의학석사 학위논문

HPV 양성 두경부암에서 방사선 저항성 예측 바이오마커 개발: TP53 mutation 및 SOX2 역할 규명

TP53 MUTATION CONFERS RADIATION RESISTANCE IN HPV POSITIVE OPSCC THROUGH SOX2 ACTIVATION

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지 도 교 수 한 명 월

이 논문을 의학석사학위 논문으로 제출함

2022 년 8 월

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김태군의 의학석사학위 논문을 인준함

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Abstract

Background: HPV-positive OPSCC patients tend to be non/low-smokers and drinkers, as well as younger and overall healthier than their HPV-negative counterparts. Given the high survivorship in HPV-positive OPSCC, there is great interest in de-intensifying therapy. However, approximately 15% of HPV-positive OPSCC patients fail standard therapy. Robust biomarkers of radiation resistance would be highly useful to exclude high-risk patients from deintensification protocols and instead direct them towards intensified treatment. The aim of this study is to validate the molecular subgroups presenting radiation resistance and investigate the mechanism of tumor aggressiveness and radiation resistance in HPV-positive OPSCC

Methods: We introduced the TP53 mutation into two novel, treatment naive HPV-positive cell line. MTT assay, migration and invasion assay and clonogenic assay was performed to compare the tumor growth, aggressiveness and radiation resistance in both parental and TP53 mutation cell line in vitro. Microarray and western blot were performed to detect robust biomarkers in TP53 mutation HPV-positive cell line compared with parental cell line. We confirmed the in vitro data through in vivo study.

Results: TP53 mutation cell line with increased ability of migration and invasiveness showed resistance to radiation compared to TP53 wild HPV-positive cell line. We found that SOX2 was expressed predominantly in the TP53 mutation cell line on microarray and confirmed the findings through western blot. And we demonstrated that SOX2 mediated the tumor migration and invasiveness and radiation resistance in TP53 mutation cell lines. Inhibition of SOX2 enhanced radiation sensitivity in the TP53 mutation cell line in vitro and in vivo study.

Conclusions: SOX2 is overexpressed in TP53 mutation HPV-positive OPSCC and plays a crucial role in the development of radiation resistance by regulating tumor invasiveness.

Keywords: human papillomavirus-associated oropharyngeal cancer, TP53 mutation, *sex-determining region Y-box 2* (*SOX2*)

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INTRODUCTION

Head and neck squamous cell carcinomas (HNSCC) comprise squamous cell carcinoma of the oral cavity, nasal cavity, pharynx (oropharynx and hypopharynx), larynx, and tongue. It is now recognized that there are two types of HNSCC, the keratinized or nonkeratinized type. The former occurs most often in elderly males and is associated with smoking and alcohol consumption, but human papillomavirus (HPV) is not involved. Conversely, nonkeratinizing squamous cell carcinoma occurs most commonly at age 40–55 years in men with little exposure to tobacco and alcohol, and HPV DNA is detected as the most characteristic feature (1).

The overall incidence of HPV-negative oropharyngeal squamous cell carcinomas (HPV− OPSCC) has decreased, probably due to the reduction in smoking populations in most high-income countries over the past 20 years(2). However, the incidence of HPV-positive squamous cell carcinomas (HPV+ OPSCC) is expected to rise continuously over the coming decades until the benefits of gender-neutral prophylactic HPV vaccination begin to become manifest. The most remarkable clinical difference between of HPV+ and HPV− tumor is higher rate of response to radiation-based treatment and HPV+ OPSCC, which, identified through PCR, in situ hybridization or P16 immunohistochemistry on tumor tissues, show significantly improved overall and disease-free survival following the standard treatment compared to HPV− OPSCC patients(3).

While there are different staging systems for HPV+ and HPV− disease, current treatment strategies are the same for both entities. The standard of care for both subtypes of OPSCC is definitive radiation therapy (RT) and concurrent chemoradiation therapy (CRT), for lower and higher stage disease, respectively. However, traditional RT and CRT are associated with severe acute and late toxicities affecting patient quality of life, such as severe mucositis, dry mouth and dysphagia. Considering that HPV+ OPSCC commonly presents in a younger, healthier patient population compared to HPV− and the good

prognosis of HPV related disease, there has been a focus on reducing treatment toxicities and optimizing quality of life while maintaining favorable oncologic outcomes. Thus, de-intensification, or the reduction in some element of the cancer treatment regimen, is of great interest in HPV+ OPSCC. As a result, several de-intensification regimens have been proposed in selected patient populations(4).

However, despite the better prognosis compared to HPV− disease, 10~20% of those with HPV+ OPSCC still develop recurrent/metastatic(R/M) disease as the high-risk subgroup of HPV+ HNSCC(3, 5). Those with HPV+ HNSCC also have unique metastatic patterns, often with multiple organs involved and atypical sites, such as the bone and liver(6). Additionally, the median time to development of distant metastases following curative treatment is longer than HPV− HNSCC(7). Furthermore, metastatic disease development after 5 years in HPV+ HNSCC patients has been described(8). Therefore, identifying the patients with high risk of R/M disease and to exclude them from de-intensification protocols remains a key challenge (9).

In a subset of heavy smokers with HPV+ disease, tobacco smoking increases the frequency of p53 mutations in a dose-dependent manner and poor prognosis is observed in these patients(10, 11). The p53 protein is a transcription factor that functions as a tumor suppressor and is the most commonly mutated gene in several human cancers, including head and neck cancer.(9, 12). Most TP53 mutations in head and neck squamous cell carcinoma (HNSCCs) are missense mutations and frequently occur within the central region of the protein that serves as the p53 DNA-binding domain(13). As the standard treatments of HNSCC, including radiation therapy and chemotherapy, are usually effective against tumors with wild-type TP53, in vitro data reveal that complete inactivation of p53 in HNSCC cell lines induces resistance to radiation therapy and more aggressive tumor behavior(9, 12-16). Metachronous recurrent HPV+ OPSCCs shared a genomic landscape with HPV- HNCs, including a high frequency of *TP53* genomic alterations. Recent reports have demonstrated that TP53 mutations can indeed occur in HPV+ cancers and seem to be

overrepresented in tumors that failed conventional treatment. However, the mechanism underlying the radiation resistance in HPV+ OPSCC cells with TP53 mutations is unclear. Thus, we hypothesized that the introduction of a TP53 mutation into HPV+ OPSCC would lead to increased aggressiveness and radiation resistance and investigate the role of SOX2(Sex determining region Y-box 2) based on the findings in which SOX2 was overexpressed in TP53 mutation cell line.

