Conjoint analysis of OPRPN and SMR3A protein expression as potential predictive biomarkers for head and neck squamous cell carcinoma after radiotherapy

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Abstract. Increased submaxillary gland androgen-regulated protein 3A (SMR3A) expression was previously shown to serve as an independent risk factor for oropharyngeal squamous cell carcinoma (OPSCC) and as a surrogate biomarker for active estrogen receptor 2 signaling in radioresistant tumor cells. In the present study, it was aimed to unravel the expression and clinical significance of another member of the opiorphin family, opiorphin prepropeptide (OPRPN), in the radiotherapy for head and neck squamous cell carcinoma (HNSCC). Expression of SMR3A and OPRPN were analyzed for the prior and post fractionated irradiation (4x2 Gy) by double immunofluorescence staining in established HNSCC cell lines as well as by immunohistochemical (IHC) staining in *ex vivo* tumor tissues. Next, in a retrospective experimental cohort study, primary tumor samples from OPSCC patients (n=96), who received definitive surgery and adjuvant radiotherapy were reviewed, and expression levels of OPRPN protein were detected by IHC. Immunoreactivity scores (IRS) were associated with pathological and clinical risk factors by Chi-square analysis. Survival analysis was performed by using the Kaplan-Meier plot, log-rank test and Cox regression analysis. The expression levels of OPRPN and SMR3A protein were both induced by fractionated irradiation *in vitro* and *ex vivo*. In primary tumor samples, IRS of OPRPN was significantly higher than scores of SMR3A expression and positively correlated with expression patterns of SMR3A. SMR3A was confirmed to serve as an unfavorable factor, while OPRPN protein had no significant association with the clinical outcome of patients with OPSCC. A combinational analysis revealed that the subgroup with $SMR3A^{high}OPRPN^{low}$ staining pattern had the worst clinical outcome among the various subgroups. Multivariate Cox regression analyses indicated that high expression of SMR3A serves as an independent unfavorable biomarker, while increased expression of OPRPN appears to exert protective function. In summary, the present study indicated that SMR3A and OPRPN serve as potential prognostic markers for HNSCC after radiotherapy.

Introduction

A total of 3 homologous genes of opiorphin, OPRPN (known as ProL1 previously), submaxillary gland androgen-regulated protein 3A (SMR3A) and SMR3B (1) are known in humans. OPRPN-derived opiorphin was reported to act as a potent inhibitor of two cell membrane-bound enkephalin-inactivating peptidases, neutral endopeptidase (NEP; CD10) and aminopeptidase N (APN; CD13) (2). CD10 and CD13 are widely distributed among a wide range of tissues and organs, which regulate signaling pathways mediating cell proliferation, survival and migration (3-5). Dysregulated expression of both proteins has been identified in various human tumor entities, such as pancreas, gastric, prostate, breast, lung and oral carcinomas (6-9).

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Head and neck squamous cell carcinoma (HNSCC) is one of the most common human malignancies worldwide with a yearly incidence of 600,000 cases (10). The standard pillars of therapy are surgery, irradiation (IR), chemotherapy or a combination of these (11). Although treatment strategies have improved in the last decades, the global 5-year-survival rate for all HNSCC sites remains low at only 40-50% (10). Tumor recurrence after radiotherapy frequently develops and significantly hampers rehabilitation (12). The identification of valuable biomarkers and radioresistant molecules contributing to poor clinical outcomes may enable patient stratification to properly select a therapeutic regimen (13). Our previous data revealed variable protein expression patterns for both CD10 and CD13 in a cohort of patients with oropharyngeal squamous cell carcinoma (OPSCC). In addition, strong SMR3A protein expression was also found in 36% of all primary OPSCC in a tissue microarray which served as an unfavorable risk factor for clinical prognosis (14). Notably, an enrichment of SMR3A-positive cells was observed in the fraction of vital HNSCC cells after fractionated IR which was dependent on estrogen receptor 2 (ESR2) signaling (15). Moreover, dysregulated OPRPN was reported to be associated with invasion in breast cancer (16). To date, the understanding of the clinical relevance of opiorphin proteins in head and neck cancer is limited.

In the present study, OPRPN protein levels were investigated by immunohistochemical (IHC) staining on primary tumor samples of OPSCC patients. The association between expression patterns of OPRPN and SMR3A with clinical and histopathological features as well as progression-free (PFS) and disease-specific survival (DSS) were addressed. A potential function of OPRPN and SMR3A after fractionated IR, in part mimicking a clinically applied treatment protocol, were also highlighted.

Materials and methods

HNSCC cell lines. Human HNSCC cell lines FaDu and Cal27 were purchased from the American Type Culture Collection (https://www.lgcstandards-atcc.org). FaDu cells were established from a hypopharyngeal SCC. Cal27, derived from the tongue, was described earlier as adenosquamous carcinoma (17), a rather aggressive subtype of oral SCC (18). Detroit562 cells originating from a metastatic pharyngeal carcinoma were purchased from CLS (CLS Cell Lines Service GmbH). By this selection of HNSCC cell lines depicting different origins/localizations the intention was to approximate the heterogeneous features of HNSCC. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.), 2 mM L-glutamine and 50 μg/ml penicillin-streptomycin antibiotics and sterile conditions with 6% CO_2 at 37˚C. Cell cultures were regularly screened to exclude mycoplasma contamination (Venor®GeM Classic Mycoplasma Detection Kit; Minerva Biolabs GmbH) according to manufacturer's recommendation, and the authentication of all cell lines was confirmed by the Multiplex Human Cell Line Authentication Test.

