High XRCC1 Protein Expression Is Associated with Poorer Survival in Patients with Head and Neck Squamous Cell Carcinoma

Mei-Kim Ang[1,10], **Mihir R. Patel**[6], **Xiao-Ying Yin**[1,6], **Sneha Sundaram**[1,8], **Karen Fritchie**[7], **Ni Zhao**[1,2], **Yufeng Liu**[9], **Alex J. Freemerman**[1,8], **Matthew D. Wilkerson**[1], **Vonn Walter**[1,2], **Mark C. Weissler**[6], **William W. Shockley**[6], **Marion E. Couch**[6], **Adam M. Zanation**[6], **Trevor Hackman**[6], **Bhishamjit S. Chera**[3], **Stephen L. Harris**[3], **C. Ryan Miller**[7], **Leigh B. Thorne**[7], **Michele C. Hayward**[1], **William K. Funkhouser**[7], **Andrew F. Olshan**[4], **Carol G. Shores**[6], **Liza Makowski**[1,8], and **D. Neil Hayes**[1,5]

[1]UNC Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina

[2]Department of Biostatistics, University of North Carolina, Chapel Hill, North Carolina

[3]Department of Radiation Oncology, University of North Carolina, Chapel Hill, North Carolina

[4]Department of Epidemiology, University of North Carolina, Chapel Hill, North Carolina

[5]Department of Medicine, Division of Hematology and Oncology, University of North Carolina, Chapel Hill, North Carolina

[6]Department of Otolaryngology, University of North Carolina, Chapel Hill, North Carolina

[7]Department of Pathology and Lab Medicine, UNC School of Medicine, University of North Carolina, Chapel Hill, North Carolina

[8]Department of Nutrition, Gillings School of Global Public Health, University of North Carolina, Chapel Hill, North Carolina

[9]Department of Statistics and Operations Research, Carolina Center of Genome Sciences, University of North Carolina, Chapel Hill, North Carolina

[10]Department of Medical Oncology, National Cancer Centre Singapore, Singapore

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.

Corresponding Author: D. Neil Hayes, UNC Lineberger Comprehensive Cancer Center, School of Medicine, CB # 7295, University of North Carolina at Chapel Hill, 450 West Drive, Chapel Hill, NC 27599. Phone: 919-966-3786; Fax: 919-966-1587; hayes@med.unc.edu.

Experimental Design—XRCC1 protein expression was assessed by immunohistochemical (IHC) staining of pretreatment 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 with 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. 73.0 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, HR 3.48; 95% CI: 1.44–8.38; $P = 0.006$). In patients treated with nonchemoradiation 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.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is diagnosed in approximately half a million individuals worldwide annually, accounting for 5% of malignancies and more than 4% of cancer deaths (1). Over the past decade, concomitant chemoradiation therapy (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 nonresponders.

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 (SNP) 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 shown. 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.

Materials and 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 to 12 weeks after treatment completion and recorded as follows: (i) complete response (CR) to nonsurgical treatment, (ii) persistent disease after nonsurgical treatment, (iii) complete surgical excision with clear margins, (iv) surgical excision with positive margins which were subsequently resected, and (v) 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.

Tissue microarray preparation

Tissue microarrays (TMA) were constructed from formalin-fixed paraffin-embedded tumor blocks. Hematoxylin and eosin sections of tumors of all patients 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 4-μm sections were cut from each TMA.

Human papillomavirus *in situ* hybridization

Slide deparaffinization, conditioning, and staining with INFORM HPV III Family 16 Probe (B; Ventana Medical Systems) were done on the Ventana Benchmark XT Autostainer according to the manufacturer's protocol. The probes have affinities to human papillomavirus (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 p16^{INK4a}

Immunohistochemical (IHC) staining of XRCC1 protein and of the CDK inhibitor p16^{INK4a} protein, a biomarker of HPV- E7 oncoprotein activity (19), was carried out in the Bond Autostainer (Leica Microsystems Inc.) 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 done for 20 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.) for 1 hour. Breast cancer tissue was used as a positive control. Antigen retrieval for p16^{INK4a} was done for 30 minutes at 100°C in Bond-Epitope Retrieval solution 1 (pH 6.0, AR9961) after which slides were incubated with p16^{INK4a} antibody (Mouse monoclonal anti-p16 antibody MAB4133, Chemicon Internationa/Millipore Corporation) for 15 minutes. Antibody detection for XRCC1 and p16^{INK4a} was done using the Bond Polymer Refine Detection System (DS9800). Image acquisition was done using ScanScope CS (Aperio Technologies). 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[INK4a] expression

XRCC1 and p16[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 (20). Using the median Allred score as the cutoff, XRCC1 expression status was dichotomized into high and low expression. Tumor p16[INK4a] expression was dichotomized as p16[INK4a] positive (strong nuclear or cytoplasmic staining in 70% or more tumor cells) or p16[INK4a] negative.