MATERIALS AND METHODS

2.1 | Cell culture

Human papillomavirus-associated oropharyngeal cancer cell lines were used in this study. Three HPV- positive UWO23 (HPV-33; wild-type *TP53*), UWO37 (HPV-16; wild-type *TP53*), UWO8 (HPV-35; mutation *TP53*) cells were provided by Dr. Anthony C. Nichols, University of Western Ontario (UWO), London Health Sciences Center, under a material transfer agreement. HPV + cell lines were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Invitrogen) and 100 µg/mL penicillin/streptomycin and incubated at 37°C in a humidified incubator containing 5% CO_2 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, 4, 8 Gy.

2.2 | Microarray expression data analysis

Microarray hybridization was performed using the standard protocol provided by Affymetrix, Inc. (Santa Clara, CA, United States). Briefly, 5 µg of total RNA from each pCDNA3.1- Control vector or *TP53*-R282W cells were reverse transcribed into cDNA using a Superscript II Double-Strand cDNA synthesis kit (Invitrogen Life Technologies, Carlsbad, CA, United States) according to the manufacturer's instructions. (Affymetrix).

2.3 | *TP53* mutation RNA interference

Human full-length, missense mutation sequences of p53 (R282W) were cloned into the the *BamHI* sites of the expression pCDNA3.1-vector. (Addgene, Inc.). Constructed plasmids (5 µg for cells incubated in a 6-cm dish) with the indicated mutations were transfected into UWO23 (HPV-33; wild-type *TP53*) and UWO37 (HPV-16; wild-type *TP53*) cells at 70–80% confluence using Opti-MEM (Thermo Fisher Scientific, Inc.) without FBS for 4–6 h according to the manufacturers' instruction. pCDNA3.1- Control

vector or *TP53*-R282W stable cells were batch-selected using puromycin in the presence of 2 μg/ml (Sigma-Aldrich Co., St Louis, MO, USA) and expanded, and gene expression was confirmed by western blotting

At 24 h prior to transfection, cells were seeded onto six well (1 □ 10^5 cells/well) and transfected with Cells were transfected with 2 μg of the shSOX2 (shRNA; sc-38408) or control shRNA (sc-108060) using shRNA transfection reagent purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cells were selected with 2ug puromycin antibiotic (sc-108071) for 10 days. The protein levels of SOX2 were evaluated via western blotting analysis. Then these stably transfected cells were used in the following *in vivo* or *in vitro* experiments.

2.4 | Western blot analysis

Total protein was extracted using RIPA protein extraction solution (Intron Biotechnology, Daejeon, Korea). Protein concentrations were determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Equal amounts of protein were separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose transfer membranes (Whatman). The membranes were incubated with primary antibodies p-p53 (#9283), SOX2 (sc-365964) and β-actin (sc-47778), followed by treatment with horseradish peroxidase (HRP)-conjugated secondary antibodies, which were detected using a Super Signal West Pico Trial kit (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions. All western blot analyses were performed at least three and the representative figures were presented.

2.5 | Clonogenic assay

HPV + HNC cell lines were plated on a 6-cm dish at a density of 100, 500, 1,000, and 4,000 cells/dish. Briefly, cells were treated with different doses of irradiation (0, 2, 4, and 8 Gy, respectively). Irradiated cells were plated in duplicates in a 6-cm dish. After 14 days, the colonies were fixed with methanol and stained with crystal violet. Colonies containing > 50 cells were counted under a microscope. Not every cell seeded would form a colony, even in the absence of irradiation, owing to factors such as errors in counting, stress of manipulation, and suboptimal growth medium. The plating efficiency (PE) was defined as the number of colonies observed divided by the number of cells plated.

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

Parallel dishes were seeded with cells that were exposed to increasing doses of radiation. The number of cells plated was increased so that a countable number of colonies were produced. Surviving fraction (SF) was defined as the colony counted divided by the number of colonies plated, with a correction for plating efficiency.

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

2.6 | Invasion and migration assay

Migration and invasion were examined in a Transwell assay using a CytoSelect™ 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 shSOX2 or control shRNA. 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 560 nm 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

2.7 | Xenograft model

Xenografts were established in 5- to 6-week-old male athymic nude mice (*nu/nu*; Harlan-Sprague-Dawley) by a subcutaneous injection of $1 \square 10^7$ sh SOX2 or control shRNA in UWO23 TP53 Mutation cells. After the tumor volume reached 50 mm^3, mice were divided into six treatment groups as follows: (a) shRNA control, vehicle; (b) shRNA control + radiation only; (c) shRNA control 1 mg/kg cisplatin (*cis*-Diamine platinum(II) dichloride, CDDP) only (Intravenous injection twice a week for 3 weeks); and (d) shRNA control cisplatin + radiation (e) shSOX2 (f) shSOX2 + radiation. Treatment of all mice began 1 week after tumor cell injection unless otherwise specified and was carried out for 3 weeks with 5 to 6 mice per group. All mice were sacrificed on day 28 of treatment. Radiation was

delivered at 4 Gy using a 6 MV therapeutic linear accelerator (CLINAC EX: Varian, Palo Alto, CA) at a does rate of 2 Gy/min; a 1.0 cm bolus was used for radiation dose build-up. Tumor volume were calculated (V= [length × Width]^2/2). During drug treatment, the mouse body weight was measured. After deparaffinization and rehydration, 4-μm thick sections were subjected to heat-induced antigen retrieval using 0.01 M citrate buffer (pH 6.0) for 1 h. Sections were incubated in aqueous 3% hydrogen peroxide (H_2O_2) for 15 min to quench endogenous peroxidase activity and washed with 1× phosphate-buffered saline (PBS). Slides were loaded into a humid chamber and blocked for 30 min with 1× universal blocking agent (10× Power Block™: BioGenex, San Ramon, CA, USA) before overnight incubation at 4°C with primary antibodies against SOX2 (1:100; Sigma-Aldrich, Inc., St. Louis, MO, USA). The following day, slides were incubated for 1 hour at room temperature and treated with Envision Reagent (Dako REAL™ EnVision™, Glostrup, Denmark) for 30 minutes. Slides were washed with PBS and treated with the chromogen DAB for 15 minutes to allow formation of brown reaction product. The slides were counterstained with Mayer's hematoxylin, dehydrated in graded alcohol, cleared in xylene, and mounted. The slides were independently interpreted by two reviewers.

