High XRCC1-protein expression is associated with poorer survival in patients with head and neck squamous cell carcinoma

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Running Title
XRCC1- expression in head and neck squamous cell carcinoma

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**Statement of Translational Relevance**
There are few established prognostic factors in head and neck squamous cell carcinoma (HNSCC). p53 mutations in HNSCC have been shown to be associated with adverse prognosis, independent of tumor primary site while Human Papillomavirus (HPV) is a favorable prognostic factor in oropharyngeal HNSCC. Recent studies have shown that treatment outcomes in HNSCC may be modulated by a combination of prognostic factors. X-ray repair complementing defective repair in Chinese hamster cells 1 (XRCC1) protein is involved in DNA base excision and single strand break repair and may therefore modulate chemoradiation outcomes by affecting efficient DNA damage repair. Our study demonstrates that high XRCC1-protein expression is associated with poorer survival in HNSCC, particularly in patients undergoing chemoradiation. Furthermore, this association was independent of HPV status, as assessed by p16 immunohistochemistry, and importantly, XRCC1-protein expression status was able to discriminate within each p16 category. Taken together, as patients with high XRCC1-expression managed with chemoradiation may have poorer outcomes while still
susceptible to the same risk of toxicity, they may benefit from alternative management strategies.

**Abstract:**

**Purpose:** We evaluated X-ray repair complementing defective repair in Chinese hamster cells 1 (XRCC1) protein in head and neck squamous cell carcinoma (HNSCC) patients in association with outcome.

**Experimental Design:** XRCC1-protein expression was assessed by immunohistochemical (IHC) staining of pre-treatment tissue samples in 138 consecutive HNSCC patients treated with surgery (n=31), radiation (15), surgery and radiation (23), surgery and adjuvant chemoradiation (17), primary chemoradiation (51) and palliative measures (1).

**Results:** Patients with high XRCC1-expression by IHC (n=77) compared to patients with low XRCC1-expression (n=60) had poorer median overall survival (OS) (41.0 months vs. OS not reached, \(P =0.009\)) and poorer progression-free survival (28.0 months vs. 73 months, \(P =0.031\)). This association was primarily due to patients who received chemoradiation (median OS of high and low XRCC1-expression patients, 35.5 months and not reached respectively, Hazard ratio (HR) 3.48; 95% Confidence interval (CI), 1.44-8.38; \(P =0.006\)). In patients treated with non-chemoradiation modalities, there was no survival difference by XRCC1-expression. In multivariable analysis, high XRCC1-expression and p16\(^{INK4a}\)-positive status were independently associated with survival in the overall study population (HR 2.62; 95% CI, 1.52-4.52; \(P <0.001\) and HR 0.21; 95%
CI 0.06-0.71; \( P = 0.012 \) respectively) and among chemoradiation patients (HR 6.02; 95% CI 2.36-15.37; \( P < 0.001 \) and HR 0.26; 95% CI, 0.08-0.92 respectively; \( P = 0.037 \)).

**Conclusions:** In HNSCC, high XRCC1-protein expression is associated with poorer survival, particularly in patients receiving chemoradiation. Future validation of these findings may enable identification of HNSCC–expressing patients who benefit from chemoradiation treatment.
Head and neck squamous cell carcinoma (HNSCC) is diagnosed in approximately half a million individuals worldwide annually, accounting for 5% of malignancies and >4% of cancer deaths\(^1\). Over the past decade, concomitant chemoradiation (CRT) has emerged as the standard of care in locally advanced HNSCC for organ preservation, treatment of unresectable disease and as adjuvant therapy in resected high risk disease\(^2\). However, a proportion of patients will not respond to CRT. Furthermore, CRT has significant short and long term treatment-related morbidities and complications. Identification of markers which predict for sensitivity to CRT will help select patients who benefit from it, while avoiding toxicity among non-responders.

Resistance to CRT may occur due to increased tolerance to DNA damage resulting from a highly efficient DNA repair capacity. X-ray repair complementing defective repair in Chinese hamster cells 1 (XRCC1) protein is involved in base excision repair (BER) and single strand break (SSB) repair by acting as a scaffold for BER/SSB repair protein complexes\(^3,4\). In preclinical studies, changes in XRCC1 gene\(^4,5\) and protein\(^6\) expression alters the sensitivity of cells to radiation and chemotherapeutic agents such as cisplatin. Clinical studies of cervical carcinoma treated with neoadjuvant platinum-based chemotherapy and laryngeal cancer treated with radiation have reported better outcomes in patients with low XRCC1-protein expression\(^7,8\). Presence of XRCC1 single nucleotide polymorphisms (SNPs) have been shown to be associated with cancer susceptibility\(^9-11\) as well as treatment outcomes after platinum-based CRT, induction chemotherapy, and radiotherapy\(^12-17\).
We therefore investigated XRCC1-protein expression and its relationship to clinical factors and treatment outcomes in HNSCC. The specificity of the XRCC1 antibody was demonstrated. In addition, 3 germline XRCC1 SNPs, which are known to be associated with HNSCC susceptibility, were evaluated to determine their association with XRCC1-protein expression and treatment outcome.