RNAi-mediated knockdown of XRCC1

UM38 head and neck cancer cell lines grown in Dulbecco's modied Eagle's medium containing 10% FBS, 100 U/mL penicillin, 100 U/mL streptomycin, and 2 mmol/L L-glutamine were used to examine XRCC1 knockdown using ON-TARGET*plus* siRNA SMART pool or scrambled control from Dharmacon, Inc. Lipofectamine LTX Plus (Invitrogen) in OptiMEM medium was used as a transfecting agent (vehicle). Cells were incubated with XRCC1 siRNA for 48 hours for mRNA and 72 hours for protein detection. Controls included untreated cells, vehicle alone treated, and scrambled siRNA-treated groups. At the end of 48 hours incubation with siRNA or controls, mRNA was isolated from the cells using the Qiagen RNeasy Mini Kit (Qiagen). After NanoDrop quantification, cDNA was prepared (iScript cDNA; BioRad). Quantitative reverse transcriptase PCR was carried out using an XRCC1 Assay on Demand (Applied Biosystems) on a LightCycler 480 (Roche Applied Science) using 18S as an internal control. For XRCC1 protein expression, at the end of 72 hours of incubation with siRNA, cells were lysed with radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors and protein content was quantified by BCA assay (Thermo Scientific). Western immunoblot analysis was carried out by probing with rabbit anti-XRCC1 primary antibody (1:5,000) (Santa Cruz Biotechnology Inc.) with an internal loading control (mouse anti-Actin, mAb1501l; 1:10,000; Chemicon, Billerica) 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 overexpressed mouse XRCC1, commercially available (Santa Cruz Biotechnology Inc.).

Immunocytochemical analysis of RNAi-mediated knockdown of XRCC1 in UM38 cells

To show 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 (1:500; Santa Cruz Biotechnology Inc.) and fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit secondary (Santa Cruz, 1:200). 4′, 6-diamidino-2-phenylindole was used to stain nuclei. At least 5 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). Two representative 20× images for each condition are shown. Images were merged using ImageJ software. Whole 20× 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 this 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

Three SNPs in XRCC1 were evaluated: RS1799782, RS25496, and RS 2682558 as part of the CHANCE study. Genomic DNA was obtained for genotyping from peripheral blood of patients 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, first, to determine the association of XRCC1 protein expression with progression-free survival (PFS) and the response to primary radiation (RT) and primary chemoradiation (CRT); and second, to determine the association of XRCC1 SNPs with protein expression and OS.

OS was defined as the time from diagnosis to date of death from all causes, or censored at the last documented follow-up date. 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 done 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 $p16^{INK4a}$ 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").

When XRCC1 and $p16^{INK4a}$ protein expression were both independently associated with mortality, we divided our study population into 4 groups on the basis of their expression status of either marker as follows: (i) $p16^{INK4a}$ negative and XRCC1 high, (ii) $p16^{INK4a}$ positive and XRCC1 high, (iii) $p16^{INK4a}$ negative and XRCC1 low, and (iv) $p16^{INK4a}$ positive and XRCC1 low. Survival of these 4 groups was evaluated.

All reported P values are 2-sided and $P < 0.05$ was considered significant. Kaplan–Meier analyses were done using R version 2.5.1, all other statistical analyses were done with SPSS software (version 18, SPSS Inc.).

Results

Patient characteristics

A total of 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 nonmetastatic 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 70 Gy (range 60–74 Gy) and 66 Gy (range 50–70 Gy), respectively. Of patients who underwent concurrent CRT, the majority (n = 64, 94%) received platinum-based chemotherapy, 2 patients received nonplatinum treatments (1 with nonplatinum chemotherapy and 1 with cetuximab), and in 2 patients (3%), information on the chemotherapy used was not available.

HPV and p16 immunostaining

There were 13 p16-positive cases in the entire cohort (9.5%), and among oropharynx cases, 11 of 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 showed specificity and sensitivity of the antibody against XRCC1 using patient samples and a head and neck cancer cell line (Supplementary Fig. S1). 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 2 bands at 90 and 75 to 80 kDa 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 with 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 showed 47% to 78% knockdown of XRCC1 bands identified in UM38 cells and 293T overexpressing XRCC1-positive controls (80 and 90 kDa, 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 in which staining is lost in cells with native XRCC1 depleted showing specificity of this antibody.

