kinaes receptor 의 가장 큰 서브 패밀리를 구성하고, ephrin으로 알려진 세포막 결합된 리간드와 상호작용한다. Eph-ephrin 의 결합은 세포의 부착과 분리, 세포 모양과 운동성, epthelial-to-mesenchymal transition (EMT)과 기능을 한다. 또한 Eph receptor의 기능은 종양발달, 세포와 세포의 부착, 침윤, 신혈관 생성, 종양 성장 및 전이를 조절한다. Eph 수용체 과발현은 다양한 범위의 종양에서 발생하며, 예후와 관련이 있다. 최근의 연구에 따르면 EphA3가 다양한 범위의 암에서 과발현되고 새로운 taget therapy 잠재성이 있음이 입증되었다. 그러나 방사선 저항성에서의 EphA3의 역할은 불분명하다.

본 연구에서는 방사선 저항성 두경부암세포주(AMC HN3R cell line)를 확립하고, EphA3가 방사선 저항성 두경부암에서 과발현되고 EMT (Epithelial mesenchymal transition) pathway를 조절함으로써 두경부암에서 방사선 저항성 발달에 중요한 역할을 함을 발견하였고, 이를 통해 방사선 저항성을 극복할 수 있는 방안이 될 수 있음을 시사되었다.

중심단어: 두경부암, EphA3, 방사선 저항, 방사선 감수성,
epthelial-to-mesenchymal transition (EMT)