IR of cell cultures and immunofluorescence (IF). A total of 10,000 cells were seeded on sterile coverslips in a 12-well plate and cultured for 24 h. Cells were irradiated on four consecutive days with a daily dose of 2 gray (Gy) using X-RAD 320 (Precision X-Ray). Controls were mock-treated. Cells were fixed with 4% paraformaldehyde for 15 min at room temperature. After being washed with phosphate-buffered saline (PBS) three times, the cells were permeabilized with 0.5% Triton X-100 buffer in PBS for 30 min, and after being rewashed three times with PBS, they were blocked with 1% BSA (Sigma-Aldrich; Merck KGaA)/0.2% Tween-20 (GERBU Biotechnik Gmbh) in 1X PBS for 30 min at room temperature. The primary antibodies anti-PROL1 (1:200; cat. no. ab169504) and anti-SMR3A (1:100; cat. no. ab97942; both from Abcam) were diluted in T-buffer with indicated concentrations and were incubated with cells for 1 h at room temperature or overnight at 4˚C. Following washing with PBS three times, the secondary antibody (1:200; cat. no. SAB4600234; Sigma-Aldrich; Merck KGaA), conjugated with Hoechst 33342 (BIOMOL International), was diluted in T-buffer and was added for 30 min at room temperature in the dark. Finally, cells were again washed with PBS three times and embedded on glass slides with Mowiol (Sigma-Aldrich; Merck KGaA). The glass slides were kept in the dark at 4˚C for at least 12 h before images were captured. Images were captured using a fluorescence microscope (model, BX50F), Olympus XC30 Camera and cellSens Entry imaging software (Olympus Soft Imaging Solutions GmbH). Images were acquired under identical imaging conditions. Mean fluorescence intensity was quantified using the ImageJ software version 1.8 (National Institutes of Health).

Patients. A total of 96 patients with primary OPSCC who were diagnosed and treated between 1990 and 2009 were comprised in the retrospective study cohort. The mean age of the cohort was 58.9 years, and 70 of the patients were male. Samples were obtained at the Department of Otorhinolaryngology, Head and Neck Surgery of Heidelberg University Hospital (Heidelberg, Germany) during diagnostic or therapeutic procedures. Biopsies of non-surgically treated patients, as well as samples of patients who underwent tumor surgery, were included. All subjects provided written informed consent for data collection as it is a standard procedure in our department. Patients with suspicious clinical findings who underwent diagnostic panendoscopy and/or patients before tumor surgery with a histologically confirmed diagnosis of OPSCC were asked to consent. The protocol was approved (approval no. 176/2002) by the Ethics Committee of the Medical Faculty of the University of Heidelberg (Heidelberg, Germany) in accordance with The Declaration of Helsinki in existing version from 1996. Experimental treatment procedures were not part of the present study. The patients were treated according to the guidelines for head and neck cancer. The final analysis was based on 96 patients with OPSCC who were treated with either definitive or post-surgical radiotherapy with or without adjuvant chemotherapy. Clinical and therapeutic follow-up of the cohort was assessed retrospectively (Table SI).

Tissue microarrays (TMAs) and IHC. TMAs were produced as previously described (19-21). In brief, H&E-stained sections were cut from each donor block to define representative tumor regions. From selected areas of each donor block, small tissue cylinders with a diameter of 0.6 mm were received using a tissue

chip microarrayer (Beecher Instruments Inc.) and transferred to a recipient paraffin block. By using standard techniques, 2 μm paraffin sections were cut from this recipient paraffin block. TMAs were stained with an Anti-OPRPN (1:300; cat. no. ab204562; Abcam) and immunostaining was visualized with the TSA Amplification Kit (PerkinElmer, Inc.) and DAB peroxidase substrate (Vector Laboratories, Inc.), according to the manufacturer's instructions. Counterstaining was performed by hematoxylin to visualize tissue integrity. Stained TMAs were scanned using the Nanozoomer HT Scan System (Hamamatsu Photonics K.K.) and were evaluated by three independent observers using the NDP Viewer software (version 1.1.27; Hamamatsu Photonics K.K.). Evaluation considered the relative amount of positive cancer cells (score 1=no positive cell, score 2 ≤33%, 33%> score 3 ≤66%, score 4 >66%) and the staining intensity (score 1=no, score 2=low, score 3=moderate, score 4=high). Both values were multiplied to calculate the final immunoreactivity score (IRS; range 1-16), and the cut-off value for further analysis was: $OPRPN^{high}$ >9 and $OPRPN^{low}$ ≤9. Data on the IRS for SMR3A were available from previous studies (22,23).

Ex vivo culture. Fresh tumor samples from the oropharynx were procured immediately after surgical resection at the Department of Otorhinolaryngology, Head and Neck Surgery, Heidelberg University Hospital (Heidelberg, Germany). Informed consent was obtained after the review of the local ethics board (ethic vote S-396/2012). Samples were processed as previously described (21). For *ex vivo* analysis of tumor response to fractionated IR, tumor sections were maintained in six-well plates with inserts (Thinsert; Greiner Bio-One) in DMEM, supplemented with 10% fetal bovine serum and antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml). After one day in culture, samples were irradiated with an intensity of 2 Gy on four consecutive days. Non-treated controls were processed in parallel. The medium was changed every second day. The tissue slices were harvested 72 h posttreatment to be evaluated for histopathological and IHC features. The sample is exemplarily depicted in Fig. 1.

