Quantification of Excision Repair Cross-Complementing Group 1 and Survival in p16-Negative Squamous Cell Head and Neck Cancers

Ranee Mehra, Fang Zhu, Dong-Hua Yang, Kathy Q. Cai, JoEllen Weaver, Mahendra K. Singh, Anna S. Nikonova, Erica A. Golemis, Douglas B. Flieder, Harry S. Cooper, Miriam Lango, John A. Ridge, and Barbara Burtness

Abstract

Purpose: Multimodality treatment of squamous cell carcinoma of the head and neck (SCCHN) often involves radiotherapy and cisplatin-based therapy. Elevated activity of DNA repair mechanisms, such as the nucleotide excision repair (NER) pathway, of which ERCC1 is a rate-limiting element, are associated with cisplatin and possibly RT resistance. We have determined excision repair cross-complementing group 1 (ERCC1) expression in human papillomavirus (HPV)-negative SCCHN treated with surgery [±adjuvant radiotherapy/chemoradiation (CRT)].

Experimental Design: We assessed ERCC1 protein expression in archival tumors using immunofluorescence staining and automatic quantitative analysis (AQUA) with three antibodies to ERCC1 (8F1, FL297, and HPA029773). Analysis with Classification and Regression Tree (CART) methods ascertained the cutoff points between high/low ERCC1 expression. Multivariable analysis adjusted for age, T, and N stage. Kaplan–Meier curves determined median survival. ERCC1 expression at initial tumor presentation and in recurrent disease were compared. Performance characteristics of antibodies were assessed.

Results: ERCC1 low/high groups were defined on the basis of AQUA analysis with 8F1/2009, FL297, and HPA029773. Among patients treated with surgery plus adjuvant radiotherapy/CRT, longer median survival was observed in ERCC1-low versus ERCC1-high tumors (64 vs. 29 months; \( P = 0.02; \) HPA029773). Data obtained with HPA029773 indicated no survival difference among patients treated only with surgery. Recurrent cancers had lower ERCC1 AQUA scores than tumors from initial presentation. Extensive characterization indicated optimal specificity and performance by the HPA029773 antibody.

Conclusions: Using AQUA, with the specific ERCC1 antibody HPA029773, we found a statistical difference in survival among high/low-ERCC1 tumors from patients treated with surgery and adjuvant radiotherapy. Clin Cancer Res; 19(23); 6633–43. ©2013 AACR.

Introduction

Squamous cell carcinoma of the head and neck (SCCHN) is diagnosed in more than 500,000 patients worldwide annually, accounting for 5% of all malignancies. In the United States, approximately 52,000 new cases occur per year (1). Risk factors for SCCHN include tobacco and alcohol use (2); mounting evidence supports a pathogenic role of infection with the human papillomavirus (HPV), especially in patients lacking the usual habitual exposures (3). p16 is a reliable surrogate biomarker for HPV-initiated oropharyngeal cancers, where p16 elevation is often associated with a favorable prognosis (4–7). In contrast, tumors that arise from other head and neck sites such as the larynx and oral cavity are not associated with HPV infection, and have a poorer prognosis. Platinum chemotherapy using agents such as cisplatin is one important treatment of SCCHN (8, 9), whereas chemoradiation is often used for patients of SCCHN with high-risk clinical features (10, 11). In view of the significant morbidity of these treatments, it is important to ensure that they are administered only to those patients who are likely to benefit.

Platinum-containing chemotherapies cause formation of platinum-DNA adducts, which interfere with DNA transcription and replication, and are typically controlled by activation of the nucleotide excision repair (NER) pathway (12, 13). Radiation typically induces double-strand breaks (DSB; ref. 14). The excision repair cross-complementing
levels had increased survival of 23 versus 12.4 months.

Among cisplatin-treated patients, those with low ERCC1
expression (NSCLC) treated with cisplatin and gemcitabine (16).

[RT-PCR] in patients with advanced non–small cell lung can-
cers (20).

suitably precise tool for quantifying DNA repair biomar-
kers (20). Moreover, their report has raised
questions of antibody quality, and of whether IHC is a
adjuvant datasets (20). Further-
more, due to the ability to localize signals associated with a
tumor mask (which eliminates signal from adjacent stroma),
or to the cytoplasmic or nuclear compartments of
tumor cells, it is possible to quantify the marker of interest
within specific subcellular regions of the most relevant
cell type. This permits more accurate quantification of
ERCC1 in the nuclear compartment, where it is known to
localize (23).

of the excision repair cross-complementing group 1
(ERCC1) enzyme, a rate-limiting element in the nucle-
otide excision repair (NER) pathway, and also acts in the
repair of double-stranded breaks (DSB). Using immu-
nofluorescence staining and automated quantitative
analysis (AQUA) to probe ERCC1 expression in archival
surgical specimens from patients who received adjuvant
radiation, as compared with those submitted only to
resection, this work suggests that SCCHN patients with
high ERCC1 levels may not receive a survival benefit
from adjuvant radiotherapy. Identification of patients
with this form of intrinsic treatment resistance will help
guide new treatment strategies in the high-risk HPV-
negative population, and allow more effective stratifica-
tion of patients in future trials.