**Methods**

**Patients and treatments**

The Carolina Head and Neck Cancer (CHANCE) study was a population-based case-control study of incident HNSCC conducted from 2002 to 2006 in North Carolina\(^{18}\). All patients enrolled in the CHANCE study and treated at the University of North Carolina at Chapel Hill (UNC-CH) were included in our study. Clinical information was abstracted from patient medical records. Survival data were obtained from patient medical records, the Social Security Death Index, and local obituaries. Treatment decisions were recommended by the UNC Head and Neck multidisciplinary team, and individualized according to patient age, tumor extent, site, comorbidities, and performance status. The response to treatment was assessed radiologically at 6-12 weeks after treatment completion and recorded as: 1) complete response to non-surgical treatment; 2) persistent disease after non-surgical treatment; 3) complete surgical excision with clear margins; 4) surgical excision with positive margins which were subsequently resected; and 5) surgical excision with positive margins which were not resected. This study was reviewed and approved by the University of North Carolina Biomedical Institutional Review Board (IRB).
Tissue microarray preparation

Tissue microarrays (TMAs) were constructed from formalin-fixed paraffin-embedded tumor blocks. Hematoxylin and eosin sections of all patients’ tumors were reviewed by one pathologist (W.K.F.) to confirm the original diagnosis, and a target area was identified in the donor block. TMAs were constructed using 1 mm cores on the manual tissue microarrayer-1 from Beecher Instruments and were made in triplicate to account for potential staining heterogeneity and potential loss of tissue during processing. Sequential 4μm sections were cut from each TMA.

Human Papillomavirus (HPV) in-situ-hybridization

Slide deparaffinization, conditioning, and staining with INFORM HPV III Family 16 Probe (B) (Ventana Medical Systems, Tucson, AZ) was performed on the Ventana Benchmark XT Autostainer according to the manufacturer’s protocol. The probes have affinities to HPV genotypes 16,18,31,33,35,39,45,51,52,56,58 and 66. Slides were scored as positive for HPV if a punctate or diffuse pattern of signal were observed in the tumor nuclei.

Immunohistochemical staining for XRCC1 and p16INK4a

Immunohistochemical staining (IHC) of XRCC1-protein and of the CDK-inhibitor p16INK4a protein- a biomarker of HPV- E7 oncoprotein activity was carried out in the Bond Autostainer (Leica Microsystems Inc., Norwell, MA) according to the manufacturer’s protocol. Briefly, slides were dewaxed in Bond Dewax solution
(AR9222) and hydrated in Bond Wash solution (AR9590). Antigen retrieval for XRCC1 was performed for twenty minutes at 100°C in Bond-Epitope Retrieval solution 2 (pH 9.0, AR9640). Slides were incubated with XRCC1 antibody (H-300; dilution 1:100, Santa Cruz Biotechnology, Inc., CA) for one hour. Breast cancer tissue was used as a positive control. Antigen retrieval for p16\textsuperscript{INK4a} was performed for thirty minutes at 100°C in Bond-Epitope Retrieval solution 1 (pH 6.0, AR9961) after which slides were incubated with p16\textsuperscript{INK4a} antibody (Mouse monoclonal anti-p16 antibody MAB4133, Chemicon\textsuperscript{®} International/ Millipore Corporation, Temecula, CA) for fifteen minutes. Antibody detection for XRCC1 and p16\textsuperscript{INK4a} was performed using the Bond Polymer Refine Detection System (DS9800). Image acquisition was performed using ScanScope\textsuperscript{®} CS (Aperio Technologies, Vista, CA). After completion of IHC, slides are stored at room temperature in our laboratory and a virtual scanned copy of all TMA slides will be kept indefinitely.

Evaluation of XRCC1-protein expression and p16\textsuperscript{INK4a}-expression

XRCC1 and p16\textsuperscript{INK4a} staining was assessed by a pathologist (K.F.) with no prior knowledge of the clinical data. The percentage of tumor cells with positive nuclei was determined by scoring 10 microscopic fields of 100 tumor cells each. Samples were also scored for the intensity of staining from 0 (no staining) to 3+ (strong staining). The Allred score was derived from the percentage of XRCC1-staining tumor cells and staining intensity\textsuperscript{20}. Using the median Allred score as the cutoff, XRCC1-expression status was dichotomized into high and low expression. Tumor p16\textsuperscript{INK4a}-
expression was dichotomized as p16\textsuperscript{INK4a}-positive (strong nuclear or cytoplasmic staining in \geq 70\% tumor cells) or p16\textsuperscript{INK4a}-negative.