Statistical analysis. SPSS 22 for Windows (IBM Corp.) and GraphPad Prism version 9.1 (GraphPad Software, Inc.) were used for statistical analysis. Fluorescence intensities were quantified using the ImageJ and compared by unpaired Student's t-test. Person and Spearman's correlation analysis between score A and B were performed before they were multiplied. Correlations between OPRPN expression and clinical and histopathological parameters (sex, age, tumor size, lymph node metastases, tumor grade) as well as risk factors (smoking, alcohol consumption, HPV status) were calculated by the cross table and chi-square test. $P<0.05$ was considered to indicate a statistically significant difference. Disease-specific survival (DSS) and progression-free survival (PFS) data were plotted by Kaplan-Meier survival curves. Differences between groups were assumed using log-rank testing. Univariate and multivariate Cox proportional hazard models were applied to define the interdependence between multiple parameters and prognosis (DSS, PFS) by using the approach 'enter'. The endpoint PFS was defined as the first appearance of an event that was counted for PFS. These are tumor progression, recurrence, metastasis and secondary tumors.

Results

Expression of OPRPN and SMR3A in HNSCC cell lines and ex vivo tumor tissues response to fractionated IR. It was previously demonstrated that SMR3A is expressed in HNSCC cell lines at low levels, but the relative amount of SMR3A-positive cells was increased after fractionated IR (15). To investigate whether OPRPN is postradiogenically upregulated and if there is a co-expression with SMR3A in HNSCC cell lines, double co-immunofluorescence staining (co-IF) was performed in FaDu, Cal27 and Detroit562 cells. Analogously to SMR3A, basal OPRPN protein expression was low and could be detected only in a sub-fraction of three cell lines (Fig. 1A). However, upon fractionated IR prominent OPRPN expression was observed in the majority of cells and was co-expressed with SMR3A (Fig. 1A). The quantitative analysis revealed that OPRPN and SMR3A protein expression were significantly induced upon fractionated IR in both FaDu and Detroit562 cells. However, the SMR3A induction as compared with control-treated cells was highly significant, while OPRPN was found without significant changes in Cal27 cells (Fig. 1B and C). To adapt these findings to the clinical setting, a rapid and cost-effective patient-derived explant *ex vivo* culture technique was developed for evaluating the therapeutic response of fresh tumor tissues from surgical resection. Elevated levels of SMR3A and OPRPN protein expression were observed by IHC staining post-fractionated IR (Fig. 1D). Thus, our *ex vivo* culture data confirmed the findings from the HNSCC cell lines.

Expression of OPRPN in primary OPSCC and correlation with clinicopathological features. Data on SMR3A staining were in part available from previous retrospective studies (14,15). To determine whether aberrant OPRPN expression is relevant for the pathogenesis and/or the clinical outcome of OPSCC, the TMAs with tissue samples of normal mucosa and OPSCC were stained by IHC. Similarly, weak staining of OPRPN was observed in basal and supra-basal keratinocytes of normal mucosa, which served as a reference. In primary OPSCC, a heterogeneous staining pattern of OPRPN protein was observed, which ranged from low to high expression (Fig. 2A). The relative number of positive tumor cells and the staining intensity were estimated by three independent observers. Both scores revealed a significant correlation (Spearman's correlation of 0.428 and Pearson's correlation of 0.437) and were multiplied to obtain a final OPRPN IRS for further analysis. The OPRPN IRS was significantly higher than that of SMR3A protein levels (Fig. 2B). A strong expression of SMR3A was significantly associated with a high OPRPN IRS, underlining the strong correlation between the two proteins (Fig. 2C).

Subsequently, OPRPN and SMR3A expression patterns and clinicopathological features were compared, including age, sex, TNM status (AJCC Cancer Staging Manual 7th ed), pathological grade, HPV status, smoking and alcohol consumption. However, these parameters were not significantly correlated with OPRPN protein levels (Table SI). These data are in line with previous findings that SMR3A has no correlation with clinicopathological features in OPSCC (14). Accordingly, the regulation of the opiorphin gene family is independent of initial events during neoplastic transformation and the malignant progression of OPSCC.