In SCCHN, ERCC1 expression levels have been common-
day in vitro applications; Santa Cruz Biotechnology; ref. 32) and a new reagent, HPA029773
(Sigma), that has not previously been characterized in
SCCHN. In addition, we supported this clinical study with
parallel biochemical analysis of these antibodies in cell line
models, to address the question of specificity for the ERCC1
protein. Our primary goal was to study the correlation of
ERCC1 and survival in a retrospective tissue analysis of
patients who were treated with either surgery or surgery plus
adjuvant radiotherapy.

Materials and Methods

Construction of tissue microarrays and annotation of
clinical data

SCCHN surgical specimens from the Fox Chase Cancer
Center (FCCC) Biosample Repository were used to con-
struct tissue microarrays (TMA). Tissue from each tumor
was placed in two unique spots on each TMA. Cases are a
random representation of tumors resected in our facility
from 1990 to 2007. All samples were obtained from primary
tumors and/or nodal metastases at the time of initial resec-
tion. A small set of recurrent squamous cell cancers was also
randomly sampled. Clinical information was available
from the repository database and extracted from clinical
databases in an anonymized fashion. At the time of tissue
acquisition, patients provided Institutional Review Board
(IRB)-approved informed consent for storing tissue and

review from a pathologist, which renders ranking of ERCC1
expression subject to variation among pathologists. This
prompted us to evaluate the ability of quantitative IHC
analysis using automated quantitative analysis (AQUA,
HistoRx) to measure ERCC1 expression levels in archival
tissue. AQUA provides a highly reproducible platform suit-
able for development as a clinical test (21, 22). Further-
more, due to the ability to localise signals associated with a
tumor mask (which eliminates signal from adjacent stroma),
or to the cytoplasmic or nuclear compartments of
tumor cells, it is possible to quantify the marker of interest
within specific subcellular regions of the most relevant
cell type. This permits more accurate quantification of
ERCC1 in the nuclear compartment, where it is known to
localize (23).

A critical concern in IHC studies is the quality of the
antibody used. A growing literature surrounds the use of
IHC analysis for ERCC1 as a biomarker in other malignan-
cies (19, 24–29). The Lab Vision 8F1 antibody has been
most widely used in retrospective series from multiple
tumor types, and has provided data with an ERCC1 cutoff
point associated with survival differences in NSCLC and
SCCHN, but recent batches of this antibody have been
criticized for lack of specificity (and for lot-to-lot variabil-
ity), making its continued use as a reagent problematic, and
calling into question the results of studies (including IALT)
during the past decade, as the quality of this
preparation has deteriorated (20, 30, 31). In this study, we
have carefully compared early batches of Lab Vision 8F1
antibody (8F1/2009), but also with FL297 (which is rep-
orted to be more specific for in vitro applications; Santa Cruz
Biotechnology; ref. 32) and a new reagent, HPA029773
(Sigma), that has not previously been characterized in
SCCHN. In addition, we supported this clinical study with
parallel biochemical analysis of these antibodies in cell line
models, to address the question of specificity for the ERCC1
protein. Our primary goal was to study the correlation of
ERCC1 and survival in a retrospective tissue analysis of
patients who were treated with either surgery or surgery plus
adjuvant radiotherapy.
reviewing clinical data. Controls included SCCHN specimens with varying levels of ERCC1 expression.

