ANTICANCER RESEARCH *40*: 2627-2635 (2020)
doi:10.21873/anticanres.14233

Periostin Plays a Key Role in Radioresistance of Head and Neck Cancer Cells *Via* Epithelial-to-Mesenchymal Transition

JUNG JE PARK[1*], YOUNG-SOOL HAH[2*], SOMI RYU[1], SO YOUNG CHEON[1], HEE YOUNG CHO[2], JIN PYEONG KIM[1], SEONG JUN WON[1], JONG SIL LEE[3], JEONG SEOK HWA[3], JI HYUN SEO[3], HYO WON CHANG[4] and SANG YOON KIM[4]

[1]*Department of Otolaryngology, Institute of Health Sciences, College of Medicine, Gyeongsang National University, Gyeongsang National University Hospital, Jinju, Republic of Korea;*
[2]*Biomedical Research Institute, Gyeongsang National University Hospital, Jinju, Republic of Korea;*
[3]*Department of Pathology, Urology and Pediatrics, Gyeongsang National University, Jinju, Republic of Korea;*
[4]*Department of Otolaryngology, Asan Medical Center, College of Medicine, University of Ulsan, Seoul, Republic of Korea*

Abstract. *Background/Aim: Head and neck squamous cell carcinoma (HNSCC) is an aggressive head and neck malignancy. The aim of this study was to elucidate the role of periostin (POSTN) in the epithelial-to-mesenchymal transition (EMT) process mediating the acquisition of radioresistance in HNSCC. Materials and Methods: The expression levels of EMT hallmark genes including POSTN and Erk/Akt signaling pathways were compared between radiosensitive and radioresistant HNSCC cells. Results: POSTN mRNA expression was higher in radioresistant HNSCC cells, and silencing POSTN significantly impaired their invasiveness under the effect of EMT process represented by up-regulation of mesenchymal markers and down-regulation of an epithelial marker. Expression levels of Erk and Akt were higher in radioresistant cells. Conclusion: POSTN in association with the Erk and Akt signaling pathways was up-regulated during the EMT process, leading to the conversion of radiosensitive to radioresistant HNSCC cells. POSTN may be a key marker for predicting the radioresistance and therapeutic target of HNSCC.*

*These Authors contributed equally to this work.

Correspondence to: Sang Yoon Kim, Department of Otolaryngology, Asan Medical Center, College of Medicine, University of Ulsan, 388-1 Pungnap-dong, Songpa-gu, Seoul 138-736, Republic of Korea. Tel: +82 230103715, Fax: +82 24892773, e-mail: sykim2@amc.seoul.kr

Key Words: Periostin, radioresistance, head and neck cancer, epithelial-to-mesenchyma transition.

Head and neck cancer is one of the most common cancer types worldwide, and squamous cell carcinoma accounts for more than 90% of all head and neck malignancies (1-2). Head and neck squamous cell carcinomas (HNSCCs) are a heterogeneous group of biologically aggressive malignancies, and effective treatments for this cancer remain to be identified (3, 4). Although various modalities, including immunotherapy and targeted therapy, have been suggested for the treatment of HNSCCs, surgery combined with radiation therapy or concurrent chemoradiotherapy remain the standard of care (5). Radiotherapy plays a critical role in advanced stage HNSCC, for which surgery alone is not sufficient (6). Local control and a strong tumoricidal effect from radiotherapy are mainly achieved through the induction of DNA double-strand breaks in the dividing cells of the patients (7, 8). However, despite the toxicity of ionizing radiation, certain patients with HNSCCs treated with radiotherapy show local recurrence or distant metastases to other organs. Increasing evidence suggests that crosstalk between cancer cells and the surrounding stroma through the extracellular matrix (ECM) plays an important role in cancer cell invasion and metastasis (9, 10).

Epithelial-to-mesenchymal transition (EMT) is involved in embryonic development and tissue generation in healthy cells in cooperation with the ECM; however, this complex developmental program also leads to the loss of epithelial features of malignant cells and the acquisition of mesenchymal properties (11, 12). Although a comprehensive understanding of the molecular and biochemical mechanisms underlying the role of ECM components in cancer cell invasiveness is still lacking, the effect of EMT on cancer cells with stem cell properties leading to tumor progression and resistance against cancer treatment have been reported (13, 14). Various factors are involved in EMT-mediated cancer cell invasiveness. Positive regulation of matrix metalloproteases (MMPs) and

various EMT markers, such as cell surface proteins (N-cadherin), cytoskeletal markers (α-SMA and vimentin), ECM proteins [periostin (POSTN) and fibronectin], transcriptional factors (Snail and ZEB), and growth factors (TGF-β) occurs concomitant with the down-regulation of E-cadherin expression (15, 16). Cadherin exchange, which is a shift from the epithelial marker E-cadherin to the mesenchymal marker N-cadherin, occurs during the EMT process (17, 18). Demonstrating up-regulation of mesenchymal markers as well as down-regulation of epithelia marker mentioned above is essential to verify the involvement EMT process in cancer development.