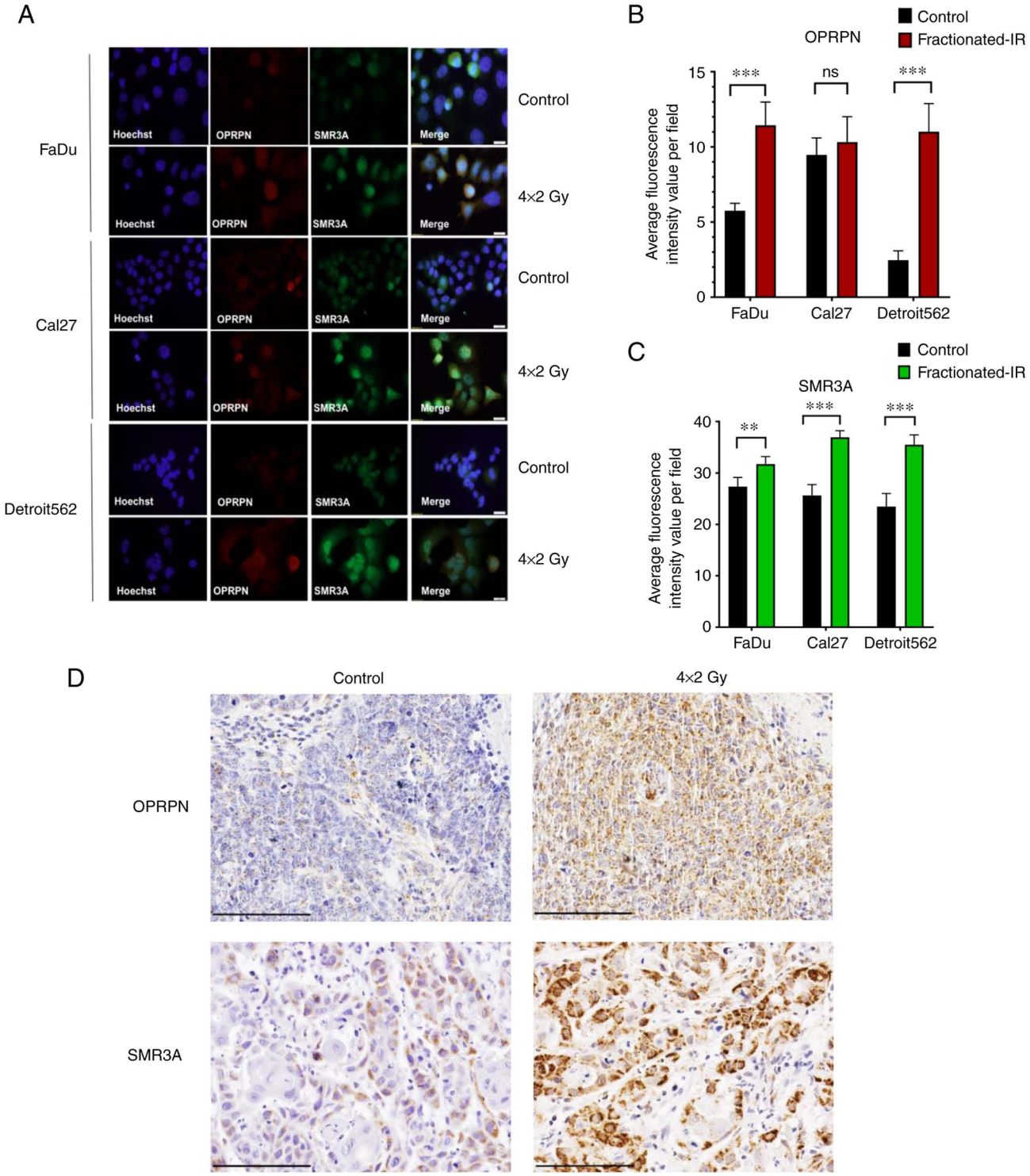

Figure 1. Elevated expression of OPRPN and SMR3A in HNSCC cell linesand *ex vivo* tumor tissues in response to fractionated irradiation. (A) Representative pictures of the co-immunofluorescence staining confirmed induced OPRPN (red signal) and SMR3A (green signal) expression upon fractionated irradiation. Cell nuclei were stained by Hoechst 33342 (blue staining). Scale bars=20 μm. (B and C) Quantitative analysis of fluorescence for (B) OPRPN and (C) SMR3A protein expression in FaDu, Cal27 and Detroit562 cells with or without fractionated irradiation. (D) Representative staining of an *ex vivo* HNSCC vital tumor sample. Distinct postradiogenic upregulation after fractionated IR scheme in both OPRPN and SMR3A was confirmed *ex vivo*. Scale bars=100 μm. **P<0.01 and ***P<0.001. SMR3A, submaxillary gland androgen-regulated protein 3A; HNSCC, head and neck squamous cell carcinoma.

Correlation of OPRPN and SMR3A expression with disease-specific survival (DSS) and PFS. Next, to investigate the prognostic value of OPRPN and SMR3A, patients were arranged into two categories according to the IRS: patients with low protein expression of OPRPN (OPRPN^{low}) and SMR3A (SMR3A^{low}) and those with high protein expression of OPRPN (OPRPN^{high}) and SMR3A (SMR3A^{high}), respectively. Patients with high IRS of SMR3A exhibited a poor DSS and PFS compared with patients with low SMR3A levels, which was highly significant (DSS, P=0.003; PFS, P=0.002) (Fig. 3A). This is in line with a previous finding that apparently opiorphin family members contribute to poor