2.8 | Statistical analysis

Statistical analyses were conducted using one-way or two-way analysis of variance (ANOVA) to identify statistical difference. Differences with P values less than 0.05 were considered statistically significant and the P value of test was provided in the figure legends.

RESULTS

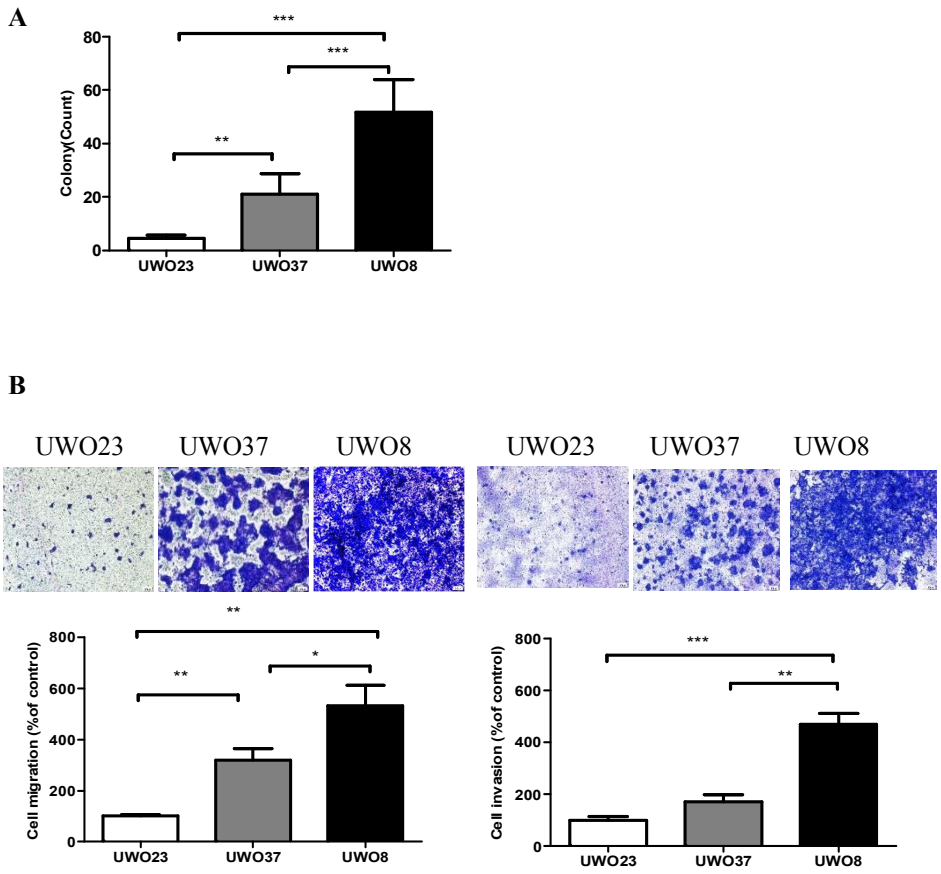
3.1 | TP53 mutation promotes cell tumorigenesis

At first, we compared the tumor aggressiveness between the natural TP53 mutation HPV + cell line and wild type cell line. TP53 mutant cell line UWO8 showed significant difference in colony formation compared with other wild type TP53 cell lines UWO23 and UWO37 ($P<0.001$, respectively) (Fig 1A). We next performed cell migration and invasion transwell assay to examine the effect on the cell migration and invasion capabilities of TP53 mutation. The results showed that TP53 mutation UWO8 cell line significantly enhanced cell migration and invasion compared to wild type TP53 cell line (Fig 1B). To confirm whether this observed correlation was associated with TP53 mutation, we used the same methods using wild type TP53 cell line with their corresponding TP53 mutant transfection cell line. Western blot analysis showed p53 protein expression increase in mutant transfected cell line $UWO23^{R248W}$ and $UWO37^{R248W}$ (Fig 1C). As expected, mutant TP53 cell line showed significant increase in colony count, cell migration and invasion rate compared to their corresponding wild type cell line (Fig 1D).

3.2 | TP53 mutation is associated with radiation-resistance in human papillomavirus-associated oropharyngeal cancer

To determine whether the OPSCC cells expressing mutant p53 were resistant to radiation, we compared the clonogenic survival of HPV+ cell lines following various doses of irradiation (0, 2, 4 and 8 Gy). Overall survival fraction decreased as radiation doses increased. UWO8 cells with mutant TP53 showed maximum resistance to radiation while both wild type TP53 cell lines UWO23 and UWO37 showed lower survival fraction (Fig 2A). Similarly, wild type TP53 cell lines and their mutant transfection cell lines were assessed, showing consistent results(Fig 2B).

Figure 1 TP53 mutation promotes cell tumorigenesis. (A) TP53 mutant cell line UWO8 showed significant increase of colony formation compared with other wild type TP53 cell lines UWO23 and UWO37 (B) Cell migration and invasion transwell assay to examine the effect on the cell migration and invasion capabilities of TP53 mutation. The results showed that TP53 mutation significantly enhanced cell migration and invasion compared to wild type TP53 cell line. (C) Western blot analysis showed p53 protein expression increase in mutant transfected cell line UWO23^{R248W} and UWO37^{R248W}. (D) As expected, mutant TP53 cell line showed significant increase in colony count, cell migration and invasion rate compared to their corresponding wild type cell line. **, P < 0.01; **, P < 0.005; ***, P < 0.0001. All experiments were carried out more than three times. The expression of the protein was quantified and compared with that of GAPDH by western blot analysis.*