Among many molecules related to the EMT process, POSTN is associated with a wide range of human tumors, such as colon, lung, breast, ovarian, prostate, and head and neck cancer (19, 20). The ECM protein POSTN, also called osteoblast-specific factor 2, is a secretory ECM protein that belongs to the superfamily of TGF-β-inducible proteins (21). It promotes integrin-mediated cell adhesion and motility in normal healthy tissues, such as bone, tooth, heart, uterus, and breast, whereas deregulation of POSTN expression affects the invasive and metastatic processes occurring during cancer progression (22, 23). The pro-tumorigenic effect of POSTN is mediated by binding to integrin, which activates intracellular signaling pathways and affects the general organization of the ECM (24).

Since POSTN overexpression is associated with radioresistance and chemoresistance (9, 25), targeting this protein may be a promising therapeutic option in certain types of cancer in which radiotherapy is an important curative modality, such as in head and neck cancer. Some reports have addressed POSTN-related radioresistance in breast cancer in association with integrin receptors or the phosphoinositide 3-kinase/AKT pathway, but the detailed underlying mechanisms remain unclear (26).

In this study, we compared POSTN expression levels between radiosensitive and radioresistant HNSCC cell lines and explored the possible underlying mechanisms, the Erk and Akt signaling pathways. It was shown that POSTN might modulate EMT by inducing ERK and Akt phosphorylation in radioresistant HNSCC cells. POSTN was knocked down using lentivirus-mediated short hairpin RNA (shRNA) to examine its role in EMT and in regulating the invasiveness of radioresistant HNSCC cells. A better understanding of the role of POSTN in promoting cancer invasiveness and radioresistance may lead to the development of novel therapies.

Materials and Methods

Cell culture and establishment of radioresistant HNSCC cell lines. The HNSCC cell lines HN3, UMSCC1, and UMSCC4 were used in this study. Cancer cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific) and 1% penicillin/streptomycin (Thermo Fisher Scientific), and were incubated at 37˚C with 5% CO_2 in a humidified incubator. Isogenic radioresistant cancer cells were established by performing serial fractionated irradiation (Figure 1). Cells grown to 50% confluency were exposed to 2 Gy of X-ray radiation generated using an X-RAD 320 irradiator (Precision X-Ray, Inc., North Branford, CT, USA). When the cells reached 70-80% confluence, they were subcultured into a new dish and irradiated with 2 Gy doses up to a cumulative dose of 70 Gy. The progeny of radioresistant cells at passages 3-7 was used.

Invasion assay. Invasion assays were performed in 24-well Transwell plates (Costar, Cambridge, MA, USA) with polycarbonate filters (8 μm pore size). Transwell plates used for invasion assays were coated with a uniform layer of BD Matrigel™ Basement Membrane Matrix (BD Biosciences, Bedford, MA, USA). The cell lines were resuspended in DMEM containing 10% FBS, seeded into the upper wells (1×10^5 cells/well), and incubated at 37˚C for 8 or 16 h. Invaded cells were fixed in 4% paraformaldehyde, stained with DAPI (D9542, Sigma-Aldrich), and manually counted under the fluorescent microscope (Nikon INTENSILIGHT C-HGFI, NIS-Elements BR 3.2) at ×100 magnification in five random fields.

Short hairpin (sh) RNA-mediated silencing of POSTN. For shRNA-mediated POSTN silencing, glycerol stocks of bacteria containing POSTN-targeting shRNA plasmid DNA (MISSION shRNA) and a non-targeting control plasmid DNA (SHC002) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lentivirus particles were used to deliver and express shRNAs against human POSTN and a non-target scrambled shRNA as the control. Lentivirus particles were generated by co-transfection of POSTN-targeting shRNA plasmids or the non-targeting control shRNA plasmid along with MISSION Lentiviral Packaging Mix (SHP001; Sigma-Aldrich) into 293FT cells (Thermo Fisher Scientific) using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's recommendations. Cell culture supernatants containing lentivirus particles were collected at 48 and 72 h post-transfection, filtered, and used to infect the HNSCC cell lines. The efficacy of each shRNA for knocking down POSTN expression was evaluated by western blot analysis of whole cell extracts.

Real-time quantitative polymerase chain reaction (qPCR). RNA was extracted from each cell preparation at the indicated times. cDNA synthesis was performed with the first-strand cDNA synthesis kit (Applied Biosystems, Framingham, MA, USA). Taqman probes used in this study (α-SMA Cat #Hs 00426835, E-cadherin Cat #Hs02758991-g1, POSTN Cat #Hs02758991-g1, Snail Cat #Hs02758991-g1, and GAPDH Cat #Hs02758991-g1) were obtained commercially (TaqMan® Gene Expression Assay, Applied Biosystems) and amplified using a kit following the manufacturer's instructions (TaqMan® Gene Expression Master Mix, Applied Biosystems). Amplification was performed as follows: i) 50˚C for 2 min, 95˚C for 10 min, ii) followed by 40 cycles of 94˚C for 15 s and iii) 60˚C for 1 min in 96-well plates using the ViiA™ 7 Real-Time PCR System (Applied Biosystems). The data were normalized to GAPDH. All experiments were independently performed at least three times.