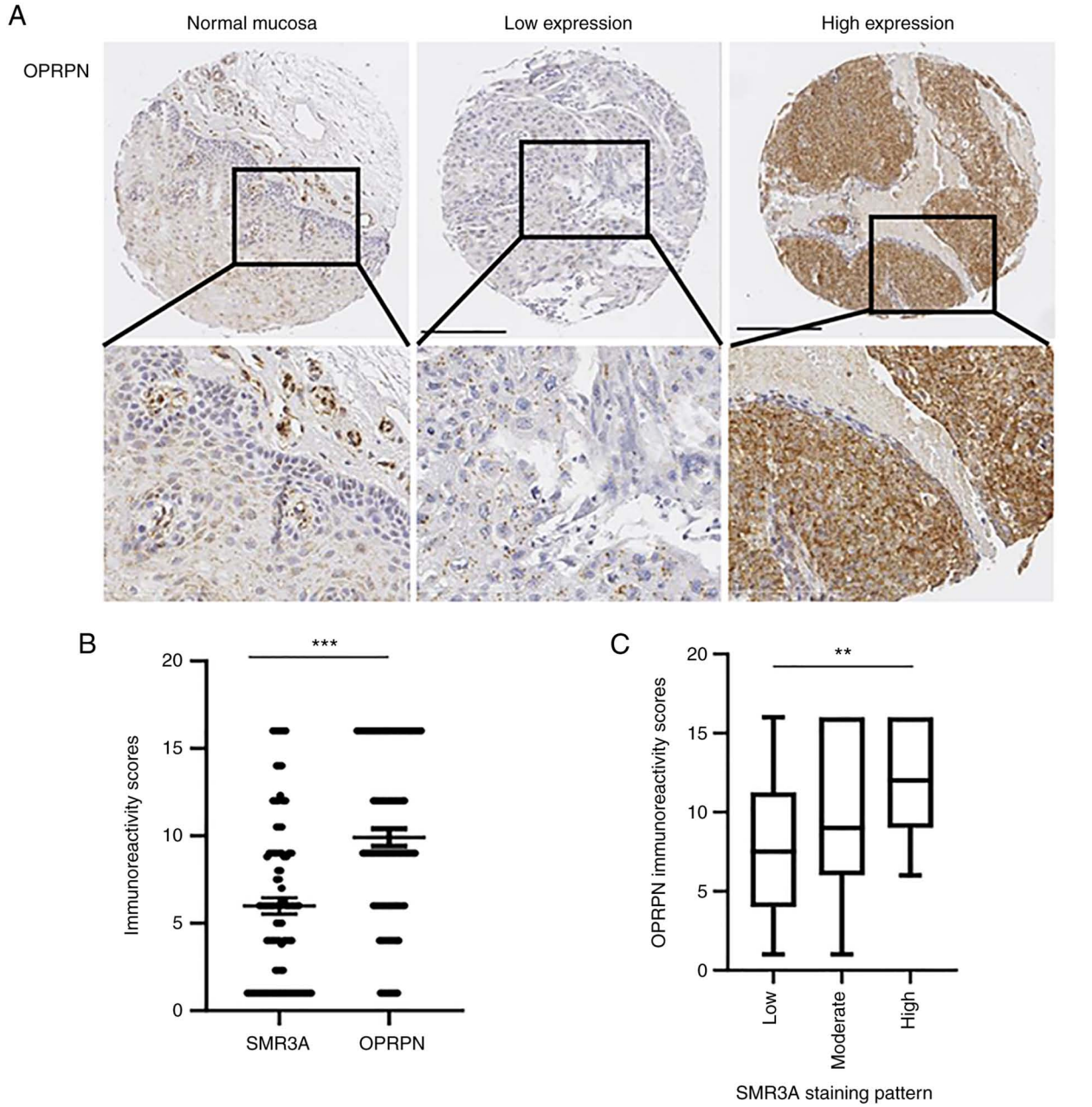

Figure 2. OPRPN expression in tumor specimens from patients with head and neck squamous cell carcinoma. (A) Representative pictures of OPRPN expression (signal in brown) in normal mucosa (left row) and primary OPSCC with low (middle row) or high (right row) protein levels as determined by immunohistochemical staining of tumor microarrays. Counterstaining of cell nuclei was performed with haematoxylin (signal in blue) to demonstrate tissue architecture. Scale bars=200 μm. (B) Immunoreactivity scores of the two proteins are summarised. (C) Box-blot depicts the OPRPN immunoreactivity scores as mean values and 5/95th percentile for individual tumors with low, moderate or high SMR3A staining pattern **P<0.01 and ***P<0.001. SMR3A, submaxillary gland androgen-regulated protein 3A.

radiosensitivity in head and neck cancer (15). SMR3A was considered as a surrogate marker for the active signaling of ESR2. The prognostic value of OPRPN expression for DSS and PFS of OPSCC patients was analyzed by Kaplan-Meier plots and log-rank testing. However, no statistically significant difference was observed for DSS and PFS (Fig. 3B). Next, a combinatorial analysis was accomplished in the subgroup of OPSCC patients, which were treated with either definitive or post-surgical radiotherapy with or without chemotherapy. Concerning DSS and PFS, SMR3A^{high}OPRPN^{high} staining pattern tumor revealed an unfavorable clinical outcome as compared with other subgroups (Fig. S1).

Notably, the subgroup of OPSCC patients with SMR3A^{high} OPRPN^{low} staining pattern displayed the worst clinical outcome in terms of the shortest DSS (P<0.001) and PFS (P<0.001) (Fig. 3C).

SMR3A but not OPRPN serves as an independent prognostic biomarker for the survival of OPSCC patients with radiotherapy. Consequently, univariate analyses revealed that SMR3A^{high} staining pattern significantly correlated with shorter DSS [hazard ratio (HR), 2.286; 95% Confidence Interval (95% CI), 1.293-4.041; P=0.004] and PFS (HR, 2.324; 95% CI, 1.327-4.071; P=0.003), which suggested the patients with