**RNAi-mediated knockdown of XRCC1**

UM38 head and neck cancer cell lines grown in DMEM medium containing 10 \% fetal bovine serum, 100 U/ml penicillin, 100 U/ml streptomycin, and 2 mM L-glutamine were used to examine XRCC1 knockdown using ON-TARGET\textsuperscript{plus} siRNA SMART pool or Scrambled control from Dharmacon, Inc. (Lafayette, CO). Lipofectamine LTX Plus (Invitrogen, Carlsbad, CA) in OptiMEM medium was used as a transfecting agent (vehicle). Cells were incubated with XRCC1 siRNA for 48h for mRNA and 72 h for protein detection. Controls included untreated cells, vehicle alone treated, and Scrambled siRNA treated groups. At the end of 48 h incubation with siRNA or controls, mRNA was isolated from the cells using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). After NanoDrop quantification, cDNA was prepared (iScript cDNA, Bio-Rad, Hercules, CA). Quantitative RT-PCR (qPCR) was carried out using an XRCC1 Assay on Demand (Applied Biosystems) on a LightCycler\textsuperscript{®} 480 (Roche Applied Science, Indianapolis, IN) using 18S as an internal control. For XRCC1 protein expression, at the end of 72 h of incubation with siRNA, cells were lysed with RIPA buffer containing protease and phosphatase inhibitors and protein content was quantified by BCA assay (Thermo Scientific, Rockford, IL). Western immunoblot analysis was carried out by probing with rabbit anti-XRCC1 primary antibody (1:5000) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) with an internal loading control (mouse anti-Actin, mAb1501) (1:10,000).
(Chemicon, Billerica, MA) followed by secondary antibodies goat anti-rabbit IgG and goat anti-mouse IgG (1:10,000), respectively. Positive control for Westerns was 293T lysate which over-expressed mouse XRCC1, commercially available (Santa Cruz Biotechnology Inc.).

**Immunocytochemical analysis of RNAi-mediated knockdown of XRCC1 in UM38 cells to demonstrate specificity of the antibody against native XRCC1**

UM38 cells were transfected as above and 72 hours after treatment, cells were spun onto slides using cytospin, permeabilized with 1% triton-X-100, and stained using anti-XRCC1 (Santa Cruz Biotechnology Inc., 1:500) and FITC conjugated goat anti-rabbit secondary (Santa Cruz, 1:200). DAPI was used to stain nuclei. At least five digital photomicrographs of each condition were captured using a Nikon Eclipse TS100 equipped with a digital camera using TSView7 software, Version 6.0.2.1 (Tucsen Imaging Technology, Fujian, China). Two representative 20X images for each condition are shown. Images were merged using ImageJ software. Whole 20X fields were quantified for fluorescent FITC intensity using ImageJ.

**Western immunoblot analysis of XRCC1 protein in patient specimens**

As additional controls for the specificity of the XRCC1 antibody used in the current study, we identified 4 cases (2 high and 2 low expression) in which patients from the TMA cohort had separately banked frozen tissue suitable for Western immunoblot
analysis of XRCC1 protein from tumor lysates. Frozen tumors from patients were lysed in RIPA, run on 10% SDS-PAGE gels, and transferred to nitrocellulose and probed along with positive control lysate as above.

**DNA extraction and genotyping**

3 SNPs in XRCC1 were evaluated: RS1799782, RS25496 and RS 2682558 as part of the CHANCE study. Genomic DNA was obtained for genotyping from patients’ peripheral blood by a salt precipitation method using Gentra’s Puregene chemistries. Genotyping was done at the UNC-CH Mammalian Genotyping Core Facility, using the Illumina GoldenGate genotyping assay. Blind duplicates of 109 samples were genotyped to verify reliability of genotype calls.

**Statistical analyses**

Baseline characteristics of patients with high versus low XRCC1-expression were compared using Fisher’s exact test for discrete variables and the independent samples T-test for continuous variables. Patients with no available XRCC1 data were excluded from all study analyses. The primary objective was to determine the association between overall survival (OS) and XRCC1-protein expression. Secondary objectives were firstly to determine the association of XRCC1-protein expression with progression-free survival (PFS) and the response to primary radiation (RT) and primary chemoradiation (CRT); secondly, to determine the association of XRCC1 SNPs with protein expression and OS; and thirdly, to evaluate the relationship of XRCC1-gene expression with OS, tumor site and treatment.
OS was defined as the time from diagnosis to date of death from all causes, or censored at the last documented follow-up date. Progression-free survival (PFS) was calculated from the date of diagnosis to the date of disease progression, or death from all causes, or censored at the last documented follow-up date. Distributions of OS and PFS were estimated using the Kaplan-Meier method and log rank statistics were used to assess differences between survival curves. Univariate and multivariable analyses of the endpoints of mortality and progression or death were performed using Cox proportional hazards logistic regression models. Factors included in the multivariable model were any variable(s) with significant univariate associations with mortality, as well as tumor site, stage, XRCC1-expression status and p16\(^{\text{INK4a}}\)-expression status. The same analyses were then repeated for the subgroups of patients treated with primary or adjuvant CRT (“CRT cohort”) and with non-CRT modalities (consisting of surgery only, radiation only or surgery plus radiation; “non-CRT cohort”).

In the event XRCC1 and p16\(^{\text{INK4a}}\)-protein expression were both independently associated with mortality, we would divide our study population into 4 groups based on their expression status of either marker as follows: 1) p16\(^{\text{INK4a}}\)-negative and XRCC1-high, 2) p16\(^{\text{INK4a}}\)-positive and XRCC1-high, 3) p16\(^{\text{INK4a}}\)-negative and XRCC1-low and 4) p16\(^{\text{INK4a}}\)-positive and XRCC1-low. Survival of these 4 groups would be evaluated.
All reported $P$-values are two-sided and $P < 0.05$ was considered significant. Kaplan-Meier analyses were performed using R version 2.5.1, all other statistical analyses were performed with SPSS software (version 18, SPSS Inc, Chicago, IL).