Western blot analysis. Cell extracts were prepared using the RIPA lysis buffer (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) supplemented with a protease inhibitor and a phosphatase inhibitor

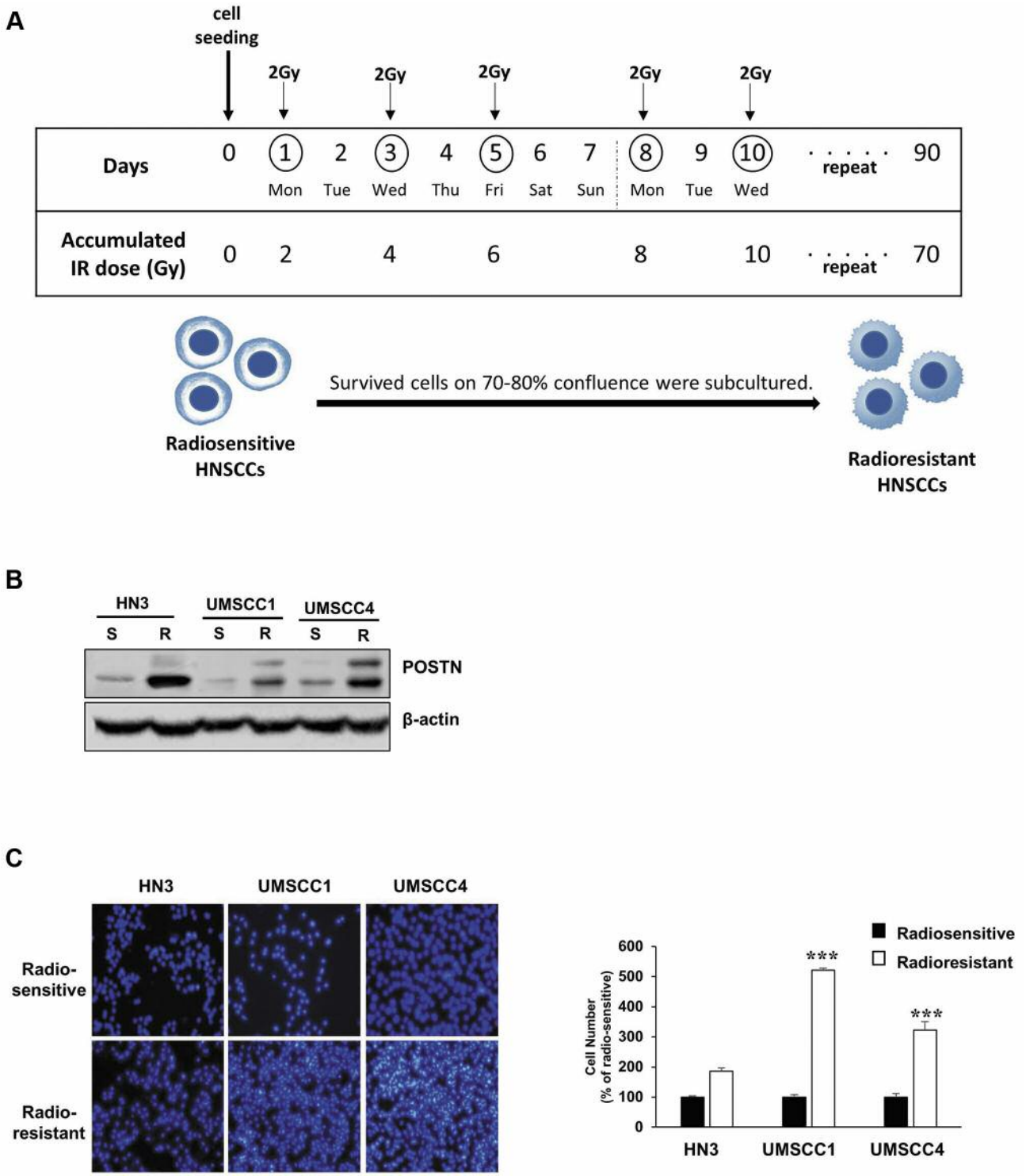

Figure 1. *Schematic representation of the isolation of radioresistant head and neck squamous cell carcinoma cell lines and up-regulated expression levels of Periostin (POSTN) in the radioresistant cell lines. (A) Isogenic radioresistant cancer cells were established by exposing cells to serial fractionated irradiation on Monday, Wednesday, and Friday for 90 days. (B) POSTN expression levels were assessed by western blotting in various parental cell lines and radioresistant head and neck cancer cell lines. β-actin was used as the loading control. (C) Invasion through Matrigel-coated Transwell plates was assessed after 16 h, and nuclei were stained with DAPI (blue). The invaded cells were photographed using Nikon NIS Elements software (original magnification, ×200). Cells were counted and normalized to control cells. The mean±SD was calculated. ***p-value<0.001 compared to control cells using Student's t-test.*