**Cell culture, siRNA, and quantitative RT-PCR**

HeLa cervical adenocarcinoma cells and FaDu SCCHN cells from the American Type Culture Collection (ATCC) and SV40-transformed XP2YO skin fibroblasts (Coriell Institute for Medical Research, Camden, NJ) were cultured as recommended by the suppliers. Transfection with siRNAs used RNAiMAX (Invitrogen). ERCC1 was depleted using Human ERCC1-ON-TARGET-plus SMARTpool (NM_001983) from Dharmacon. The four siRNAs in this pool bind areas in common between all reported ERCC1 isoforms and, therefore, are predicted to deplete all ERCC1 species. Negative scrambled control was purchased from Dharmacon. For evaluation of ERCC1 knockdown, total RNA was extracted using the RNaseasy Mini Kit (Qiagen) 48 hours after transfection, reverse-transcribed using standard approaches, and analyzed by Taqman chemistry using Assay-on-Demand Hs01012158_m1 for ERCC1. Expression was normalized to the housekeeping gene POLR2F, for which the primers and probe sequences were TGCCGATGGAAGACCTCAAGG, TCACTGCTCCCATCTGGCCAG, and 6fam-CCCCCATCATCA-TCGCCGTTAC-bhq1. The assays were validated with a 4-fold 4-point dilution curve of cDNA.

**Western blot and immunoprecipitation analyses**

Cells were lysed in the CellLytic M lysis buffer from Sigma-Aldrich supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Scientific). Tumor tissues were homogenized in T-PER Tissue protein extraction reagent from (Thermo Scientific) supplemented with Halt protease and phosphatase inhibitor cocktail on ice, then cleared by centrifugation. Immunoprecipitation samples were incubated overnight with antibody at 4°C, then incubated for 2 hours with protein A/G-Sepharose (Thermo Scientific), washed, and resolved by SDS-PAGE. Western blotting used standard procedures, and was developed by chemoluminescence using Luminata Western HRP substrates (Classico, Crescendo, and Forte) from EMD Millipore. Primary antibodies included anti-ERCC1 (FL-297) from Santa Cruz Biotechnology, anti-ERCC1 (8F1) from Neomarker, anti-ERCC1 (HPA0297733) from Sigma-Aldrich, and mouse anti-ercc1 conjugated to horseradish peroxidase (HRP; #ab49900) from Abcam. Secondary anti-mouse and anti-rabbit HRP-conjugated antibodies from GE Healthcare were used at a dilution of 1:10,000. Image analysis was done using ImageJ (NIH, Bethesda, MD), with signal intensity normalized to β-actin.

**Fluorescent immunohistochemistry**

Tissue sections were deparaffinized in xylene and rehydrated in a graded series of ethanol. The sections were subjected to antigen retrieval by boiling in 0.01 mol/L sodium citrate buffer (pH 6.0) in a steamer for 20 minutes, and then allowed to cool in the buffer for 20 minutes. Endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide in absolute methanol for 15 minutes and washed once with distilled water and 3 times with PBS. After limiting nonspecific binding with blocking reagent (Protein Block Serum-Free, 162 DAKO) for 30 minutes, the sections were incubated overnight with ERCC1 antibody (1-100, 8F1/2009, Lab Vision; 1:400 FL297/2010, Santa Cruz Biotechnology; or 1:5,000 HPA0297731/2011, Sigma) and pan-cytokeratin (tumor mask) in antibody diluent Da Vinci Green (PD900, Biocare Medical) at 4°C overnight. The pan-cytokeratin was probed with an Alexa Fluor 555 dye–labeled secondary antibody (Invitrogen). The primary antibody was targeted with Envision reagents (DAKO), which provides multiple HRP moieties, intensifying signal. Target amplification and visualization was accomplished using a Cy-5-tetramethyl signal amplification system (TSA; Cat. AI705A, PerkinElmer): the Cy-5 (far-red) emission peak is well outside the green-orange spectrum of tissue autofluorescence. Prolong Gold mounting medium (P36931; Molecular Probes) containing 4,6-diamidino-2-phenylindole (DAPI) was used to stain tissue nuclei. Positive and negative controls, discussed in the Results section, were stained simultaneously.

**Image acquisition and AQUA analysis**

Automated image capture was performed by the HistoRx PM-2000 (HistoRx), using the AQUAvision software. High-resolution monochromatic digital images of the cytokeratin staining visualized with AF555, DAPI, and target staining with Cy5 were captured and saved for each tumor histospot. Tumor mask was created from the cytokeratin image of each histospot, representing areas of the epithelial tumor. Histospots were excluded if the tumor mask represented less than 5% of the total histospot area. DAPI immunoreactivity defined the nuclear compartment. The cytoplasmic compartment was defined by the tumor mask. Images were visually inspected and cropped for unfavorable factors such as “out of focus,” debris, or damaged specimen before automatic analysis (33). An AQUA score was generated by dividing the sum of target signals within the tumor mask. AQUA scores were normalized to the exposure times and bit depth at which the images were captured, allowing scores collected at different exposure times to be compared directly. The nuclear scores from two nonoverlapping images were averaged for each case.