**Results**

**Patient Characteristics**

143 patients were treated at UNC-CH during the CHANCE study. Five patients with no available XRCC1 data were excluded. The characteristics of the remaining 138 patients are listed in Table 1. Of these, 137 patients with non-metastatic disease were treated with curative intent, 1 patient had metastatic (stage IVc) disease and was treated palliatively. This patient was excluded from further analyses. Patients who underwent primary or adjuvant CRT/RT received a median radiation dose of 70Gy (range 60-74Gy) and 66Gy (range 50-70Gy) respectively. Of patients who underwent concurrent CRT, the majority (n=64, 94%) received platinum-based chemotherapy, 2 patients received non-platinum treatments (1 with non-platinum chemotherapy and 1 with cetuximab), and in 2 patients (3%), information on the chemotherapy used was not available.

**HPV-ISH and p16 immunostaining**

There were 13 p16-positive cases in the entire cohort (9.5%), and amongst oropharynx cases, 11/38 (28.9%) were p16-positive. P16 status was significantly associated with oropharyngeal tumor site ($P<0.001$). HPV infection and p16 expression status were significantly associated, agreeing in 125 cases (92%; $P<0.001$).
Evaluation of XRCC1 antibody

We demonstrated specificity and sensitivity of the antibody against XRCC1 using patient samples and a head and neck cancer cell line (Supplemental Figure 1). Using anti-XRCC1 in Western immunoblot analysis of XRCC1 protein from frozen patient tumor lysates identified from TMA data as high or low XRCC1 expressers (and confirmed by mRNA expression analysis, data not shown), we detected two bands at 90 and 75-80kDa which correlated with bands in positive control 293T cells overexpressing XRCC1. Elevated levels of XRCC1 are confirmed in tumors a priori identified as “high” XRCC1 expressers, which supports IHC presented herein. Next, XRCC1-specific mRNA knockdown was achieved with an 89% reduction compared to scrambled siRNA in UM38 head and neck cells using RNAi-mediated knockdown reagents. Using this experimental paradigm, immunoblot and immunocytochemical (ICC) detection of XRCC1 was undertaken in control and siRNA- knockdown conditions in UM38 cells. Western immunoblot demonstrated 47-78% knockdown of XRCC1 bands identified in UM38 cells and 293T over-expressing XRCC1 positive controls (80 and 90kDa, respectively). Anti-XRCC1 ICC clearly detected XRCC1 positive FITC staining in vehicle (not shown) and Scrambled RNAi-treated UM38 cells. Most striking, XRCC1 positive staining is reduced to essentially undetectable levels in cells with XRCC1 knockdown. Taken together, these data suggest that anti-XRCC1 is indeed identifying XRCC1 protein in human tumor samples and human head and neck protein lysates, as well as conditions where staining is lost in cells with native XRCC1 depleted demonstrating specificity of this antibody.
Clinical pathological data and XRCC1-protein expression

Using IHC, XRCC1 showed baseline nuclear staining in almost all patients (n=135, 97.8%). XRCC1-protein expression was more prominent in the parabasal cells in the lower half of the epithelium at lower staining intensities (1+, 2+), while at high staining intensity (3+), XRCC1-expression was present throughout the epithelium (Figure 1A). The median percentage of positive tumor cells was 86.7% and median staining intensity was 3+. The distribution of Allred scores was heavily skewed with a median Allred score of 8 (range 0-8, Figure 1B). Using this median score as a cutoff, there were 77 and 61 patients in the high (score = 8) and low (score < 8) XRCC1-expression groups respectively. There was no significant difference in age, gender, smoking status, tumor site, and stage between the high and low XRCC1-expression groups (Table 1).

Treatment modality and XRCC1-expression

Patients in the CRT group (n=68), as compared with the non-CRT group (n=69), were younger (age <60yrs, 69% vs. 46%, \( P =0.009 \)), more likely to be male (78% vs. 58%, \( P =0.017 \)), and had more advanced stage tumors (93% vs. 43%, \( P <0.001 \)). Compared with the non-CRT group, more CRT group patients had oropharynx or hypopharynx tumors, while less had oral cavity tumors (\( P <0.001 \)). There was a trend towards a higher proportion of CRT group patients having high XRCC1-expression compared with non-CRT group patients (65% vs. 48% respectively, \( P =0.058 \), Supplementary Data Table 1).

Treatment response and XRCC1-protein expression
46 of 66 (70%) patients treated with primary CRT and primary RT had a complete response (CR) to treatment; 16 (24%) had persistent disease. Response status was unknown in 4 patients. There was no relationship between treatment response and XRCC1-expression status (CR 73% and 75% for low and high XRCC1-expression respectively, \( P =0.488 \)). Assessing primary CRT patients independently, there was still no relationship between XRCC1-expression and response (CR 73% and 78% for low and high XRCC1-expression, \( P =0.540 \)).

Survival and XRCC1-protein expression

The median follow-up was 66.0 months (range 39.0-87.0 months). In the overall study population, median OS was 73.0 months (95% confidence interval (CI) 47.0 months – not reached (NR)), 5-year OS was 58% and median PFS was 47.0 months (95% CI 29.0 months- NR). High expression of XRCC1 was significantly associated with adverse OS. Median OS for patients with high XRCC1-expression was 41.0 months (95% CI 28.0 months- NR) while median OS was not reached (95% CI 73.0 months- NR) for patients with low XRCC1-expression (\( P =0.009 \)). Stratifying the cohort by treatment modality (CRT vs. non-CRT), we found the association of XRCC1-expression with OS was related to the treatment group. Of patients who received CRT, median OS for patients with high XRCC1-expression was 35.5 months (95% CI 28.0-66.0 months) while median OS was not reached (95% CI 68.0 months- NR) for patients with low XRCC1-expression (\( P =0.003 \)). 5-year OS was 35% and 79% in high and low XRCC1-expression groups respectively. In contrast, in patients treated with non-CRT modalities, there was no significant difference in OS between high and low XRCC1-expression patients, with 5-
year OS of 58% and 60% respectively ($P = 0.674$). Figures 2A, 2B and 2C show the Kaplan-Meier curves for OS in the overall, CRT and non-CRT cohorts respectively.