cocktail (Thermo Fisher Scientific). Cells were sonicated for 2 min and centrifuged at 12,000 g for 10 min at 4˚C to remove insoluble cell debris. Protein concentration in cell lysates was determined with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). A total of 50 μg protein from each cell preparation was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA). After blocking with 5% nonfat dry milk, membranes were incubated with primary antibodies against Snail (Abcam), slug (Abcam), E-cadherin (Santa Cruz Biotechnology, Inc.), vimentin (Santa Cruz Biotechnology, Inc.), POSTN (Santa Cruz Biotechnology, Inc.), MMP-2 (Santa Cruz Biotechnology, Inc.), MMP-9 (Abgent), Akt, phospho-Akt, Erk1/2, and phospho-Erk1/2 (Cell Signaling Technology, Danvers, MA, USA). The blots were subsequently incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin (Ig) G or anti-mouse IgG (Cell Signaling Technology, Inc.). Signals were visualized using the Supersignal Chemiluminescent Substrate (Pierce). Images were acquired with the ChemiDoc Touch Imaging System (Bio-Rad, Hercules, CA, USA). β-actin was used as a loading control in the stripped blot.

Immunocytochemistry. UMSCC4 and UMSCC4R cells were seeded on cover slips at a density of 5×10^4 cells. Cells were fixed with 4% (w/v) paraformaldehyde for 15 min, and permeabilized with phosphate-buffered saline containing 0.2% Triton X-100 for 5 min at room temperature. Then, cells were blocked with 1% bovine serum albumin in PBS for 30 min and incubated with primary antibodies against E-cadherin (cat. No. sc-21791; Santa Cruz Biotechnology, Inc.) and Snail (cat. No. NBP1-80022; Novus biologicals, Ltd.) for 2 h. After two additional washes, cells were further incubated with Alexa Fluor 488-conjugated goat anti-mouse lgG (cat. No. A-11001; Thermo Fisher Scientific, Inc.) and Alexa Fluor 594-conjugated goat anti-rabbit lgG (cat. No. A-11012; Thermo Fisher Scientific, Inc.) secondary antibodies for 45 min at room temperature, and mounted onto glass microscope slides with ProLong® Gold anti-fade mounting medium with DAPI (cat. No. P36941; Life Technologies). Fluorescent images were obtained for analyses at the confocal microscope system (FV1000, Olympus Life Science) with excitation/emission wavelength of 488/520 nm (Alexa Fluor 488) or 591/614 nm (Alexa Fluor 594).

Statistics. GraphPad Prism (version 5.01; GraphPad Software, San Diego, CA, USA) was used for statistical analysis. Data are shown as the mean±SD. Student's *t*-test and one-way ANOVA were used to analyze the differences between groups and among groups, respectively. p-Values<0.05 were considered significant.

Results

POSTN expression is higher in radioresistant than in radiosensitive HNSCC cells. To investigate the potential role of POSTN in HNSCC after irradiation, we established isogenic radioresistant cancer cell lines through exposure to cumulative irradiation (Figure 1A) and analyzed their POSTN profiles (Figures 1B-1C). As shown in Figure 1B, POSTN protein levels were significantly higher in the radioresistant head and neck cancer cell lines. Also, it was shown that invasive ability was higher in radioresistant than in radiosensitive head and neck cancer cells (Figure 1C).

POSTN plays a functional role in tumor cell invasion of radioresistant HNSCCs. To determine whether POSTN is involved in tumor cell invasiveness, invasive radioresistant head and neck cancer cells (UM1SCCR and UM4SCCR) were infected with shPOSTN or the control lentivirus shGFP. As shown in Figure 2A, shPOSTN strongly impaired the invasive activities of UM1SCCR and UM4SCCR cells. Also, marked down-regulation of POSTN expression in UMSCC1R and UMSCC4R expressing shPOSTN was shown in a time-dependent manner (Figure 2B).

Mesenchymal markers including POSTN are up-regulated while epithelia marker is down-regulated in radioresistant HNSCC cells. Involvement of EMT process in radioresistance of HNSCC was proved by showing up-regulation of mesenchymal markers and down-regulation of the epithelial marker E-cadherin. The results showed that radioresistant cells expressed higher levels of POSTN (approximately 5-fold and 13-fold in UM1SCCR and UM4SCCR cells, respectively) and Snail1 (approximately 4-fold in UM4SCCR cells), and lower levels of E-cadherin compared to what radiosensitive cells expressed (Figure 3A). α-SMA expression varied according to the type of cancer cell, with lower levels in UM1SCCR cells and higher levels in UM4SCCR cells. Western blot analysis showed that POSTN expression was markedly higher in UM1SCCR and UM4SCCR cells compared to the corresponding radiosensitive lines (Figure 3B). The epithelial marker E-cadherin was down-regulated in UM4SCCR cells, whereas the mesenchymal markers vimentin, ZEB1, Snail, and Snail2 were up-regulated. Confocal immunofluorescent images also revealed that Snail1 is up-regulated while E-cadherin is down-regulated in radioresistant cells (Figure 3C).