**Standard IHC for ERCC1 and p16**

Immunohistochemical staining was performed on 5-μm slides. Deparaffinization, rehydration, epitope retrieval, blocking of peroxidase activity, and nonspecific protein binding, and primary antibody incubation were as for fluorescent IHC (F-IHC). Primary antibodies included ERCC1 (1:100, 8F1/2009, Lab Vision) and p16 (1:3,000, E6H4, MTM Laboratories), with mouse immunoglobulin G (IgG) as negative control. Immunodetection was performed with the Dako Envision® system. The antigen–antibody immunoreaction was visualized using 3’-3’-diaminobenzidine as the chromogen. The slides were washed, counterstained with hematoxylin, dehydrated with alcohol, cleared
in xylene, and mounted. Patient samples that were shown previously to express high levels were used as positive controls.

**Standard IHC evaluation (H-score)**

The slides were viewed and scored by a single pathologist. Immunoreactivity was semiquantitatively scored using a well-established immunoreactivity score system (H-Score) by multiplying both the percentage of positive tumor cells (0%–100%) and the intensity of staining (0–3). H-score range is 0 to 300.

**Statistical analysis**

Only HPV-negative primary tumors were included in the final statistical analysis (all oropharyngeal primary tumors in which p16 was positive or unknown were excluded). Nuclear ERCC1 AQUA scores were analyzed for all antibodies used. Survival time was measured as the number of months between diagnosis and death from any cause. Individuals who were alive at time of last follow-up were considered censored. Overall survival time distributions were plotted by stage and level of nuclear ERCC1 expression using Kaplan–Meier curves and median survival times were estimated. Classification and Regression Tree (CART) methods for failure time data (34) were used to identify optimal cutoff points for nuclear ERCC1 expression levels with respect to the association with survival time within the population treated with adjuvant radiotherapy, separately for each antibody. Next, Cox proportional hazards models were used to compare survival times between nuclear ERCC1 expression-level groups (above or below the optimal cutoff point) while adjusting for patient age, gender, and T/N stage. These analyses were performed separately for surgery-only and adjuvant radiotherapy groups using the same ERCC1 expression cutoff point.

To assess the validity of our method, we randomly sampled 75% of the entire dataset as a training set and used the remaining 25% as a test set. The Harrell C index was computed on the basis of the multivariable Cox model on the test set (35). The Harrell C index measures the agreement of predictions with observed failure order. It is defined as the proportion of all usable subject pairs in which the predictions and outcomes are concordant. The C-index ranges between 0 and 1 with a value of 0.5 indicating no predictive ability. We repeated this procedure 50 times, and the C-index ranged between 0.70 and 0.89 with a mean of 0.78, indicating good predictive ability of the CART method.

**Quantitative IHC analysis**

To optimize the AQUA-based assay with increased reliability, we analyzed these TMAs with three different ERCC1 expression levels.

**Table 1. Patient characteristics of initial and expanded TMAs**

<table>
<thead>
<tr>
<th>Primary site</th>
<th>Initial TMA</th>
<th>Expanded TMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral cavity</td>
<td>46</td>
<td>51</td>
</tr>
<tr>
<td>Tongue</td>
<td>33</td>
<td>42</td>
</tr>
<tr>
<td>Tonsil</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Glottic</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td>Pyriform sinus</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Other</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>66</td>
<td>76</td>
</tr>
<tr>
<td>Female</td>
<td>39</td>
<td>42</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>95</td>
<td>107</td>
</tr>
<tr>
<td>Non-Caucasian</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>T stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>14</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>23</td>
<td>33</td>
</tr>
<tr>
<td>3</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>32</td>
<td>38</td>
</tr>
<tr>
<td>Unknown</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td>N stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>40</td>
<td>41</td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>31</td>
<td>41</td>
</tr>
<tr>
<td>Unknown</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surgery alone</td>
<td>33</td>
<td>38</td>
</tr>
<tr>
<td>Radiotherapy</td>
<td>65</td>
<td>63</td>
</tr>
<tr>
<td>Chemoradiation</td>
<td>7</td>
<td>17</td>
</tr>
<tr>
<td>Total</td>
<td>105</td>
<td>118</td>
</tr>
</tbody>
</table>

**Results**

**TMA patient characteristics**

TMAs were initially constructed from tissue obtained from 105 patients at the time of surgery at the FCCC for newly diagnosed SCCHN. Primary sites included tongue, tonsil, glottis, pyriform sinus, and non-tongue oral cavity (Table 1). The patient population is representative of SCCHN disease, as illustrated by the overall survival curves of the patients analyzed across all stages. (Fig. 1) The limited number of oropharyngeal tumors reflects institutional practice in which such patients were predominantly treated with definitive chemoradiation. Thirty-three patients were treated with surgery alone and 72 received adjuvant radiotherapy; a minority (n = 7) were treated with platinum-based chemoradiation (CRT). Standard indications for adjuvant therapy included multiple involved lymph nodes, close or positive margins, or extracapsular spread of disease.