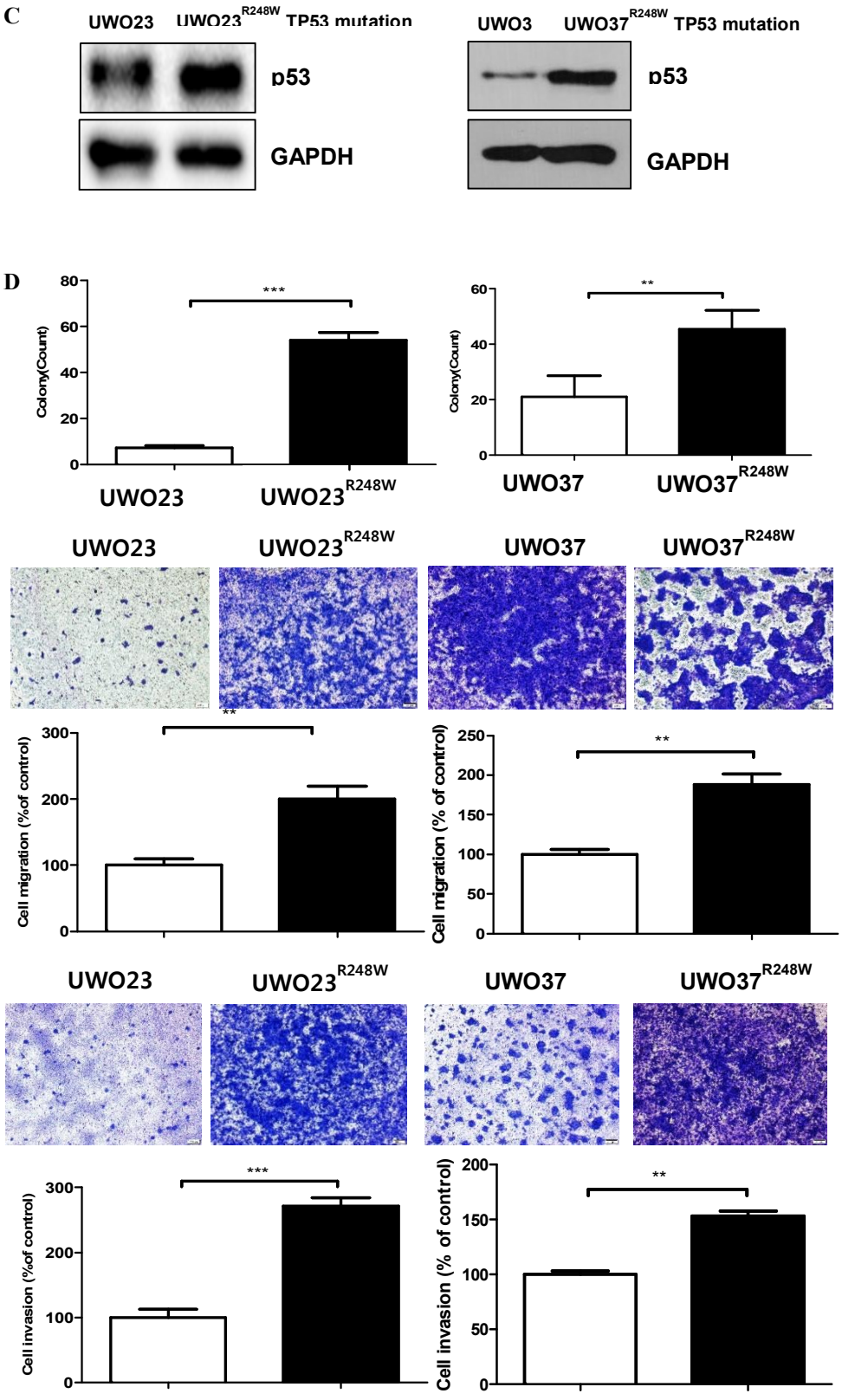
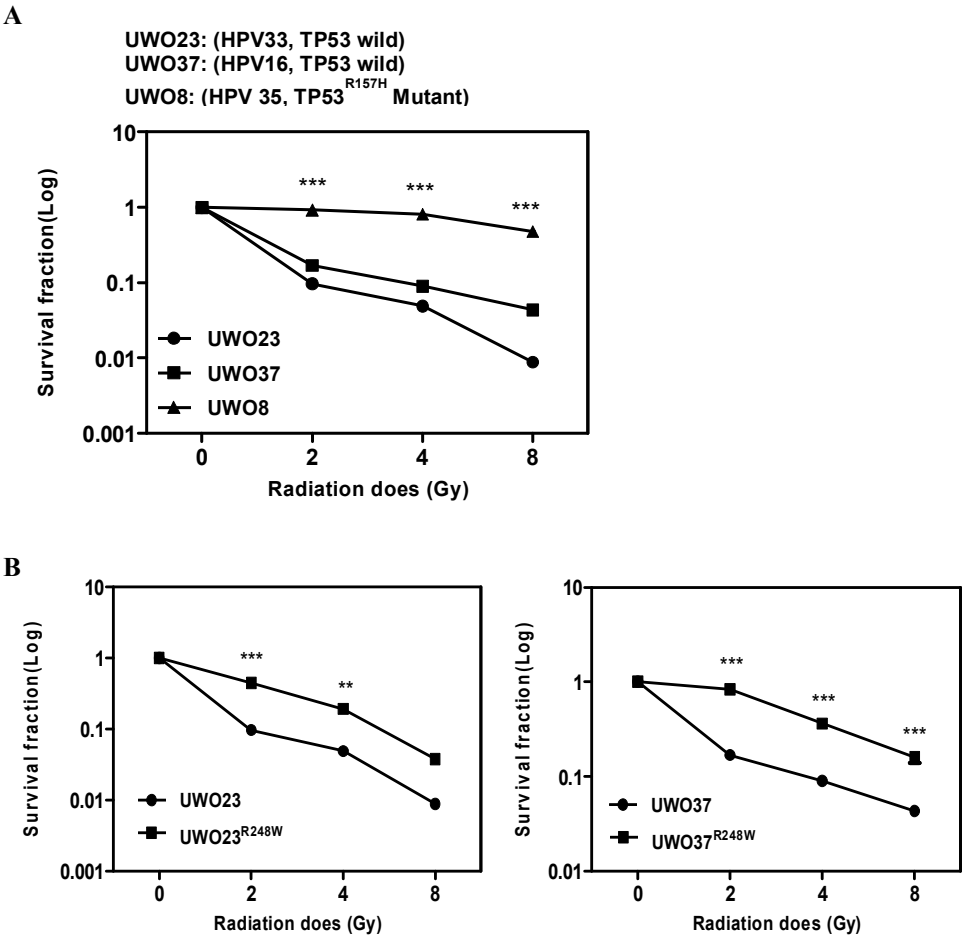