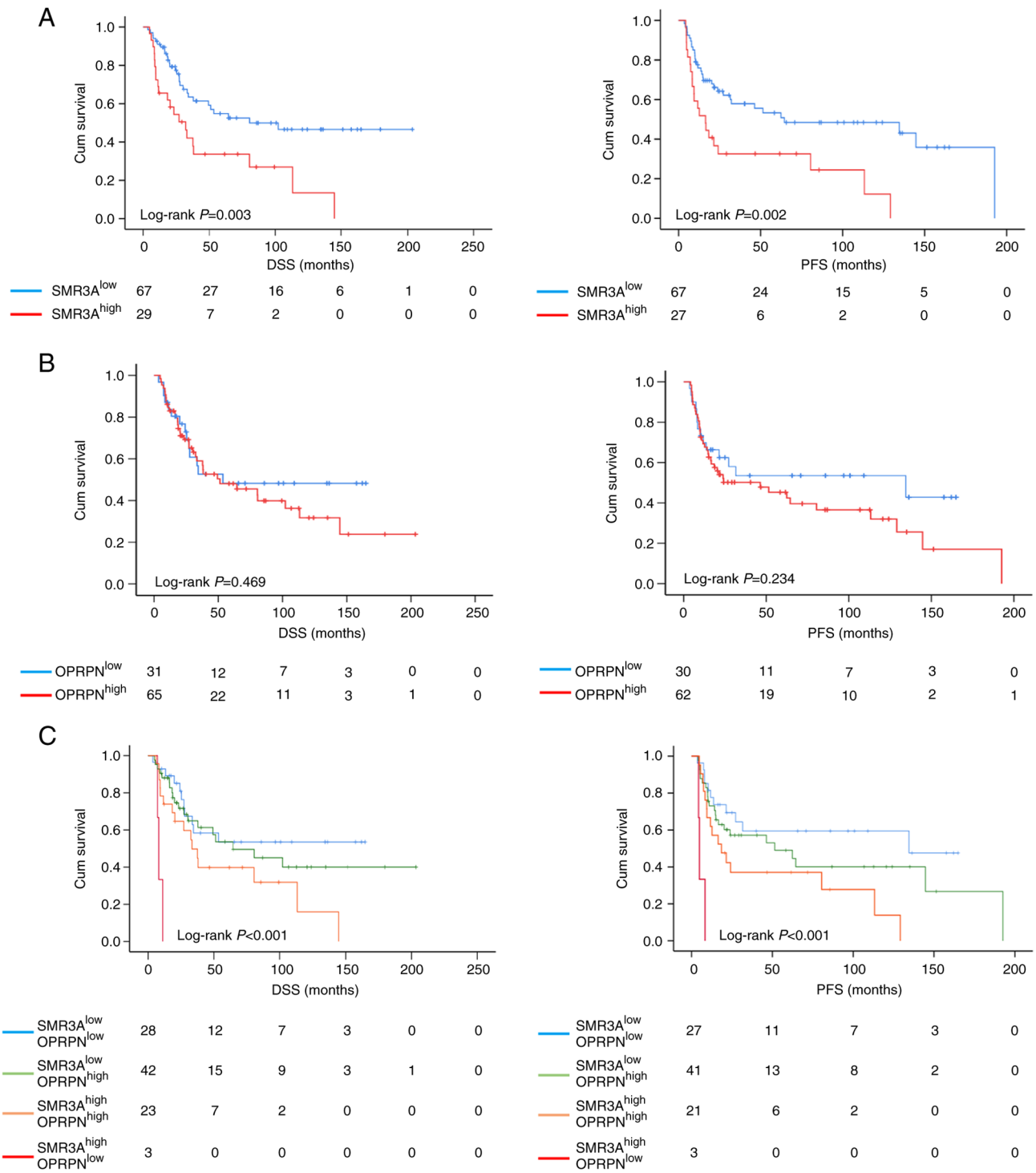

Figure 3. Correlation of OPRPN and SMR3A expression with DSS and PFS. (A-C) Kaplan-Meier plots demonstrated differences in DSS and PFS between subgroups with (A) low (blue line) and high (red line) SMR3A staining, (B) low (blue line) and high (red line) OPRPN staining and (C) the subgroups of tumors co-expressing high or low levels of OPRPN and SMR3A. SMR3A, submaxillary gland androgen-regulated protein 3A; DSS, disease-specific survival; PFS, progression-free survival.

elevated SMR3A expression are at increased risk for treatment failure, presumably due to resistance against IR. It is worth noting that combinational analysis with $OPRPN^{high}$ was mitigating the predictive effect of SMR3A. $OPRPN^{high}SMR3a^{high}$ remained significant on univariate analyses concerning DSS (HR, 1.826; 95% CI, 1.019-3.272; P=0.043) and PFS (HR, 1.869; 95% CI, 1.047-3.338; P=0.034) (Fig. 4). To adjust for all available clinical parameters, a multivariate Cox regression model was applied to confirm that patients with $SMR3A^{high}$ staining pattern had an unfavorable DSS (HR, 2.440; 95% CI, 1.187-5.018; P=0.015) and PFS (HR, 2.323; 95% CI, 1.181-4.570; P=0.015) (Fig. 5). Notably, $OPRPN^{low}$ $SMR3A^{high}$ staining pattern serves as the most unfavorable independent prognostic biomarker (Table SII). These data indicated that SMR3A and OPRPN serve as potential prognostic markers for HNSCC after definitive surgery and adjuvant radiotherapy.

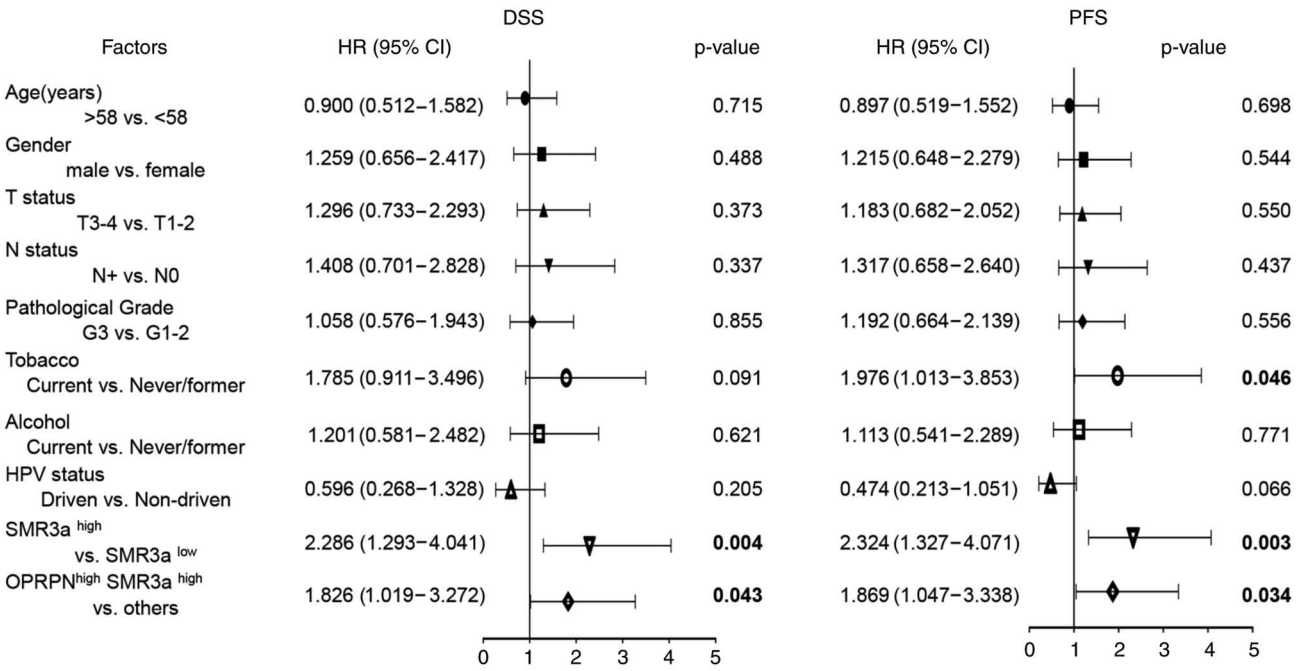

Figure 4. Univariate Cox regression analysis for DSS and PFS (n=96). DSS, disease-specific survival; PFS, progression-free survival; CI, confidence interval; SMR3A, submaxillary gland androgen-regulated protein 3A.