As with OS, patients with high XRCC1-expression had poorer median PFS compared to patients with low XRCC1-expression (28.0 months (95% CI 20.0-66.0 months) and 73.0 months (95% CI 47.0- NR) respectively, $P = 0.031$; Figure 2D). This association of XRCC1-expression with PFS was once again accounted for by the subgroup of patients who received CRT. In this group, PFS was 28.0 months (95%CI 17.0–62.0 months) in patients with high XRCC1-expression and was not reached (95% CI 59.0 months- NR) in patients with low XRCC1-expression ($P = 0.022$, Figure 2E). 5-year PFS was 33% and 62% in XRCC1 high and low expression groups respectively. In patients treated with non-CRT modalities, there was no significant difference in PFS between high and low XRCC1-expression patients, with 5-year PFS of 48% and 52% respectively ($P = 0.577$, Figure 2F).

**Univariate and Multivariable analyses**

The univariate relationships with mortality are shown in Table 2. In the whole cohort, T3-T4 tumors, advanced stage (stage III/IV) disease and high XRCC1-protein expression were associated with increased risk of death (HR 1.94; $P = 0.010$; HR 1.79; $P = 0.049$ and HR 1.97; $P = 0.011$ respectively). In the CRT cohort, only XRCC1-protein expression was associated with mortality (HR 3.48; $P = 0.006$). There was no association of disease stage with survival, however, only 5 patients had early stage disease in the CRT group.
In a multivariable Cox regression model (Table 3), in the overall study population, high XRCC1-expression was independently associated with mortality (HR 2.62; 95% CI 1.52-4.52; \( P < 0.001 \)). Late stage disease (stage III/IV) and positive \( p16^{\text{INK4a}} \) status were also associated with mortality (HR 1.86, 95% CI 1.02-3.39; \( P = 0.043 \) and HR 0.21; 95% CI 0.06-0.71; \( P = 0.012 \) respectively). In addition, high XRCC1-expression was independently associated with increased risk of disease progression or death (HR 2.04; 95% CI 1.25-3.33; \( P = 0.004 \)).

Restricting the multivariable analysis to the CRT cohort, XRCC1-expression status remained strongly associated with the endpoints of mortality as well as disease progression or death, independent of stage, tumor site and \( p16^{\text{INK4a}} \)-expression status (HR 6.02; 95% CI, 2.36-15.37; \( P < 0.001 \) and HR 3.37; 95% CI 1.57-7.23; \( P = 0.002 \) respectively, Table 3). In contrast, in the non-CRT cohort, there was no association of XRCC1-expression status with mortality (HR 1.69; 95% CI, 0.77-3.72; \( P = 0.189 \)).

Relationship of XRCC1 and \( p16^{\text{INK4a}} \)-expression status with survival

Amongst the 4 groups as previously defined, survival was lowest in the \( p16^{\text{INK4a}} \)-negative/ XRCC1-high group (n=64), and highest in the \( p16^{\text{INK4a}} \)-positive/ XRCC1-low group (n=2) (5-year OS 35% and 100% respectively, \( P = 0.001 \)). Among patients who were either \( p16^{\text{INK4a}} \)-positive/XRCC1-high (n=11) or \( p16^{\text{INK4a}} \)-negative/XRCC1-low (n=59), 5-year OS was similar and intermediate (5-year OS 70% and 67% respectively, Figure 3). 1 patient with no available \( p16^{\text{INK4a}} \) data was excluded from this analysis.
SNP analysis

The frequency of the different gene polymorphisms are shown in Supplementary Table 2. No patients had minor alleles for RS25496. There was no relationship of RS1799782, a SNP encoding an amino acid change at position 194, and XRCC1-protein expression, as measured by the Allred score. Presence of at least one minor allele for RS2682558 was associated with lower XRCC1-protein expression compared with the presence of only common alleles, suggesting that perhaps this allele located in the 3′UTR is associated with a regulatory element for the gene (Allred score 7.31 vs. 6.56, \( P = 0.038 \)). 27/137 (19%) patients did not have available data for SNP analysis.

There was no relationship of RS1799782 with mortality in the whole cohort. Among patients who underwent CRT, RS1799782 showed a trend towards increased mortality (Table 2) and was significantly associated with disease progression or death (HR 2.77; 95% CI 1.05 -7.29; \( P =0.040 \)). While these data are insufficient to support that the Arg194Trp allele encodes a protein with increased activity, the phenotype associated with Arg194Trp mirrors that of increased protein abundance. In parallel, patients with at least one variant allele for RS2682558 had lower mortality (Table 2) and lower disease progression or death compared with patients with only common alleles, consistent with lower XRCC1-protein expression, but this was not statistically significant (median OS 69m vs. NR respectively, \( P =0.233 \) for whole cohort and OS 63m vs. NR respectively, \( P =0.190 \) for CRT cohort).