C
UWO23
UWO23R248W
TP53 mutation
p53
GAPDH
UWO3
UWO37R248W
TP53 mutation
p53
GAPDH
D
Colony(Count)
UWO23
UWO23R248W
UWO37
UWO37R248W
Cell migration (%of control)
Cell migration (% of control)
Cell invasion (%of control)
Cell invasion (% of control)

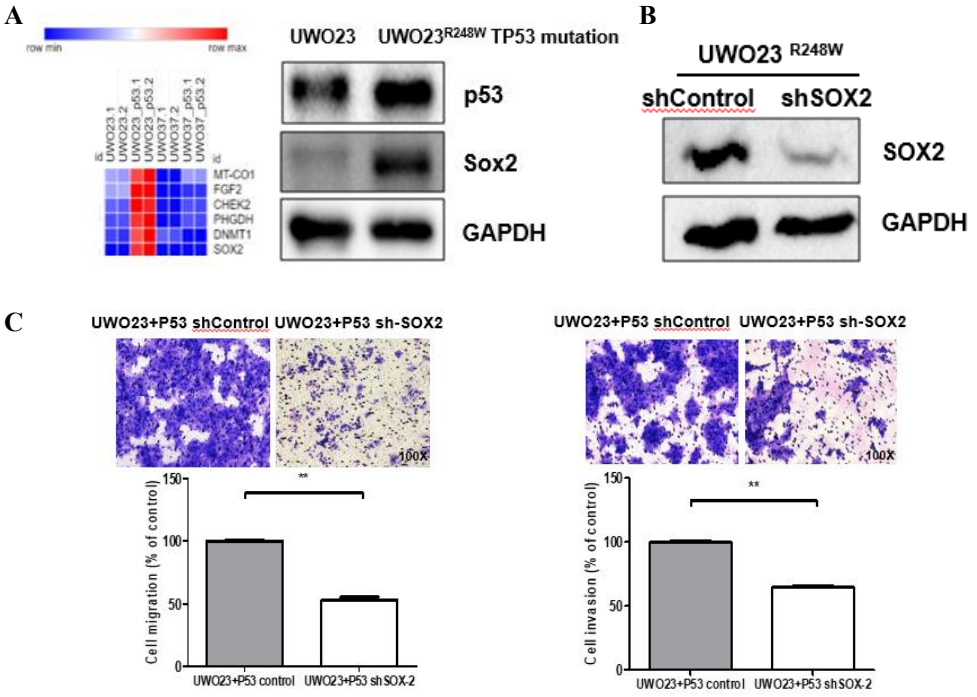
Figure 2 TP53 mutation is associated with radiation resistance in human papillomavirus-associated oropharyngeal cancer. (A) We compared the clonogenic survival of HPV positive cell lines following various doses of irradiation(0, 2, 4 and 8 Gy). Overall survival fraction decreased as radiation doses increased. UWO8 cells with mutant TP53 showed maximum resistance to radiation while both wild type TP53 cell lines UWO23 and UWO37 showed lower survival fraction. Significant difference was observed with each doses of radiation. (B) Wild type TP53 cell lines and their mutant transfection cell lines were assessed, showing consistent results except for 8 Gy radiation in UWO23 cell line. **, $P < 0.01$; **, $P < 0.005$; ***, $P < 0.0001$. All experiments were carried out more than three times. The survival fraction is presented as the mean of three experiments and the standard error is indicated.*



3.3 | Gain-of-function mutant p53 upregulates SOX2 expression

To investigate the effect of TP53 mutation on radiation resistance, microarray expression data analysis revealed SOX2 overexpression among several target molecules overexpressed in TP53 mutant transfection cells(Fig 3A). To confirm the upregulatory role of SOX2 on radiation resistance, we used shRNA technology to silence SOX2 in the UWO23^{R248W} cell line. Efficient SOX2 knockdown was confirmed using western blotting(Fig 3B). Migration and invasion transwell assay revealed significant lower capabilities of cell migration and invasion in SOX2 silenced cell line compared with their corresponding control cell line(Fig 3C). Next, we assessed the effect of SOX2 inhibition on the response to irradiation in cancer cells. The regulatory effect of the SOX2 inhibitor(shRNA of SOX2) were assessed by cell survival. Colony formation was significantly higher in shControl(Fig 3D). We observed the sensitivity of TP53 mutant cells to radiation after silencing SOX2. After 14 days of exposure to various doses of irradiation(0, 2, 4 and 8 Gy), clonogenic assay was performed. SOX2 shRNA-treated UWO23^{R248W} cell line showed significantly lower survival fraction compared with their corresponding control cell line(Fig 3E). Taken together, these results support that inhibition of SOX2 enhances radiation sensitivity in radiation resistant cancer cells and reduce tumor cell migration and invasion. Reaching a conclusion that SOX2 can mediate radiation resistance and tumor aggressiveness in OPSCC.

Figure 3 Gain-of-function mutant p53 upregulates SOX2 expression. (A) To investigate the effect of TP53 mutation on radiation resistance, microarray expression data analysis revealed several target molecule overexpression in TP53 mutant transfection cells including SOX2. (B) To confirm the upregulatory role of SOX2 on radiation resistance, we used shRNA technology to silence SOX2 in the UWO23^{R248W} cell line. Efficient SOX2 knockdown was cofirmed using western blotting. (C) Migration and invasion transwell assay revealed significant lower capabilities of cell migration and invasion in SOX2 silenced cell line compared with their corresponding control cell line. (D) Colony formation was significantly higher in shControl. (E) We observed the sensitivity of TP53 mutant cells to radiation after silencing SOX2. After 14 days of exposure to various doses of irradiation(0, 2, 4 and 8 Gy), clonogenic assay was performed. SOX2 shRNA-treated UWO23^{R248W} cell line showed significantly lower survival fraction compared with their corresponding control cell line. **, P < 0.01; **, P < 0.005; ***, P < 0.0001. All experiments were carried out more than three times. The survival fraction is presented as the mean of three experiments and the standard error is indicated.*