POSTN mediates the activation of the Akt and Erk pathways in radioresistant HNSCC. To identify the signaling mechanisms involved in POSTN-related EMT in radioresistant head and neck cancer cells, we assessed the expression of the phosphorylated forms of Erk and Akt because certain EMT markers, such as Snail are involved in the Erk and Akt pathways (27). The expression of Erk and Akt phosphorylated proteins was markedly higher in UM4SCCR cells compared to the parental lines (Figure 4A). Silencing POSTN with recombinant lentivirus expressing shPOSTN in UM4SCCR cells decreased the expression levels of phosphorylated Erk and Akt (Figure 4B). Also, the up-regulated expression levels of pErk and pAkt were dominant after 1h of POSTN treatment in UMSCC4R cells (Figure 4C).

Discussion

Because head and neck cancer involves various subpopulations of cells and multi-gene interactive processes,

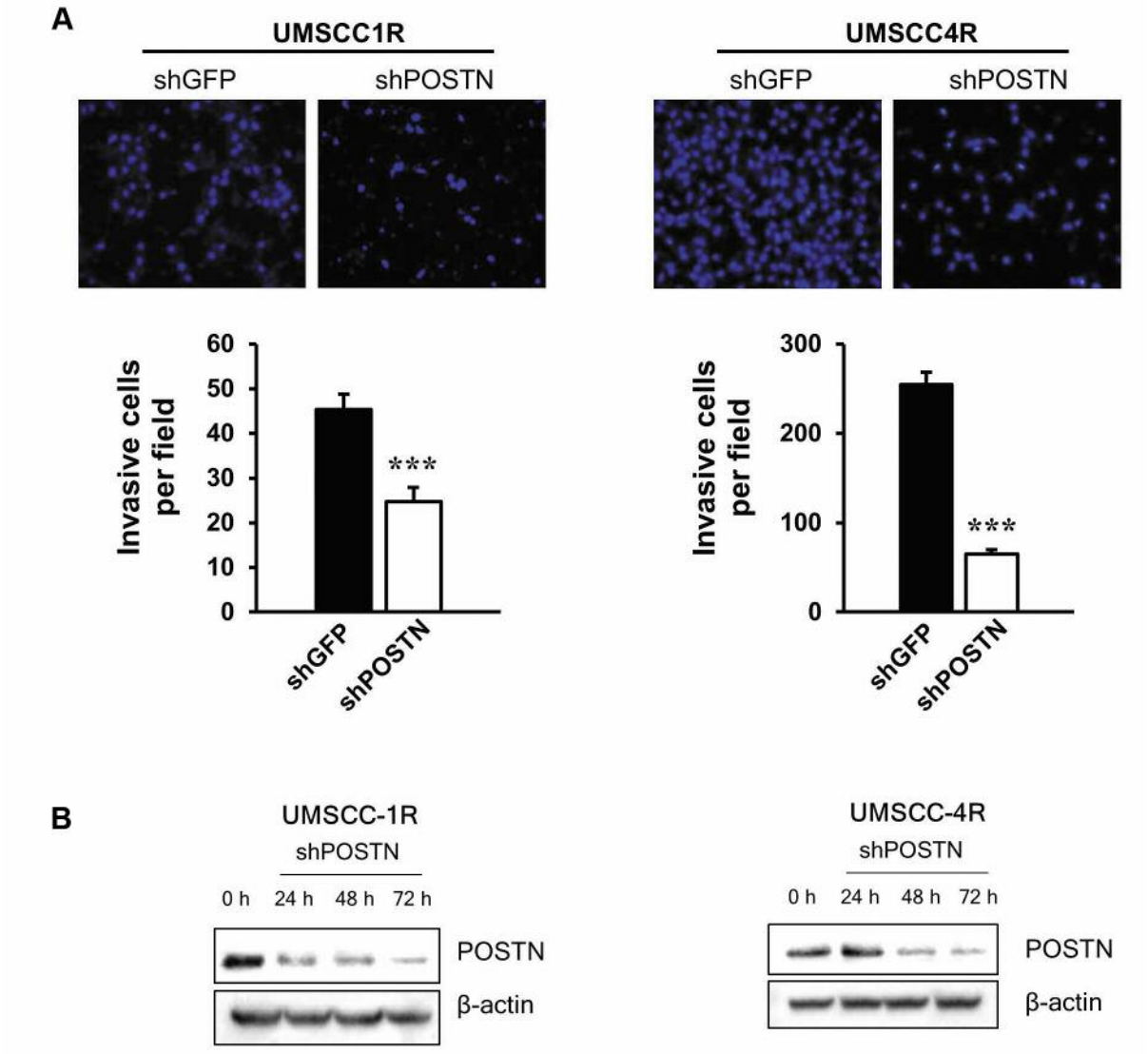

Figure 2. *Periostin knockdown suppresses invasion of radioresistant head and neck squamous cell carcinoma cell lines. (A) Invasion of UMSCC1R and UMSCC4R expressing shGFP and shPOSTN was analyzed by Matrigel invasion assays (magnification, x200). Quantitative data from two independent experiments performed in duplicates are shown. Results are presented as the mean±SD. ***p-Value<0.001 compared to shGFP control cells. (B) Time-dependent changes in POSTN expression levels of UMSCC1R and UMSCC4R expressing shPOSTN up to 72 h.*

not only within the tumor itself but also from the surrounding microenvironment, the efficacy of existing treatment modalities against HNSCC is limited. Although advances in surgery, radiotherapy, and chemotherapy have improved the control of localized cancer cells and overall survival, a high proportion of patients experience tumor relapse and metastasis associated with treatment failure. Radiotherapy is used as a primary treatment as well as an adjuvant to surgery in many cases of HNSCC, and improving our understanding of the molecular mechanisms of radioresistant cancer cells is critical, as is developing novel strategies to decrease radioresistance.

Close interactions between the tumor itself and the surrounding cellular environment are required for cancer growth and invasion (28). Among the wide range of components of the surrounding microenvironment, we identified POSTN as a potentially important molecule for radioresistance. POSTN is upregulated in various cancers including head and neck, colon, breast, and ovarian cancers; however, the role of POSTN in radioresistance of head and neck cancer cells has not been reported previously (28, 29). Cumulative findings showing the protumorigenic effect of POSTN led us to target POSTN as a key molecule involved in the conversion of HNSCC cells from a radiosensitive to a radioresistant state (28-30).