Discussion
In this study, we investigated the expression of XRCC1 in HNSCC patients and its association with clinicopathological factors and outcome. Our results demonstrate that XRCC1-protein expression is common in HNSCC, and that high XRCC1-protein expression, regardless of primary tumor site, stage and p16INK4a status, confers poorer survival as compared to low XRCC1-expression. Furthermore, this association was strongest in the subgroup of patients who received CRT.

XRCC1 facilitates efficient DNA damage processing and is therefore especially pertinent in patients undergoing CRT\textsuperscript{3,4,21}. Ionizing radiation kills cells by inducing DNA damage such as base damage, SSB, double-strand breaks, and inter-strand DNA cross-links. Chemotherapy, particularly platinum-based chemotherapy, binds to DNA forming DNA adducts, which distort DNA structure, causing damage and cell death. Combining chemotherapy with radiation greatly increases the overall cytotoxic effect of radiotherapy by inducing further DNA damage and interfering with DNA repair. This is mitigated to a certain extent by non-specific DNA repair systems such as nucleotide excision repair (NER) and BER multi-step enzymatic complexes. Therefore, high XRCC1-expression may increase the DNA repair capacity of tumor cells leading to increased tolerance to DNA damage from CRT. Our study findings of high XRCC1-protein expression being associated with poorer survival after CRT are consistent with this hypothesis as well as previous reports\textsuperscript{7,8}. Our study also demonstrates that the coding SNP Arg194Trp is associated with treatment outcome amongst CRT patients, whilst the non-coding RS2682558 in the 3’ UTR may have a regulatory role and thus affect XRCC1-protein
expression levels. Other studies have shown that XRCC1 Arg194Trp was associated with treatment response to platinum-based chemotherapy, and may predict for PFS\textsuperscript{16,17}.

Despite the strong association between XRCC1-protein expression status and survival, we were not able to demonstrate any relationship between XRCC1-expression and response to primary CRT or RT. Response to CRT/RT was assessed 6-12 weeks after treatment completion, and categorized as either complete response or persistent disease. From a clinical standpoint, this was to facilitate planning for early surgical salvage of patients with residual disease. However, assessment of response is often difficult in HNSCC where imaging may not always be accurate, particularly in the immediate post-radiation period. Furthermore, some patients take longer to respond completely to CRT/RT and may therefore have been inappropriately categorized as persistent disease at the 6-12 week post-treatment time point.

Presence of HPV has been established as a favorable prognostic factor in oropharyngeal HNSCC\textsuperscript{19,22,23}. p16\textsuperscript{INK4a} IHC has been shown to correlate with HPV status\textsuperscript{19,22} and patients with HPV-DNA positive and p16-expressing tumors in particular, have a favorable prognosis\textsuperscript{23}. Consistent with previous studies, our results showed that p16\textsuperscript{INK4a}-positive status was associated with reduced risk of death. Interestingly, XRCC1-expression status was able to further discriminate within each p16\textsuperscript{INK4a} category. Among p16\textsuperscript{INK4a}-positive patients, who were expected to have good prognosis, patients with high XRCC1-expression had poorer survival compared to patients with low XRCC1-expression. Furthermore, survival was similar between patients who were p16\textsuperscript{INK4a}-
negative/XRCC1-low and p16\textsuperscript{INK4a}-positive/XRCC1-high, implying XRCC1-expression status has significant prognostic implications. These findings are consistent with recent reports of subsets of HPV-positive patients with differing survival outcomes, depending on factors such as Bcl2 expression, EGFR expression, and smoking history\textsuperscript{19,22,24-26}.

Nevertheless, other DNA repair enzymes may also influence treatment outcomes and require consideration. Poly (ADP-ribose) polymerase (PARP)-1 is involved in DNA repair, and is required for the assembly and stability of XRCC1\textsuperscript{27,28}. ERCC1 is one of the key rate-limiting enzymes involved in NER and studies in HNSCC have shown that low ERCC1 levels are associated with good outcomes from CRT\textsuperscript{39} and induction chemotherapy\textsuperscript{30}. Enzymes that modulate the availability of platinum compounds (such as glutathione S-transferase \(\pi\)), or modulate cell death and apoptosis (Bcl-2 and p53) have also been reported to affect the treatment outcomes in HNSCC\textsuperscript{27,31-35}.

This study had several limitations. First, the study was retrospective, and comprised a heterogeneous cohort of HNSCC patients of different stages, tumor sites, and treatments, although the baseline characteristics of patients in the high and low XRCC1-expression cohorts were similar. Second, the study population was divided into high and low XRCC1-expression cohorts using the population median score as the cutoff point. We believe that this use of the Allred score is clinically relevant because it defines two distinct patient populations with different IHC staining patterns of XRCC1-expression: 1) homogenous and intense staining throughout the epithelium; and 2) heterogeneous (average of <67% of tumor cells stained) and/or lower intensity staining (average
intensity <3). This makes XRCC1 staining evaluation a potentially useful biomarker in HNSCC as there is little ambiguity with regards to high versus low XRCC1-expression status. Third, only one antibody was used for XRCC1 IHC, however, we demonstrated its specificity for XRCC1 using Western immunoblot of patient tumors, and Western and immunocytochemical analysis of RNAi-mediated knockdown of XRCC1 in a head and neck cancer cell line. Our data demonstrating loss of native XRCC1 detection after RNAi support that this antibody indeed is detecting specifically XRCC1. Fourth, the non-CRT cohort consisted of patients treated with three different treatment modalities and thus it may have been inappropriate to analyze them as a single group. Fifth, we did not have information on tumor differentiation and were unable to correlate XRCC1-expression to tumor differentiation or assess its effect on survival.