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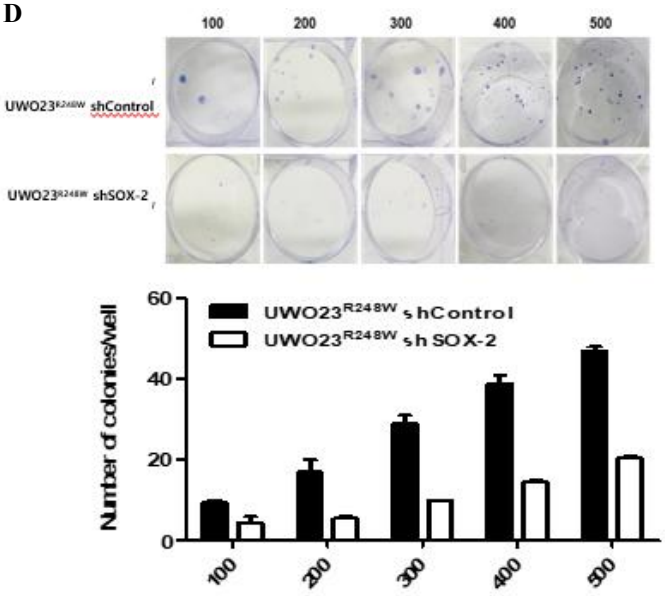
100
200
300
400
500
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UWO23R248W shSOX-2
Number of colonies/well
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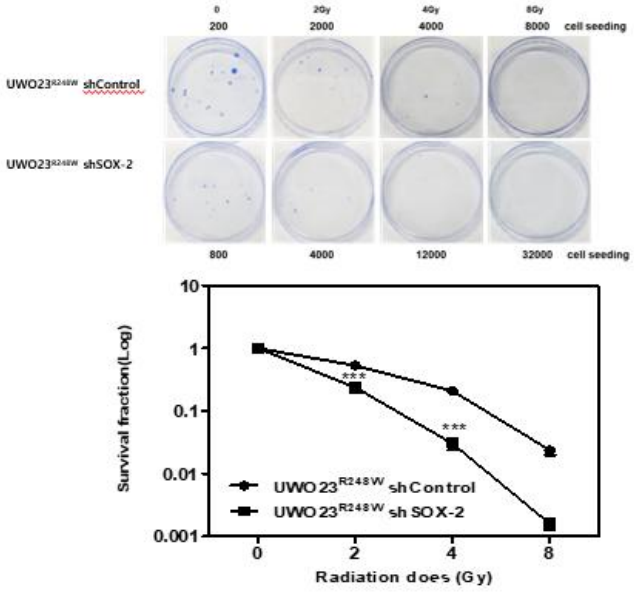
40
20
0
UWO23R248W shControl
UWO23R248W shSOX-2
100
200
300
400
500

E

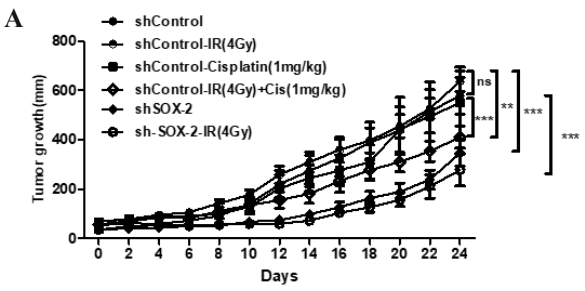

0
2Gy
4Gy
8Gy
200
2000
4000
8000
cell seeding
UWO23R248W shControl
UWO23R248W shSOX-2
800
4000
12000
32000
cell seeding
Survival fraction(Log)
10
1
0.1
0.01
0.001
***
***
UWO23R248W shControl
UWO23R248W shSOX-2
0
2
4
8
Radiation does (Gy)

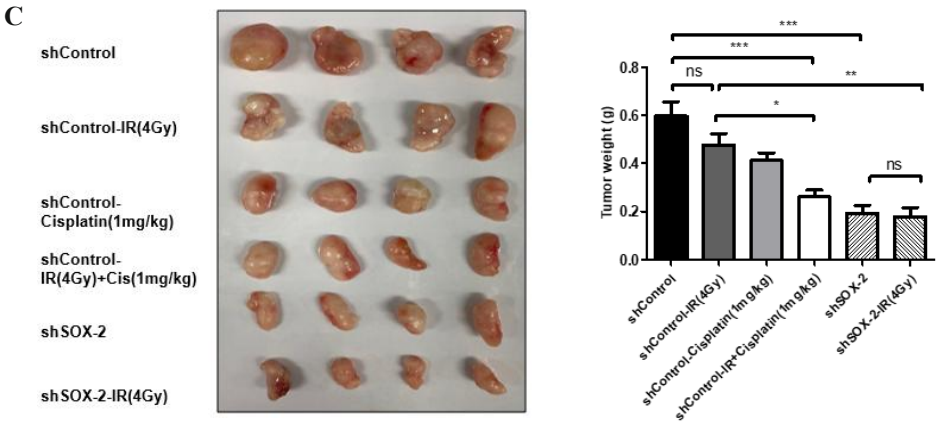
3.4 | SOX2 downregulation reduces tumor growth of xenograft derived from radiation resistant TP53 mutation cell line

Having established the in vitro effects of SOX2 inhibition on sensitivity of radiation resistant cancer cells and tumor behavior, the biologic efficacy of combined treatment was tested in tumor growth delay using UWO23^{R248W} xenografts established in nude mice. Tumor mice (each group with 5 to 6 mice) were treated in 6 different conditions and were sacrificed after approximately 24 days of treatment. Groups were divided into; shRNA control vehicle, shRNA control cell line with radiation, shRNA control cell line with cisplatin only, shRNA control cell line with cisplatin and radiation combined, shSOX2, shSOX2 with radiation. (Fig 4A). Radiation was delivered at 4 Gy at a dose rate of 2 Gy/min, cisplatin was delivered via intravenous injection twice a week for 3weeks. SOX2 inhibition reduced tumor growth: There was no significant tumor growth difference between shControl and shControl with radiation, however, shSOX2 and shSOX2 with radiation resulted in a significant delayed tumor growth and decreased tumor wight at 24 days of treatment, compared to shControl and shControl with radiation(Fig 4B). Mouse body weight monitoring suggested that all treatments were relatively well tolerated(Fig 4C). These data suggest that SOX2 inhibition can enhance the radiation sensitivity and submissive tumor behavior in irradiated UWO23^{R248W} xenografts.