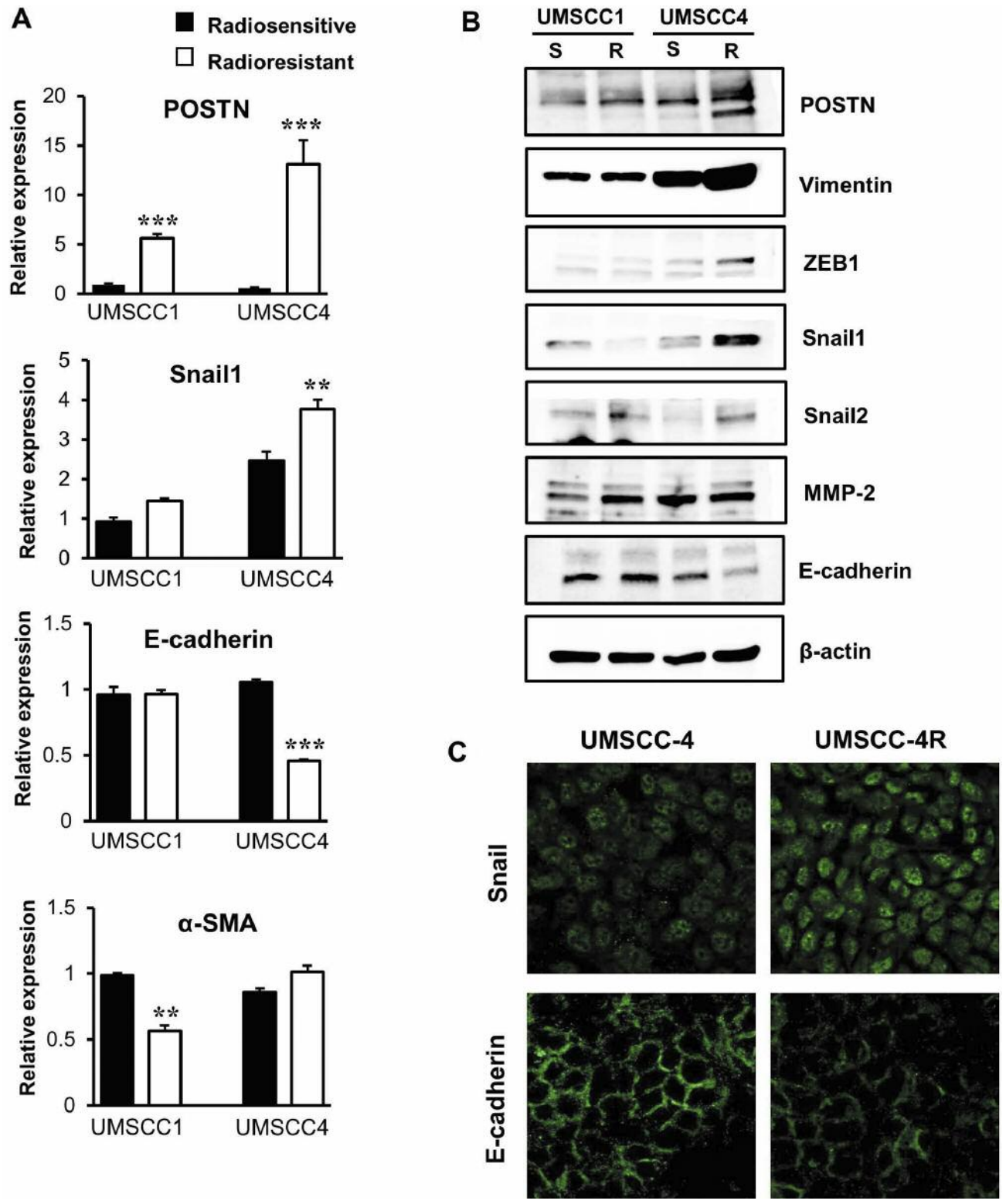

Figure 3. *Periostin regulates the expression of EMT hallmark genes. (A) Changes in the mRNA expressions levels of EMT-related genes were measured by qPCR. The expression levels of EMT-related genes were normalized to GAPDH. **p-Value<0.01, ***p-Value<0.001 compared to each parental cell control. (B) The levels of EMT-related biomarkers were detected by western blotting using antibodies against the indicated proteins. All experiments were performed at least in triplicates, and representative results are shown. (C) Fluorescent images showing up-regulated levels of Snail1 (nuclear; green) and down-regulated levels of E-cadherin (cell membrane; green)in radioresistant cells were obtained with confocal images (magnification ×800).*