Clinical pathologic 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+), whereas at high staining intensity (3+), XRCC1 expression was present throughout the epithelium (Fig. 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, Fig. 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 <60 years, 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, whereas

less had oral cavity tumors ($P< 0.001$). There was a trend toward 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 Table S1).

Treatment response and XRCC1 protein expression

Forty-six of 66 (70%) patients treated with primary CRT and primary RT had a 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% 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), whereas 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), whereas median OS was not reached (95% CI: 68.0 months–NR) for patients with low XRCC1 expression ($P= 0.003$). Five-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–C 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 with 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; $P= 0.031$; Fig. 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 ($P= 0.022$; Fig. 2E). Five-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 ($P= 0.577$; Fig. 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^{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^{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^{INK4a} expression status with survival

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

SNP analysis

The frequency of the different gene polymorphisms are shown in Supplementary Table S2. 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 1 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′-untranslated region (UTR) is associated with a regulatory element for the gene (Allred score 7.31 vs. 6.56, $P = 0.038$). Twenty-seven of 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 toward increased mortality (Table 2) and was significantly associated with disease progression or death (HR = 2.77; 95% CI: 1.05–7.29; $P = 0.040$). Although 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 1 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 69 months vs. NR, respectively, $P = 0.233$ for whole cohort and OS 63 months 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 clinicopathologic factors and outcome. Our results show that XRCC1 protein expression is common in HNSCC and that high XRCC1 protein expression, regardless of primary tumor site, stage, and p16^{INK4a} status confers poorer survival as compared with 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 pertinentinpatientsundergoing CRT (3, 4, 21). Ionizing radiation kills cells by inducing DNA damage such as base damage, SSB, double-strand breaks, and interstrand 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 nonspecific DNA repairsystemssuchasnucleotideexcisionrepair(NER) and BER multistep 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 areconsistentwiththishypothesisaswellaspreviousreports (7, 8). Our study also shows that the coding SNP Arg194Trp is associated with treatment outcome among CRT patients, whereas the noncoding 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 (16, 17).

Despite the strong association between XRCC1 protein expression status and survival, we were not able to show any relationship between XRCC1 expression and response to primary CRT or RT. Response to CRT/RT was assessed 6 to 12 weeks after treatment completion, and categorized as either CR 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 in which imaging may not always be accurate, particularly in the immediate postradiation 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- to 12-week posttreatment time point.

Presence of HPV has been established as a favorable prognostic factor in oropharyngeal HNSCC (19, 22, 23). p16^INK4a IHC has been shown to correlate with HPV status (19,22) and patients with HPV-DNA positive and p16 expressing tumors, inparticular, have a favorable prognosis(23). Consistent with previous studies, our results showed that p16^INK4a-positive status was associated with reduced risk of death. Interestingly, XRCC1 expression status was able to further discriminate within each p16^INK4a category. Among p16^INK4a-positive patients, who were expected to have good prognosis, patients with high XRCC1 expression had poorer survival compared with patients with low XRCC1 expression. Furthermore, survival was similar between patients who were p16^INK4a negative/XRCC1 low and p16^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, epidermal growth factor receptor expression, and smoking history (19, 22, 24–26).