Figure 4 SOX2 downregulation reduces tumor growth of xenograft derived from radiation resistant TP53 mutation cell line. Biologic efficacy of combined treatment was tested in tumor growth delay using UWO23^{R248W} xenografts established in nude mice. (A) Tumors were regularly measured and the relative tumor volume was tracked for each animal. (B) Body weight of each mouse was regularly evaluated. (C) Tumor weight was examined in each treatment group at 24 days. The photographs show representative tumors from each mouse of approximately 24 days (each group: 5-6 mice). (D) β-gal staining was performed using tumor sections. The photographs show representative tumor staining of β-gal. (E) SOX2 expression was assessed using western blotting with tumor specimen of mouse

**, P < 0.01; **, P < 0.005; ***, P < 0.0001.*





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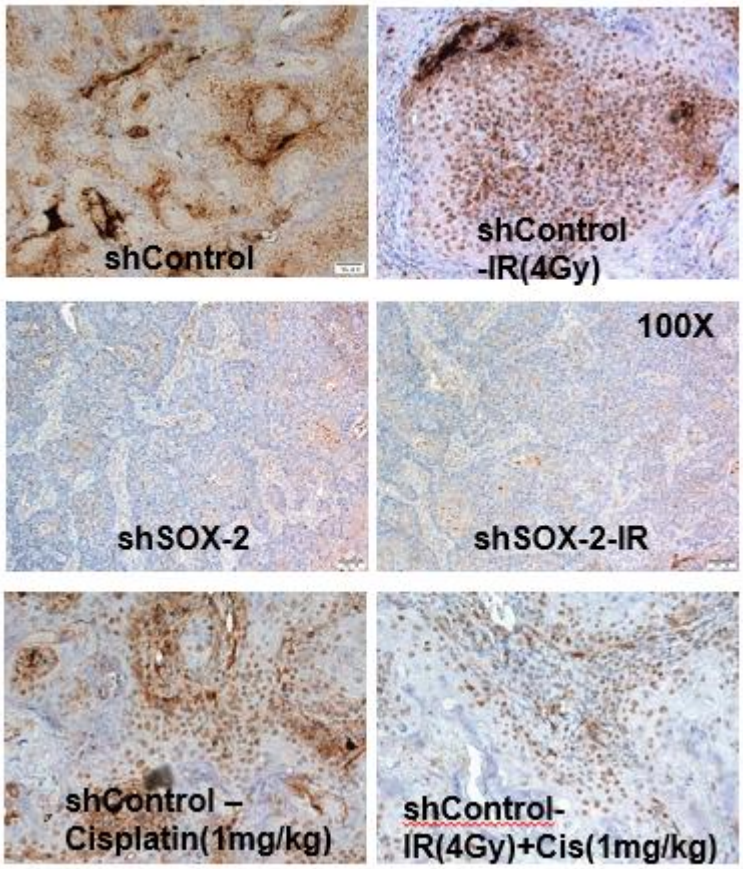
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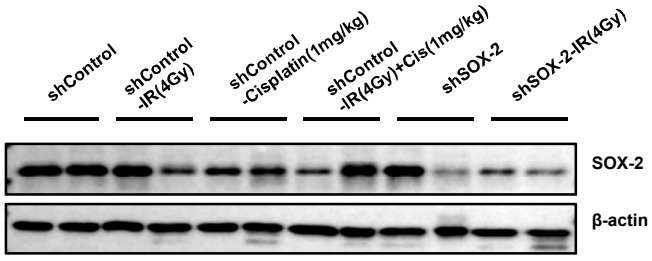
E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SOX-2
β-actin

DISCUSSION

The molecular and clinical differences of HPV+ and HPV− OPSCCs are substantial; they are now largely considered as two distinct cancers despite their histological similarities. HPV+ OPSCC, like those of other HPV associated malignancies, is caused by the various oncogenic functions of high-risk HPV E6 and E7, which may efficiently compensate for common oncogenic driver mutations that lead to OPSCC carcinogenesis(18, 19). Indeed, The Cancer Genome Atlas (TCGA) data demonstrate significantly higher mutation loads in HPV− OPSCC than in HPV+ OPSCC(20). The coding sequences of several tumor suppressors in HPV− HNSCC contain driver mutations that are not observed in HPV+ HNSCC. One of the most striking contrasts is p53, which is mutated in the vast majority of HPV− HNSCC, but rarely in HPV+ HNSCC(13, 20). In addition, a comparison of HPV+ and HPV− tumor samples from patients of a similar age and tumor site using microarray-based comparative genomic hybridization (maCGH) showed a significantly higher number of chromosomal alterations in HPV− tumors compared to HPV+ tumors(21, 22). Four chromosomal regions, the smallest of which spanned four megabases, were found to be significantly altered in HPV− tumors, whereas no change or a change in the opposite direction were found in HPV+ tumors(22). In contrast, distinct chromosome aberrations are associated with viral integration sites in HPV+ HNSCC, contributing to cancer progression(23, 24). In HPV+ OPSCC it is likely that expression of the viral oncogenes E6 and E7 is sufficient to inactivate many of the critical tumor suppressor pathways, promoting carcinogenesis without generating somatic mutations in tumor suppressor genes. TP53 gene is the most frequently mutated gene in HPV– OPSCC, occurring in at least 75% of patients, while rarely observed in HPV+ OPSCC almost certainly owing to the inhibition of p53 function by E6 and thus an ability of the virus to phenocopy this genetic alteration(15). However, compared with primary HPV+ tumors, recurrent and metastatic HPV+ tumors has higher rates of TP53 mutation (25-27). The degree of chromosomal

instability in TP53 mutated HPV+ tumors was significantly higher than in TP53 wild type tumors (25). Mutations in the TP53 gene increase the risk for chromosomal rearrangements, such as copy number alterations, which are involved in the development and progression of many human malignancies(28). Amplifications or deletions in the fragile sites harboring important transcription factors may further advance the process of carcinogenesis(29). These suggests that complete inactivation of p53 has functional sequelae beyond those resulting from incomplete degradation by E6/E7(25). Comparison of mutant TP53 cell line with wild type TP53 in our study confirmed that mutant TP53 show significantly higher radiation resistance and aggressive tumor behavior.