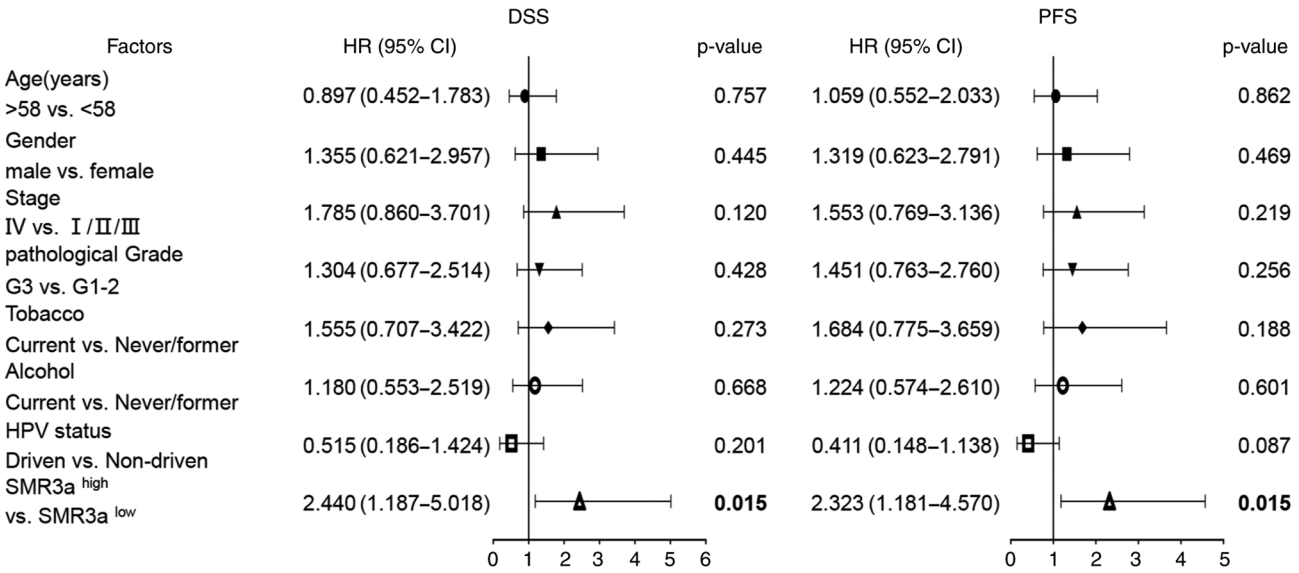

Figure 5. Multivariate Cox regression analysis for DSS and PFS (n=96). DSS, disease-specific survival; PFS, progression-free survival; CI, confidence interval; SMR3A, submaxillary gland androgen-regulated protein 3A.

Discussion

The present study is the first report associating elevated expression levels of opiorphin members with prognosis in OPSCC cohorts with radiotherapy. The present data suggested that the expression levels of OPRPN together with SMR3A correlate with treatment failure after radiotherapy. At present, treatment of head and neck cancer has been significantly improved by novel radiotherapy techniques and protocols (24). Radiotherapy is applied as primary or as adjuvant therapy after surgery and ~75% of HNSCC patients will benefit (25). However, intrinsic and acquired radioresistance remains a major barrier to curative therapeutic approaches in HNSCC. It is crucial to unravel the molecular mechanisms of radioresistance and to identify new biomarkers for HNSCC patients at high risk for treatment failure.

A previous study demonstrated that the mouse homolog of human SMR3A gene, Smr1 is differentially expressed in primary and recurrent tumors of an orthotopic mouse xenograft model for oral cancer (26). Upon fractionated IR SMR3A was shown to be prominently expressed in vital tumor cells (15). It was assumed as a surrogate for resistant tumor cells which are a putative source for relapse after radiotherapy. High SMR3A expression was furthermore described as a risk factor for unfavorable PFS and overall survival in OPSCC

patients (14). Nevertheless, no impact of ectopic SMR3A expression was identified on tumor-relevant processes under normal growth conditions, which suggested that SMR3A has no major impact on tumor cell physiology under normal growth conditions. It was hypothesized that SMR3A serve as a biomarker for a subpopulation of resistant cells as a putative source for tumor relapse after radiotherapy. The findings also explain that high SMR3A expression revealed an unfavorable outcome in a cohort of OPSCC patients.

It is worth noting that an increased transcript level of Muc10, the mouse homolog of the human OPRPN gene, another member of the opiorphin gene family, was detected in recurrent tumors following surgical resection as compared with their matching primaries. This finding indicated a general principle of regulation and function of opiorphin family members in recurrence progression and treatment failure (26). So far, the opiorphin family members have been linked to various physiological and pathological conditions, such as erectile dysfunction (ED), colonic motility and nociception, pain and mood disorders and hypoxic response (1,27-30). For instance, it was reported that the pentapeptide opiorphin is a potent analgesic as it inhibits pain perception. The pain-suppressive efficacy is equal to morphine in the behavioral rat model, suggesting opiorphin may act as a potential initiator to develop a novel candidate drug for pain control (2). Opiorphin has been identified as a potent inhibitor of enkephalin-degrading enzymes, namely CD10 and CD13 (6,9,31-34). Moreover, positive regulation of opiorphin family members by hormone signaling has been reported in several studies (35,36). This is in line with our previous findings that suggested ESR2 signaling regulates SMR3A expression and plays an important role in radioresistance (15).