**Individuals who were alive at time of last follow-up were considered censored. Overall survival time distributions were plotted by stage and level of nuclear ERCC1 expression using Kaplan–Meier curves and median survival times were estimated. Classification and Regression Tree (CART) methods for failure time data (34) were used to identify optimal cutoff points for nuclear ERCC1 expression levels with respect to the association with survival times within the population treated with adjuvant radiotherapy, separately for each antibody. Next, Cox proportional hazards models were used to compare survival times between nuclear ERCC1 expression-level groups (above or below the optimal cutoff point) while adjusting for patient age, gender, and T/N stage. These analyses were performed separately for surgery-only and adjuvant radiotherapy groups using the same ERCC1 expression cutoff point. To assess the validity of our method, we randomly sampled 75% of the entire dataset as a training set and used the remaining 25% as a test set. The Harrell C index was computed on the basis of the multivariable Cox model on the test set (35). The Harrell C index measures the agreement of predictions with observed failure order. It is defined as the proportion of all usable subject pairs in which the predictions and outcomes are concordant. The C-index ranges between 0 and 1 with a value of 0.5 indicating no predictive ability. We repeated this procedure 50 times, and the C-index ranged between 0.70 and 0.89 with a mean of 0.78, indicating good predictive ability of the CART method. These permutations were performed using HPA029773 (Sigma) expression data derived from patients treated with adjuvant radiotherapy.

The Spearman correlation coefficient was computed to assess the association between nuclear AQUA and H-scores measured on the same tissue sample. Wilcoxon ranked sum tests were used to compare ERCC1 expression values between primary and recurrent tissue samples. All test were two-sided with a 5% type-I error. Statistical analyses were conducted using Stata version 12 (Stata Corporation, College Station, Texas).
antibodies: 8F1/2009, FL297, and HPA029773 (Supplementary Fig. S1A). A 2009 lot of the antibody 8F1 (Lab Vision), produced before the time of reported deterioration in performance (20), represents a standard that is much reported in the literature. However, given increasing concerns about reproducibility and specificity with the 8F1 antibody (30), we evaluated the expression of nuclear ERCC1 on these TMAs with additional antibodies. FL297 (32) is a rabbit polyclonal IgG developed against full-length ERCC1. This reagent has been reported to be highly specific for ERCC1 by Western blot and immunoprecipitation analyses (32), although it has not been certified by its manufacturer for IHC. Finally, HPA029773 is a more recently developed polyclonal affinity-isolated antibody that targets ERCC1 with reported high specificity (36). These three antibodies indicate a wide expression range of ERCC1 between different tumor specimens, indicating a potentially useful dynamic range. In most specimens, 8F1/2009 and HPA029773 staining was largely restricted to the nuclear compartment. In contrast, FL297 showed nuclear staining in some cases, but in other specimens, the staining was ambiguous with considerable cytoplasmic staining, raising some concerns (Supplementary Fig. S1A).

**AQUA analysis**

The cutoff point for nuclear ERCC1 expression (8F1/2009) based on CART was established at the 23rd percentile of AQUA scores from this cohort of tissue (172 in the nuclear compartment; Fig. 2A and B). With this value, in a multivariable analysis adjusted for age, gender, and T and N stage, a higher median survival (133 months) was observed in ERCC1-low tumors compared with ERCC1-high (26 months; \( P = 0.036, HR = 2.35 \)) in SCCHN treated with adjuvant radiotherapy. At this cutoff point, there was a significantly increased survival among the patients who were treated with surgery alone as well (\( P = 0.014 \)). However, we note that in this cohort there were only seven patients in the ERCC1-low group, and merely two events. As an additional control, specimens stained with 8F1 antibody were also assigned H-scores using standard IHC; these results indicated a strong correlation between AQUA and H-scores (\( r = 0.69 \)), indicating robust performance of the AQUA technology (Supplementary Fig. S1B).

In comparison, FL297 did not provide a cutoff point for high/low ERCC1 expression that was associated with a significant survival difference (Fig. 2C and D). With this antibody, a trend toward improved survival among the patients who had surgery plus radiation was observed at the 28th percentile cutoff point (2,492 in the nuclear compartment). However, this was not statistically significant (\( P = 0.19 \)) after adjusting for patient age, gender, and T/N stage.