A distinct chromosomal region, which has recently been suggested to be critically involved in HNSCC progression, is the chromosomal arm 3q. A chromosomal CGH analysis found that overrepresentation of chromosomal bands 3q21- q29 was associated with decreased overall and disease-free survival in HNSCC(30). Subsequent studies defined several candidate proto-oncogenes inside this chromosomal region. SOX2, a proto-oncogene located inside the chromosome arm 3q, is a frequent subject of DNA copy number gain in OPSCC. TP53 has been demonstrated to down-regulate several stem cell-associated genes, including SOX2, following DNA damage in embryonic stem cells(31). SOX2 is a stem cell-associated gene known for its crucial role in maintaining the stem cell-like phenotype in cancer cells and its overexpression is generally associated with aggressive disease and poor outcome in several different tumor types(17). In general, SOX2 positively or negatively regulates proliferative or anti-proliferative signaling pathways, respectively, leading to enhanced proliferation, survival, and tumorigenesis(12, 16). SOX2 copy number gain results in an increase of SOX2 transcriptional activity, which might be critically involved in OPSCC initiation and progression(32).

In the present study, data are in line with previous studies indicating the role of SOX2 in carcinogenesis. Microarray expression data showed SOX2 was overexpressed in mutant TP53 cell line and we hypothesized that it could be one of the target molecules that mediate

radiation resistance and tumor aggressiveness in HPV+ OPSCC. In vitro results showed that SOX2 affects tumorigenesis. Overexpression of SOX2 increased cell migration and invasion, colony formation, furthermore it increased radiation resistance. While silenced SOX2 cells showed impaired cell growth and radiation sensitivity.

Treatment for early stage HNSCC often starts with surgical resection followed by observation in some situations. Radiation alone is also an acceptable option for early stage oropharyngeal, hypopharyngeal and laryngeal tumors(33). However, these cancers represent the minority of patients, as the most present with advanced disease. Multimodality therapy is therefore required for the majority of these patients. In general, adjuvant therapy with radiation therapy is indicated for those with larger primary tumors or those with positive lymph nodes. In patients who undergo surgical resection, but have high risk features, as defined as lymph node extracapsular extension or positive margins, adjuvant chemoradiation therapy is recommended based on findings from the Meta-analysis of chemotherapy in head and neck cancer(MACH-NC)(34). For those who present with surgically unresectable tumors or are at risk of irreversible organ damage with associated poor post-surgical functional outcomes, definitive CRT remains the standard-of-care(35). Consistent with our in vitro results, mice in the control group showed no significant tumor growth difference with irradiated group confirming that TP53 mutation induce radiation resistance in HPV+ OPSCC. However, significant growth restriction was observed just by silencing SOX2.

We investigated the effects of SOX2 knockdown on radiation resistance of mutant TP53 HPV+ OPSCC. When SOX2 was silenced with shRNA, in vitro results showed that tumor migration and invasion was decreased with increased radiation sensitivity in TP53 mutant cell line. Furthermore, in vivo results showed that group of mice with silenced SOX2 showed reduced tumor growth compared to groups treated with radiation or cisplatin. These results indicate that SOX2 plays a crucial role in the development of radiation resistance by regulating tumor invasiveness. However, further studies are needed to analyze the

mechanisms behind TP53 induced regulation of SOX2 expression and its contribution to radiation resistance in HPV+ OPSCC.

CONCLUSION

In conclusion, our results suggest that SOX2 is overexpressed in radiation resistant HPV+ OPSCC and plays a crucial role in the development of radiation resistance by regulating tumor migration and invasion. Further studies will help to fully clarify the potential role of SOX2 in cancer treatment. Moreover, the better understanding of these mechanisms will help in the rational design of combination regimens and sequential treatment algorithms to improve clinical outcomes and 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.

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국문 요약

배경: 인간 유두종바이러스 양성 구개인두편평세포암 환자들은 그들의 대조군인 인간 유두종바이러스 음성 구개인두편평세포암 환자들에 비해 대체로 흡연, 음주력이 낮고 더 젊고 건강한 경향이 있다. 인간 유두종바이러스 양성 구개인두편평세포암의 더 높은 생존률에 의거하여 기존 치료보다 강도를 낮춘 치료에 대한 관심이 커지고 있다. 하지만, 기존 치료에도 약 15%의 환자는 실패한다. 따라서 이런 고위험군 환자들을 강도를 낮춘 치료가 아닌 보다 더 강도 높은 치료를 받을 수 있게 하는 방사선 저항성에 대한 핵심 분자 표지자를 알아내는 것이 중요할 것이다. 이 연구의 목적은 인간 유두종바이러스 양성 구개인두편평세포암에서 방사선 저항성과 종양 공격성을 예측할 수 있는 분자 표지자를 확인하고 그 기전을 확인하고자 한다.

방법: 조작을 가하지 않은 인간 유두종바이러스 양성 구개인두편평세포암 세포에 TP53 돌연변이를 가하였다. 세포 독성 실험, 세포 이동 및 침습 분석, 세포 집락 형성 검사를 실행하여 종양 성장과 공격성 그리고 방사선 저항성을 모체 세포 및 TP53 돌연변이 세포에서 비교하였다. 염색체 마이크로어레이 검사와 웨스턴 블롯 검사를 이용하여 모체 세포와 TP53 돌연변이 세포의 분자 표지자 차이를 비교하였다. 세포 내 검사 결과를 바탕으로 마우스를 이용하여 생체 내 검사를 진행하였다.

결과: TP53 돌연변이 세포는 모체 세포에 비교하여 세포 이동 및 침습 능력이 증가하였으며 방사선 저항성을 띄었다. 염색체 마이크로어레이 검사를 통해 TP53 돌연변이 세포에서 SOX2 전사 인자의 발현이 증가되어 있는 것을 알 수 있었고 이를 웨스턴 블롯 검사를 통해 확인하였다. 또한 shRNA 기법을 이용하여 SOX2 가 TP53 돌연변이 세포에서 세포 이동 및 침습 능력과 방사선 저항성을 야기함을 확인하였다. 세포 내 및 생체 내 검사에서 SOX2를 억제하였을 때 방사선에 대한 민감도가 증가하는 것을 확인하였다.

결론: SOX2는 TP53 돌연변이 인간 유두종바이러스 양성 구개인두편평세포암에서 과발현되어 있으며 종양 침윤성을 조절하여 방사성 저항성의 획득에 중요한 역할을 한다.

핵심용어: 인간 유두종바이러스 양성 구개인두편평세포암, TP53 돌연변이, sex-determining region Y-box 2 (SOX2)