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# EphA3 maintains radioresistance in head and neck cancers through epithelial mesenchymal transition

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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.

## 1. 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 a direct proinvasive effect of radiation treatment on tumor cells [8].

The Eph receptors constitute the largest subfamily of receptor tyrosine kinases, and interact with cell membrane bound ligands, known

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as ephrins. Eph-ephrin mediated cell communication controls biological 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.

## 2. Materials and methods

### 2.1. 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 100 μg/mL of penicillin/streptomycin and incubated at 37 °C with 5% $CO_2$ in a humidified incubator. Head and neck cancer cells were grown to approximately 50% confluence 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 2 Gy or 4 Gy. Cell lines were kept in continuous culture for < 10 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 (2 Gy) at 2-day intervals were successively delivered. After receiving a cumulative dose of 70 Gy, 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.

### 2.2. 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, 300 ng of total RNA from each sample was converted to double-strand cDNA using a random hexamer incorporating a T7 promoter; amplified 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).

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

### 2.4. RNA interference

At 24 h before transfection, AMC HN3R cells were plated into 6-well plates ($1 \times 10^5$ cells per well). Cells were transfected with 30 nM 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 72 h prior to western blot analysis.

### 2.5. 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 1 μg 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 > 3 times. The level mRNA expression was presented as the mean of three experiments and the standard error was indicated.

### 2.6. Western blot analysis

Total protein was extracted using PRO-PREP protein extraction solution (Intron Biotechnology). Protein concentrations were determined using the Pierce™ 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) and β-actin (sc-47778), which were purchased from Cell Signaling Technology (Danvers, MA, USA) and horseradish peroxidase-conjugated secondary antibodies, followed by detection with a SuperSignal