We then used HPA029773 to stain 97 of the original specimens, augmented with 21 additional clinical samples obtained from the FCCC Biosample Repository, for a total of 118 cases (see Table 1). Thirty-eight patients were treated with surgery alone; 80 received adjuvant treatment, either with radiation or chemoradiation. At a cutoff point of 2,136, which was the 28th percentile, there were 24 ERCC1-low cases and 94 ERCC1-high cases (Fig. 2E and F). For this new reagent, in an additional assessment, we found that the ERCC1-low specimens showed the same level of cytokeratin and DAPI staining as the ERCC1-high specimens, indicating that the difference of ERCC1 expression is intrinsic and not due to differences in tissue preservation (Supplementary Fig. S2). Using the HPA029773 antibody on the AQUA platform, there was a statistically significant difference in survival (\( P = 0.02, HR = 2.72 \)) after adjusting for patient age, gender, and T/N stage, with an overall median survival of 64 months in the low ERCC1 group and 29 months in the high ERCC1 group among patients receiving adjuvant radiotherapy/chemoradiation therapy. Among patients treated with surgery alone, there was no difference in overall survival between the two ERCC1 groups (\( P = 0.23 \)).

On the basis of AQUA nuclear scores, there was a weak to moderate relationship of ERCC1 high/low assignment using the CART-based cutoff points between all three antibodies, with 82% concordance between 8F1 and FL297, 75% agreement between 8F1 and HPA029773, and 73% between FL297 and HPA029773.

**Recurrence disease**

Tissues were available from a cohort of patients with SCCHN with recurrent cancer. In further analysis with HPA029773, we sought to determine if there was a difference in nuclear ERCC1 levels among previously untreated cancers and tumor samples from sites of recurrent disease (Fig. 3). Specimens from recurrences were available from oral cavity, oropharyngeal, and laryngeal tumors. Initial treatment was heterogeneous and included radiotherapy, chemoradiation, surgery, and surgery plus radiotherapy (Supplementary Table S1). This series includes 15 nonrecurrent nodal metastases, which were removed at the time of the original presentation of malignancy, 11 recurrent nodal metastases, 20 recurrences at the primary site, and 43...
untreated primary cancers (which were randomly selected from the original TMA). There was an insufficient number of matched previously untreated and recurrent specimens from the same patient for direct intrapatient comparison. Overall, the ERCC1 AQUA values were lower in recurrent tumors \((P < 0.01)\) with a mean score of 1,243 compared with 2,879 for previously untreated tumors. Nonrecurrent nodes, recurrent nodes, and primary tumors all had similar values.

Comparison of antibodies by Western blot and immunoprecipitation analyses

Our data demonstrated nonequivalent behavior of three different commercial reagents for detection of ERCC1 in predicting clinical outcome. Several recent studies have emphasized changes in behavior in the erstwhile gold standard 8F1 affecting antibody lots produced in recent years (20, 31), whereas FL297 and HPA029773 have not been as extensively characterized. To address this issue, we compared (i) the early batch of 8F1/2009 used here for AQUA and IHC on tumors, (ii) a more recent batch of 8F1 (from 2010), (iii) FL297, and (iv) HPA029773, using Western blot and immunoprecipitation analyses (Fig. 4). As a test set for evaluation of antibody specificity, we used the HeLa, FaDu, and XP2YO cell lines (in this last cell line, a mutation eliminating the ERCC1 partner XPF destabilizes the ERCC1 protein; ref. 37), transfected with an siRNA to deplete ERCC1 or a negative-control–scrambled siRNA. Control assessment using quantitative RT-PCR confirmed very effective depletion of ERCC1 mRNA using the ERCC1 siRNA (Fig. 4A). Western blot analysis indicated that 8F1/2009 and FL297 both identified a single species with a molecular weight corresponding to the reported gel mobility of ERCC1 (\(\sim 35-38\) kD) whether analyzed by Western blot (Fig. 4B) or immunoprecipitation analyses (Figures 4C, D). HPA029773 not only identified unambiguously a single specific ERCC1 species, but also identified a prominent cross-reacting species (Fig. 4B and C). In contrast, the recent batch lot of 8F1 did not detect a specific species either by Western blot or by immunoprecipitation analysis (Fig. 4B–D), concurring with other recent studies indicating a loss of performance (20, 31).