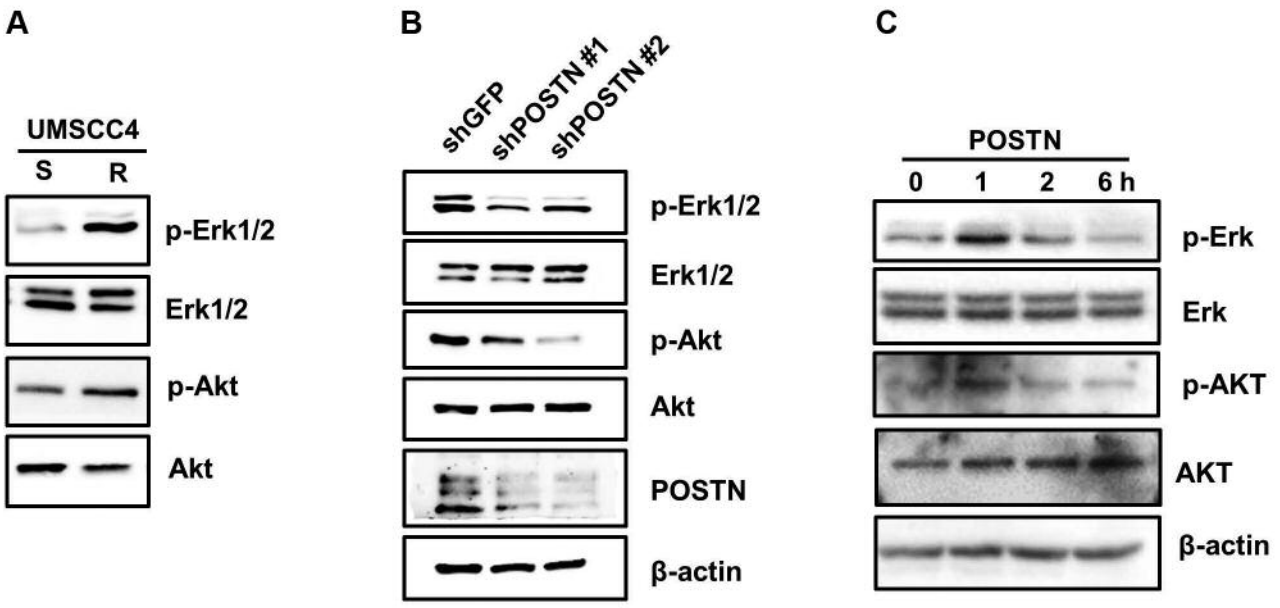

Figure 4. *Periostin mediates the activation of the Akt and Erk pathways in radioresistant head and neck squamous cell carcinoma lines. (A) The protein expression levels of phosphorylated Erk and Akt were compared between radioresistant and radiosensitive cells by western blotting (S=radiosensitive, R=radioresistant). (B) Western blot assays of Erk and Akt protein levels in UMSCC4R cells treated with shPOSTN. Data of two independent experiments performed in duplicates (#1 and #2) are shown. (C) Western blot assays of Erk and Akt protein levels in UMSCC4R cells after 1, 2, 6 h of POSTN treatment. Beta-actin was used as the loading control.*