Taken together, our findings presented herein suggest that XRCC1 may be an important biomarker in HNSCC. Assessment of XRCC1-protein expression status by IHC may be useful in clinical decision-making. Patients with high expression may have poorer outcomes from CRT while still being subject to toxicity and thus could be directed to alternative treatment modalities and/or clinical trials. Conversely, patients with low XRCC1-expression may benefit from CRT, particularly if they are also p16INK4a-positive. Future studies will be required to define the role of XRCC1, as a prognostic or predictive marker, in different tumor sites and for different treatment modalities; and also to address the interactions and roles that different repair proteins and prognostic markers may have in determining the outcome of CRT in HNSCC.
References


Figures

Figure 1A. Representative examples of XRCC1 immunostaining in head and neck squamous cell carcinoma at different staining intensities. (A) intensity 0 (B) intensity 1+ (C) intensity 2+ (D) intensity 3+. Magnification 200x.

Figure 1B. Distribution of XRCC1 Allred score (range 0-8) in the overall study population

Figure 2. Kaplan-Meier estimates of the probability of overall survival (A-C) and progression-free survival (D-F) according to XRCC1-expression in whole cohort (A,D), CRT cohort (B,E), and non-CRT cohort (C,F).

Figure 3. Kaplan-Meier estimates of the probability of overall survival in patients who are 1)p16INK4apositive, XRCC1-low; 2)p16INK4apositive, XRCC1-high; 3)p16INK4anegative, XRCC1-low and 4) p16INK4apositive, XRCC1-high.
Table 1. Patient characteristics in whole cohort and by XRCC1-expression status

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>All patients (n=138)</th>
<th>Low XRCC1 (n= 61)</th>
<th>High XRCC1 (n= 77)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number (%</td>
<td>Number (%)</td>
<td>Number (%)</td>
<td></td>
</tr>
<tr>
<td><strong>Age years</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>80 (58)</td>
<td>33 (54)</td>
<td>47 (61)</td>
<td>0.488</td>
</tr>
<tr>
<td>≥60</td>
<td>58 (42)</td>
<td>28 (46)</td>
<td>30 (39)</td>
<td></td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>94 (68)</td>
<td>43 (70)</td>
<td>51 (66)</td>
<td>0.713</td>
</tr>
<tr>
<td>Female</td>
<td>44 (32)</td>
<td>18 (30)</td>
<td>26 (34)</td>
<td></td>
</tr>
<tr>
<td><strong>Mean no. pack years smoked</strong></td>
<td>40 pk yrs</td>
<td>40 pk yrs</td>
<td>39 pk yrs</td>
<td>0.842</td>
</tr>
<tr>
<td>Smoking history</td>
<td>Yes</td>
<td>126 (91)</td>
<td>56 (92)</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>12 (9)</td>
<td>5 (8)</td>
<td></td>
</tr>
<tr>
<td><strong>Alcohol</strong></td>
<td>Yes</td>
<td>93 (67)</td>
<td>46 (75)</td>
<td>0.100</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>45 (33)</td>
<td>15 (25)</td>
<td></td>
</tr>
<tr>
<td><strong>Site</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larynx</td>
<td>35 (25)</td>
<td>12 (20)</td>
<td>23 (30)</td>
<td></td>
</tr>
<tr>
<td>Oral Cavity</td>
<td>57 (41)</td>
<td>31 (51)</td>
<td>26 (34)</td>
<td>0.235</td>
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<tr>
<td>Oropharynx</td>
<td>38 (28)</td>
<td>15 (24)</td>
<td>23 (30)</td>
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<tr>
<td>Hypopharynx</td>
<td>8 (6)</td>
<td>3 (5)</td>
<td>5 (6)</td>
<td></td>
</tr>
<tr>
<td><strong>Tumor status</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1-2</td>
<td>67 (49)</td>
<td>30 (49)</td>
<td>37 (48)</td>
<td>1.000</td>
</tr>
<tr>
<td>T3-4</td>
<td>71 (51)</td>
<td>31 (51)</td>
<td>40 (52)</td>
<td></td>
</tr>
<tr>
<td><strong>Nodal status</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0-1</td>
<td>81 (59)</td>
<td>37 (61)</td>
<td>44 (57)</td>
<td>0.729</td>
</tr>
<tr>
<td>N2-3</td>
<td>57 (41)</td>
<td>24 (39)</td>
<td>33 (43)</td>
<td></td>
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<tr>
<td><strong>Stage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early (I,II)</td>
<td>44 (32)</td>
<td>20 (33)</td>
<td>24 (31)</td>
<td>0.856</td>
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<tr>
<td>Late (III,IV)</td>
<td>94 (68)</td>
<td>41 (67)</td>
<td>53 (69)</td>
<td></td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surgery</td>
<td>31 (22)</td>
<td>18 (29)</td>
<td>13 (17)</td>
<td>0.090</td>
</tr>
<tr>
<td>Radiation</td>
<td>15 (11)</td>
<td>7 (11)</td>
<td>8 (10)</td>
<td></td>
</tr>
<tr>
<td>Surgery + RT</td>
<td>23 (17)</td>
<td>11 (18)</td>
<td>12 (16)</td>
<td></td>
</tr>
<tr>
<td>Surgery + CRT</td>
<td>17 (12)</td>
<td>9 (15)</td>
<td>8 (10)</td>
<td></td>
</tr>
<tr>
<td>Primary CRT</td>
<td>51 (37)</td>
<td>15 (25)</td>
<td>36 (47)</td>
<td></td>
</tr>
<tr>
<td>Palliative</td>
<td>1 (1)</td>
<td>1(2)</td>
<td>0</td>
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</table>