In AQUA analysis, low ERCC1 expression as measured with FL297 did not predict an increased survival benefit, in...
and primary tumors have similar values. By 8F1 and HPA029773. That is part of a larger pool of cross-reacting species detected indicating the presence of a common, prognostic protein. Taken in sum, these data are most simply interpreted as specific signal for HPA029773 in AQUA analysis (Fig. 4F).

A specific cross-reacting band, again supporting the idea of a specific signal. In reciprocal analysis, in Western blotting by reduced HPA029773 signal levels, consistent with a siRNA depletion or with the XP2YO genotype was reflected in pellets prepared from some of the cell lines used for Western blot and immunoprecipitation analyses (Fig. 4E). In this setting, the reduced expression of ERCC1 associated with siRNA depletion or with the XP2YO genotype was reflected by reduced HPA029773 signal levels, consistent with a specific signal. In reciprocal analysis, in Western blotting and immunoprecipitation of tumor lysates from tumors characterized by AQUA using the HPA029773 antibody as high or low for ERCC1, signal variance detected by the FL297 and HPA029773 reflected ERCC1, rather than the nonspecific cross-reacting band, again supporting the idea of a specific signal for HPA029773 in AQUA analysis (Fig. 4F).

In summary, our AQUA data indicate that low nuclear ERCC1 expression detected by either 8F1/2009 or current HPA029773 antibodies is associated with a survival benefit contrast to data generated with 8F1/2009 or with HPA029773. One potential explanation suggests that FL297 does not recognize ERCC1 following preparation of fresh-frozen paraffin embedded (FFPE) tissue and, therefore, AQUA data with this antibody reflect nonspecific cross-reactivity, whereas early-batch 8F1 and HPA029773—despite having partial cross-reactivity with non-ERCC1 proteins detectable by Western blot—predominantly react with ERCC1 in IHC and AQUA analyses. If this hypothesis is correct, ERCC1 is predictive of overall survival. An alternative possibility is that FL297 accurately and specifically identifies ERCC1 in IHC and AQUA analyses, whereas early-batch 8F1 and HPA029773 do not. In that scenario, ERCC1 expression does not predict overall survival. To discriminate between these models, we used AQUA and IHC to analyze FFPE cell pellets prepared from some of the cell lines used for Western blot and immunoprecipitation analyses (Fig. 4E). In this setting, the reduced expression of ERCC1 associated with siRNA depletion or with the XP2YO genotype was reflected by reduced HPA029773 signal levels, consistent with a specific signal. In reciprocal analysis, in Western blotting and immunoprecipitation of tumor lysates from tumors characterized by AQUA using the HPA029773 antibody as high or low for ERCC1, signal variance detected by the FL297 and HPA029773 reflected ERCC1, rather than the nonspecific cross-reacting band, again supporting the idea of a specific signal for HPA029773 in AQUA analysis (Fig. 4F).

In summary, our AQUA data indicate that low nuclear ERCC1 expression detected by either 8F1/2009 or current HPA029773 antibodies is associated with a survival benefit among patients with HPV-negative SCCHN who received adjuvant radiotherapy after surgery. Because HPV-negative tumors are known to be less sensitive to chemoradiation, this association provides a potential mechanistic explanation for this well-established clinical finding. Importantly, this work includes a cohort of patients treated with surgery alone; ERCC1 biomarker data have not been studied previously in such patients, because patients in all earlier reports received chemoradiation. With HPA029773, there was no appreciable difference in survival associated with ERCC1 expression among these patients with a more favorable prognosis who needed no adjuvant therapy and were treated with resection alone. Our finding that low ERCC1 expression correlates with improved treatment outcomes is consistent with previous reports (24, 25, 35). The finding that recurrent disease shows lower ERCC1 expression levels was unexpected and potentially reflects altered DNA repair capacity in response to prior therapy; this warrants study in a larger series. Our retrospective, nonrandomized series suggests, but does not prove, the hypothesis that ERCC1 is a predictive marker for radiation sensitivity in SCCHN. Similarly, it suggests, but does not prove the hypothesis that ERCC1 expression has no influence on the prognosis of SCCHN, a factor independent of treatment.

Other retrospective studies of ERCC1 levels and correlation to chemotherapy response and survival in SCCHN have been reported (24, 25, 38). In one of the larger series, Handra-Luca and colleagues studied specimens from 107 patients with SCCHN who had received cisplatin-based induction therapy. In this report, which was published in 2007, ERCC1 expression was also analyzed with an older batch of 8F1 and assigned an H-score with standard IHC; 71% of tumors had high-level expression, whereas 29% had low-level expression. This later group derived a 4-fold greater response and benefit to cisplatin-based induction therapy, as well as an increase in median survival (24). However, additional series yielded conflicting results, thus engendering controversy whether ERCC1 is truly a marker for platinum resistance and if IHC is the best method to interrogate this marker (38, 39).