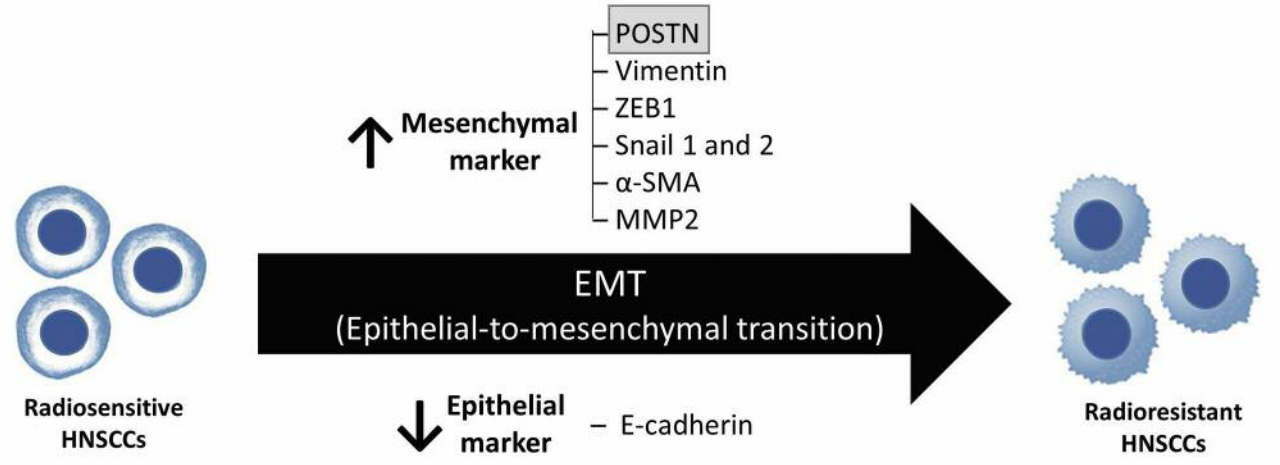

Figure 5. *Schematic overview of regulation of EMT hallmark genes in radioresistant HNSCC. EMT process represented by upregulation of POSTN and other mesenchymal markers (vimentin, ZEB1, Snail 1/2, α-SMA, and MMP2) and downregulation of epithelial markers, such as E-cadherin, plays a key role in radioresistance of HNSCC cells.*

The mRNA and protein expression levels of POSTN were higher in several radioresistant HNSCC cell lines compared to the radiosensitive ones, confirming that POSTN expression is involved in the resistance of cancer cells to radiotherapy. Knockdown of POSTN using shRNA technology in radioresistant malignant cells markedly suppressed their invasive activities. To assess the radioresistant characteristics of other ECM molecules, their expression levels were compared between radiosensitive and radioresistant HNSCC lines. Certain EMT hallmark genes that were tested, including Snail, vimentin, ZEB1, and MMP2 as well as POSTN, were overexpressed at the mRNA

and protein levels in radioresistant cells, whereas E-cadherin was downregulated. Lower expression of E-cadherin in radioresistant cells was consistent with the well-known concept of cadherin exchange, a shift from the epithelial marker E-cadherin to the mesenchymal marker N-cadherin that occurs during the EMT process (31).

Upregulated expression of POSTN activates the Erk and Akt/NF-κB signaling pathways. We showed that the expression levels of phosphorylated Erk and Akt were markedly increased in radioresistant cells. The protumorigenic effects of Erk and Akt/NF-κB have been widely demonstrated, and they are likely to play an important role in cancer cell resistance to radiotherapy similar to what has been observed during skin inflammation (32, 33). Because inactivation of the Erk and Akt pathways has generated a growing interest in cancer therapy, Erk/Akt inhibition may be used as part of a promising strategy for HNSCCs that recur after radiotherapy.

One limitation of this study is that not all the radioresistant HNSCC cells tested showed significantly higher expression levels of POSTN; therefore, further studies focusing on clinical practice, including *in vivo* studies, are required. In addition, detailed analyses of the molecular mechanisms by which POSTN is associated with tumor radioresistance besides the Erk and Akt/NF-κB pathways are needed.

Conflicts of Interest

There are no actual or potential conflicts of interest.

Authors' Contributions

Conceptualization was done by JJP, YSH and SYK. Data curation was done by SR, SYC, HYC and SJW. Formal analysis was done by JPK, JSL, JSH and JHS. Funding acquisition was done by JJP, YSH and JHS. Literature search was done by HWC, JJP, YSH, SYK and SR. SYC, SR, HYC and HWC performed molecular experiments. Supervision of the study was done by SYK. Validation was done by JJP and SYL. Writing, review and editing were done by JJP, SR, YSH, JSL, JSH, JHS and SYK.

Acknowledgements

This work was supported by the National Research Foundation of Korea, Grant Award Numbers: NRF-2020R1A2C1012038 and NRF-2015R1D1A1A01057644 and by the Biomedical Research Institute fund (GNUHBRIF-2017-0008, GNUHBRIF-2018-0012) from the Gyeongsang National University Hospital.

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Received March 11, 2020
Revised March 24, 2020
Accepted April 1, 2020