A cell culture model of fractionated IR was now presented, providing evidence for the existence and expansion of a subpopulation of tumor cells, which are characterized by IR-induced OPRPN and SMR3A expression. The prognostic significance of another member of the opiorphin gene family, OPRPN (formerly known as ProL-1), and its potential role in mediating radioresistance were also investigated. Therapy response is affected by pronounced intratumorigenic heterogeneity in HNSCC. The selection of radioresistant tumor cell subclones after fractionated radiotherapy as clinically applied is thereby facilitated. However, numerous previous studies made statements on molecular mechanisms of radioresistance after single-dose IR considerably exceeding a dosage of 2 Gy (37-40). The clonal selection of radioresistant tumor cells under fractionated-IR is hereby not taken into account. Therefore, a clinically applied fractionated-IR protocol was mimicked. Norm fractionation with 5x2 Gy (37) applied over 6-7 weeks, considered standard treatment in HNSCC OPRPN, was found to be distinctly upregulated in both cell lines (FaDu and Detroit562) as well as in the *ex vivo* tumor culture after fractionated IR. Notably, the characteristics of the cell lines that affect the opiorphin protein expression regulated by IR should be clarified in more head and neck cancer cell lines.

Experimental data of our research group demonstrated that SMR3A is expressed in HNSCC cell lines at low levels but was postradiogenically upregulated (15). Similar to SMR3A, basal OPRPN protein expression was low and detected only in a sub-fraction of both cell lines. However, upon fractionated IR, prominent OPRPN expression was identified in the majority of cells and was co-expressed with SMR3A. In a recent study, OPRPN was regarded as a favorable predictive factor in OPSCC as the expression of OPRPN was associated with consistently increased survival rates (41), which is in line with the present findings. The subgroup displaying high SMR3A but low OPRPN expression levels is the one with the worst clinical outcome, while patients whose tumors express both high are only the second-worst. These data may indicate that SMR3A and OPRPN are inverse prognosticators of the clinical outcome, but SMR3A is the more powerful prognosticator. However, in our retrospective study patient cohort is small. A larger number of patients would be recruited in the future prospective study to confirm the predictive value of human opiorphin proteins for HNSCC after radiotherapy.

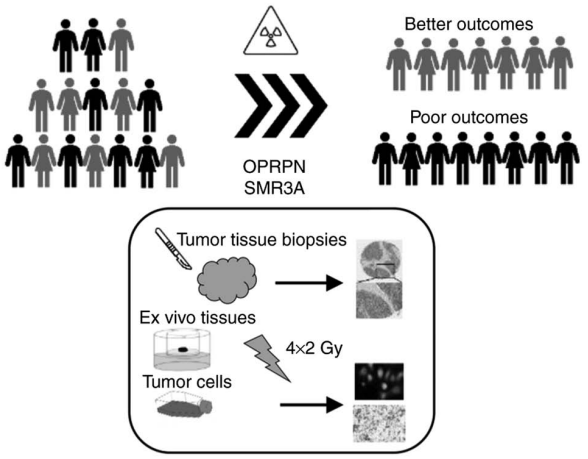

Figure 6. Graphical summary. SMR3A and OPRPN serve as potential prognostic markers for head and neck squamous cell carcinoma after radiotherapy. Black colour: subgroup of $SMR3A^{high}$ $OPRPN^{high}$ has poor DSS and PFS; Grey colour: other subgroups have improved DSS and PFS. SMR3A, submaxillary gland androgen-regulated protein 3A; DSS, disease-specific survival; PFS, progression-free survival.

Nevertheless, the predictive power of OPRPN appears to be minor and mitigates the impact of SMR3A. Furthermore, an upregulation of both the opiorphin family members was observed, which means that both markers are affected by standard HNSCC treatment such as fractionated IR and respond by upregulation of expression levels. The correlation between OPRPN expression pattern and clinical outcome of patients with OPSCC missed statistical significance. However, combined expression of OPRPN and SMR3A was significantly associated with unfavorable clinical prognosis post definitive or adjuvant radiotherapy, indicating opiorphin-related genes serve as a surrogate marker for HNSCC cells with intrinsic radioresistance (Fig. 6).

In conclusion, to the best of our knowledge, this is the first study to provide the experimental evidence for a predictive but probably antagonistic role of opiorphin genes in OPSCC after radiotherapy. Although the patient number was small, the subgroup with low OPRPN and high SMR3A expression presented the worst outcome in terms of DSS and PFS. SMR3A and OPRPN are likely to serve as potential prognostic markers in HNSCC. As there is a severe lack of stable and reliable predictive and prognostic biomarkers in HNSCC due to the heterogeneity of this entity, validating the impact of OPRPN proteins is also a worthy subject for studies with larger patient collectives.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

CR, PKP and AA conceptualized and designed the study. PKP and AA supervised the study. CR, JG, JT, CLP, GM, DH, GD, JK, AL, CS, NR and AA curated raw data and wrote-reviewed and edited the manuscript. CR and AA confirm the authenticity of all the raw data. JG and JT conducted experimental investigation. CR and AA performed data analyses and visualization. All authors have read and agreed to the published version of the manuscript.

Ethics approval and consent to participate

Informed consent was obtained from all patients after approval (approval no. 176/2002) by the Ethics Committee of the Medical Faculty of the University of Heidelberg (Heidelberg, Germany).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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