Nevertheless, other DNA repair enzymes may also influence treatment outcomes and require consideration. PARP-1 is involved in DNA repair and is required for the assembly and stability of XRCC1 (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 (29) and induction chemotherapy (30). Enzymes that modulate the availability of platinum compounds (such as glutathione S-transferase π) or modulate cell death and apoptosis (Bcl-2 and p53) have also been reported to affect the treatment outcomes in HNSCC (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 2 distinct patient populations with different IHC staining patterns of XRCC1 expression: (i) homogenous and intense staining throughout the epithelium; and (ii) 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 regard to high-versus low-XRCC1 expression status. Third, only one antibody was used for XRCC1 IHC, however, we showed its specificity for XRCC1 using Western immunoblot of patient tumors, as well as Western and immunocytochemical analysis of RNAi-mediated knockdown of XRCC1 in a head and neck cancer cell line. Our data showing 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 3 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, although 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 p16^{INK4a}-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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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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, whereas 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 shows 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, although still susceptible to the same risk of toxicity, they may benefit from alternative management strategies.
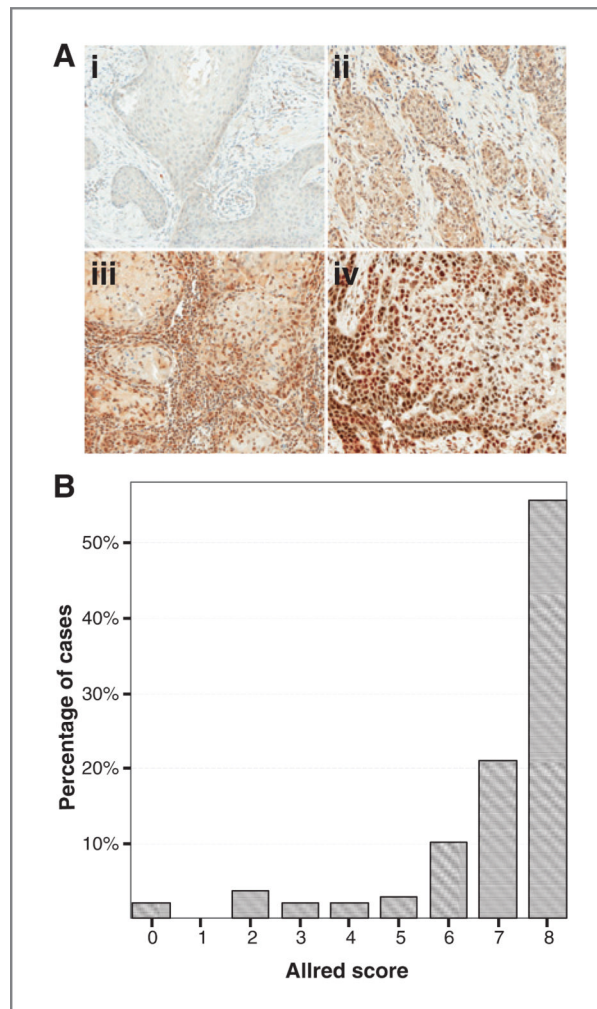
Figure 1.
A, representative examples of XRCC1 immunostaining in head and neck squamous cell carcinoma at different staining intensities. i, intensity 0; ii, intensity 1+; iii, intensity 2+; and iv, intensity 3+.
Magnification 200×. B, distribution of XRCC1 Allred score (range 0–8) in the overall study population.
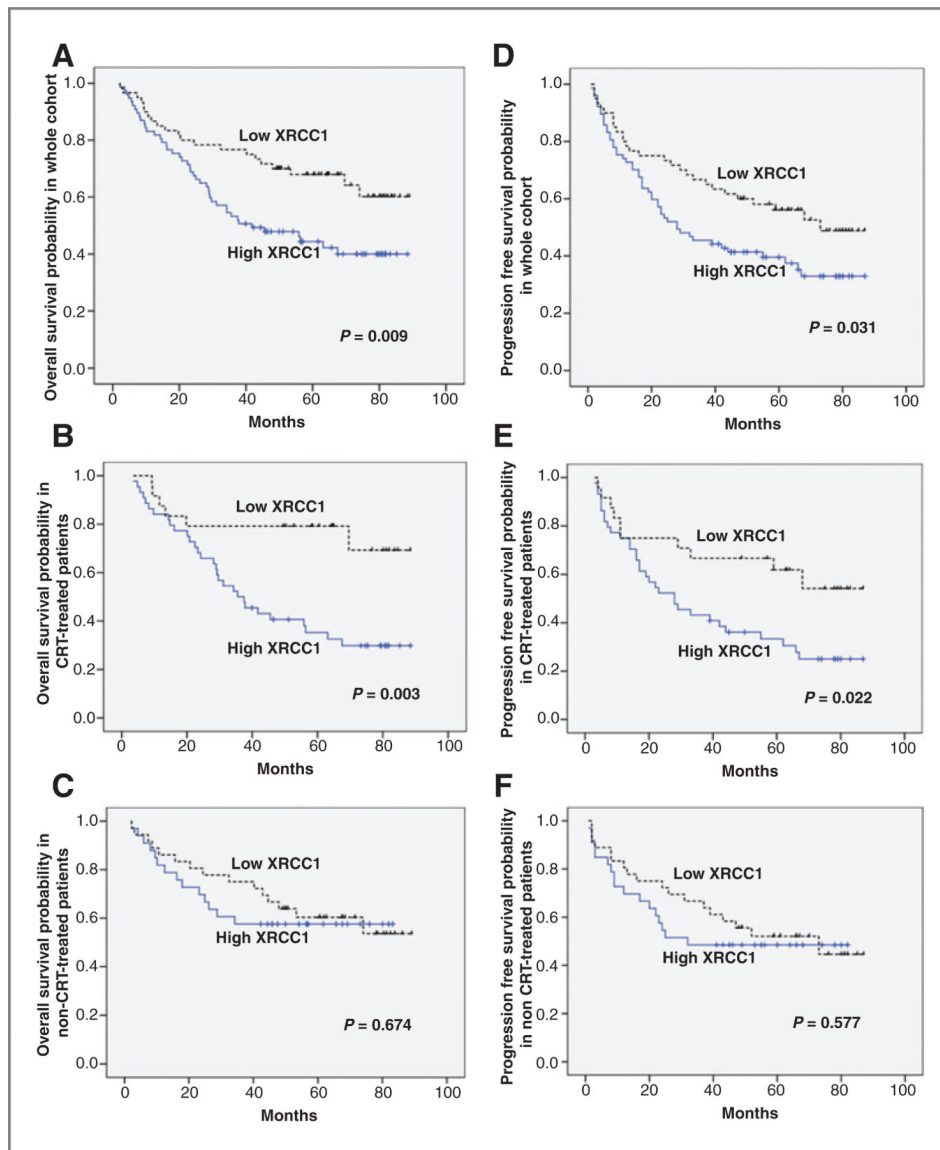
Figure 2.
Kaplan–Meier estimates of the probability of overall survival (A–C) and PFS (D–F) according to XRCC1 expression in whole cohort (A, D), CRT cohort (B, E), and non-CRT cohort (C, F).