Abbreviations: Pk yrs- pack years, RT- radiation, CRT- chemoradiation
Figure 1B. Distribution of XRCC1 Allred score (range 0-8) in the overall study population.
**Figure 2 (A-F)**
Kaplan-Meier estimates of the probability of overall survival (A-C) and progression-free survival (D-F) according to XRCC1-expression in whole cohort (A,D), CRT cohort (B,E), and non-CRT cohort (C,F).
A

Overall survival probability in whole cohort

Low XRCC1

High XRCC1

P = 0.009

Months

0

40

60

80

100
Overall survival probability in CRT-treated patients with low or high XRCC1 expression. The graph shows a significant difference between the two groups, with a p-value of 0.003.
Progression-free survival probability in non-CRT treated patients

F

Low XRCC1

High XRCC1

P=0.577
Table 2. Univariate analyses of prognostic factors for mortality in the overall study population and CRT cohort

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Overall study population</th>
<th>CRT cohort</th>
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<tr>
<td></td>
<td>HR</td>
<td>95% CI</td>
</tr>
<tr>
<td>Age (years)</td>
<td>≥60/ &lt;60</td>
<td>1.11</td>
</tr>
<tr>
<td>Smoking status</td>
<td>Smoking history/ Nonsmoker</td>
<td>1.26</td>
</tr>
<tr>
<td>p16^{INK4a} Positive/ Negative</td>
<td>0.34</td>
<td>0.11-1.07</td>
</tr>
<tr>
<td>Site</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larynx</td>
<td>1.03</td>
<td>0.52-2.04</td>
</tr>
<tr>
<td>Oral Cavity</td>
<td>1.24</td>
<td>0.67-2.29</td>
</tr>
<tr>
<td>Hypopharynx</td>
<td>2.67</td>
<td>1.04-6.82</td>
</tr>
<tr>
<td>Oropharynx</td>
<td>1.0 (reference)</td>
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<tr>
<td>T stage</td>
<td>T3-T4/ T1-T2</td>
<td>1.94</td>
</tr>
<tr>
<td>N stage</td>
<td>N2-N3/ N0-N1</td>
<td>1.54</td>
</tr>
<tr>
<td>Stage</td>
<td>Late (III-IV)/ Early (I-II)</td>
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<tr>
<td>XRCC1 expression</td>
<td>High/ Low</td>
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<td>XRCC1 SNP</td>
<td>RS1799782</td>
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<td></td>
<td>RS2682558</td>
<td>0.69</td>
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</table>

Abbreviations: CRT-chemoradiation, HR- hazard ratio, CI- confidence interval, T- tumor, N- nodal
Table 3. Multivariate Cox regression analysis for mortality (‘OS’) and progression or mortality (‘PFS’) endpoints in the overall study population and CRT cohort

<table>
<thead>
<tr>
<th>Factor</th>
<th>Overall study population</th>
<th>CRT cohort</th>
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<tbody>
<tr>
<td></td>
<td>OS</td>
<td>PFS</td>
</tr>
<tr>
<td></td>
<td>HR (95%CI)</td>
<td>P</td>
</tr>
<tr>
<td>Site</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larynx</td>
<td>0.73 (0.36-1.51)</td>
<td>0.087</td>
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<tr>
<td>Oral Cavity</td>
<td>1.28 (0.66-2.47)</td>
<td>0.462</td>
</tr>
<tr>
<td>Hypopharynx</td>
<td>2.40 (0.93-6.20)</td>
<td>0.071</td>
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<tr>
<td>Oropharynx</td>
<td>1.00 (reference)</td>
<td>1.00</td>
</tr>
<tr>
<td>Late Stage</td>
<td>1.86 (1.02-3.39)</td>
<td>0.043</td>
</tr>
<tr>
<td>p16INK4a</td>
<td>0.21 (0.06-0.71)</td>
<td>0.012</td>
</tr>
<tr>
<td>High XRCC1</td>
<td>2.62 (1.52-4.52)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Abbreviations: CRT- chemoradiation, HR- hazard ratio, CI- confidence interval
Figure 3. Kaplan-Meier estimates of the probability of overall survival in patients who are (1) P16 positive, XRCC1-low; (2) P16 positive, XRCC1-high; (3) P16 negative, XRCC1-low and (4) P16 negative, XRCC1-high.
High XRCC1-protein expression is associated with poorer survival in patients with head and neck squamous cell carcinoma

Mei-Kim Ang, Mihir R Patel, Xiaoying Yin, et al.

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