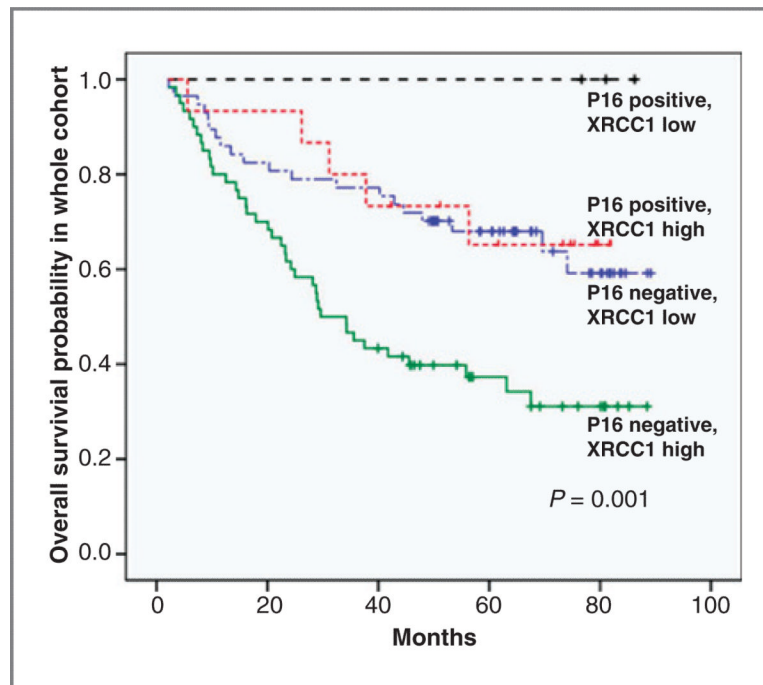**Figure 3.**
Kaplan–Meier estimates of the probability of overall survival in patients who are (i) p16^{INK4a} positive, XRCC1 low; (ii) p16^{INK4a} positive, XRCC1 high; (iii) p16^{INK4a} negative, XRCC1 low; and (iv) p16^{INK4a}-negative, XRCC1-high.

Table 1

Patient characteristics in whole cohort and by XRCC1 expression status

Characteristics	All patients (*n* = 138)	Low XRCC1 (*n* = 61) Number (%)	High XRCC1 (*n* = 77)	*P*
Age, y	57 (median)	Mean 59	Mean 56	0.105
<60	80 (58)	33 (54)	47 (61)	0.488
60	58 (42)	28 (46)	30 (39)	
Sex				
Male	94 (68)	43 (70)	51 (66)	0.713
Female	44 (32)	18 (30)	26 (34)	
Mean no. pack-years smoked	40	40	39	0.842
Smoking history				
Yes	126 (91)	56 (92)	70 (91)	1.000
No	12 (9)	5 (8)	7 (9)	
Alcohol				
Yes	93 (67)	46 (75)	47 (61)	0.100
No	45 (33)	15 (25)	30 (39)	
Site				
Larynx	35 (25)	12 (20)	23 (30)	
Oral cavity	57 (41)	31 (51)	26 (34)	0.235
Oropharynx	38 (28)	15 (24)	23 (30)	
Hypopharynx	8 (6)	3 (5)	5 (6)	
Tumor status				
T1–2	67 (49)	30 (49)	37 (48)	1.000
T3–4	71 (51)	31 (51)	40 (52)	
Nodal status				
N0–1	81 (59)	37 (61)	44 (57)	0.729
N2–3	57 (41)	24 (39)	33 (43)	
Stage				
Early (I, II)	44 (32)	20 (33)	24 (31)	0.856
Late (III, IV)	94 (68)	41 (67)	53 (69)	
Treatment				
Surgery	31 (22)	18 (29)	13 (17)	0.090
Radiation	15 (11)	7 (11)	8 (10)	
Surgery + RT	23 (17)	11 (18)	12 (16)	
Surgery + CRT	17 (12)	9 (15)	8 (10)	
Primary CRT	51 (37)	15 (25)	36 (47)	
Palliative	1 (1)	1 (2)	0	

Table 2

Univariate analyses of prognostic factors for mortality in the overall study population and CRT cohort

Characteristics	Overall study population			CRT cohort		
	HR	95% CI	P	HR	95% CI	P
Age, y						
60/<60	1.11	0.68–1.81	0.678	0.89	0.44–1.80	0.738
Smoking status						
Smoking history/Nonsmoker	1.26	0.51–3.14	0.621	1.65	0.40–6.88	0.492
p16INK4a						
Positive/Negative	0.34	0.11–1.07	0.064	0.37	0.11–1.19	0.095
Site			0.197			0.167
Larynx	1.03	0.52–2.04	0.933	1.10	0.46–2.66	0.833
Oral cavity	1.24	0.67–2.29	0.490	2.08	0.91–4.79	0.083
Hypopharynx	2.67	1.04–6.82	0.041	2.53	0.89–7.22	0.083
Oropharynx	1.00 (reference)			1.00 (reference)		
T stage						
T3-T4/T1-T2	1.94	1.17–3.21	0.010	1.75	0.80–3.85	0.162
N stage						
N2-N3/N0-N1	1.54	0.95–2.50	0.084	0.97	0.49–1.89	0.918
Stage						
Late (III–IV)/early (I–II)	1.79	1.00–3.19	0.049	1.63	0.39–6.77	0.504
XRCC1 expression						
High/low	1.97	1.17–3.32	0.011	3.48	1.44–8.38	0.006
XRCC1 SNP						
RS1799782	1.62	0.79–3.34	0.191	2.66	0.90–7.85	0.076
RS2682558	0.69	0.38–1.28	0.238	0.63	0.28–1.43	0.267

Abbreviations: 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

Factor	Overall study population				CRT cohort			
	OS		PFS		OS		PFS	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
Site		0.087		0.143		0.018		0.043
Larynx	0.73 (0.36–1.51)	0.399	0.76 (0.39–1.47)	0.411	0.62 (0.24–1.59)	0.320	0.64 (0.27–1.54)	0.321
Oral cavity	1.28 (0.66–2.47)	0.462	0.99 (0.54–1.81)	0.963	2.44 (0.99–6.00)	0.052	1.87 (0.81–4.32)	0.143
Hypopharynx	2.40 (0.93–6.20)	0.071	2.19 (0.92–5.24)	0.078	2.77 (0.93–8.22)	0.066	2.51 (0.94– 6.71)	0.066
Oropharynx	1.00 (reference)		1.00 (reference)		1.00 (reference)		1.00 (reference)	
Late Stage	1.86 (1.02–3.39)	0.043	1.29 (0.76–2.19)	0.340	1.58 (0.37–6.75)	0.540	1.17 (0.35–3.90)	0.795
p16^{INK4a}positive	0.21 (0.06–0.71)	0.012	0.25 (0.09–0.73)	0.011	0.26 (0.08–0.92)	0.037	0.34 (0.11–1.01)	0.052
High XRCC1	2.62 (1.52–4.52)	0.001	2.04 (1.25–3.33)	0.004	6.02 (2.36–15.37)	<0.001	3.37 (1.57–7.23)	0.002