Many previous reports used the 8F1 ERCC1 antibody, with specific batch undefined; most reports in the past 2 years probably involve 8F1 batches that have lost specificity for ERCC1 (24, 25, 35). In a 2012 American Society of Clinical Oncology (ASCO) report, Austin and colleagues analyzed tissue from 84 platinum-treated patients who were enrolled in a clinical trial for locally advanced SCCHN. In standard IHC reported as an H-score, and cutoff point established at the median, 8F1-based determination of high ERCC1 expression (46%) was not associated with an inferior response rate or progression-free survival (PFS) whereas, in FL297-based determination, high ERCC1 levels were associated with a significantly inferior PFS (40). Similarly, in 2012 Hao and colleagues reported a retrospective AQUA IHC using 8F1 and FL297 to analyze 35 SCCHN tumors from patients who underwent chemoradiation (38).

Discussion

In summary, our AQUA data indicate that low nuclear ERCC1 expression detected by either 8F1/2009 or current HPA029773 antibodies is associated with a survival benefit among patients with HPV-negative SCCHN who received adjuvant radiotherapy after surgery. Because HPV-negative tumors are known to be less sensitive to chemoradiation, this association provides a potential mechanistic explanation for this well-established clinical finding. Importantly, this work includes a cohort of patients treated with surgery alone; ERCC1 biomarker data have not been studied previously in such patients, because patients in all earlier reports received chemoradiation. With HPA029773, there was no appreciable difference in survival associated with ERCC1 expression among these patients with a more favorable prognosis who needed no adjuvant therapy and were treated with resection alone. Our finding that low ERCC1 expression correlates with improved treatment outcomes is consistent with previous reports (24, 25, 35). The finding that recurrent disease shows lower ERCC1 expression levels was unexpected and potentially reflects altered DNA repair capacity in response to prior therapy; this warrants study in a larger series. Our retrospective, nonrandomized series suggests, but does not prove, the hypothesis that ERCC1 is a predictive marker for radiation sensitivity in SCCHN. Similarly, it suggests, but does not prove the hypothesis that ERCC1 expression has no influence on the prognosis of SCCHN, a factor independent of treatment.

Other retrospective studies of ERCC1 levels and correlation to chemotherapy response and survival in SCCHN have been reported (24, 25, 38). In one of the larger series, Handra-Luca and colleagues studied specimens from 107 patients with SCCHN who had received cisplatin-based induction therapy. In this report, which was published in 2007, ERCC1 expression was also analyzed with an older batch of 8F1 and assigned an H-score with standard IHC; 71% of tumors had high-level expression, whereas 29% had low-level expression. This later group derived a 4-fold greater response and benefit to cisplatin-based induction therapy, as well as an increase in median survival (24). However, additional series yielded conflicting results, thus engendering controversy whether ERCC1 is truly a marker for platinum resistance and if IHC is the best method to interrogate this marker (38, 39).

Many previous reports used the 8F1 ERCC1 antibody, with specific batch undefined; most reports in the past 2 years probably involve 8F1 batches that have lost specificity for ERCC1 (24, 25, 35). In a 2012 American Society of Clinical Oncology (ASCO) report, Austin and colleagues analyzed tissue from 84 platinum-treated patients who were enrolled in a clinical trial for locally advanced SCCHN. In standard IHC reported as an H-score, and cutoff point established at the median, 8F1-based determination of high ERCC1 expression (46%) was not associated with an inferior response rate or progression-free survival (PFS) whereas, in FL297-based determination, high ERCC1 levels were associated with a significantly inferior PFS (40). Similarly, in 2012 Hao and colleagues reported a retrospective AQUA IHC using 8F1 and FL297 to analyze 35 SCCHN tumors from patients who underwent chemoradiation (38).
with 8F1 indicated no difference in survival, whereas, with FL297, there was a significant relationship between low ERCC1 expression and increased overall survival ($P = 0.004$). This relationship remained significant among the HPV-negative tumors, but not the HPV-positive group, which comprised half of their study population. This work, in general, supports the results of our study, conducted entirely in an HPV-negative group. However, although our studies with FL297 suggest a survival difference predicted by this reagent, statistical significance was not reached. Why FL297 would be prognostic in the article by Hao and colleagues but not our series is unclear. In prior analyses, and consistent with our experience with automated platforms, FL297 nuclear staining was weak and cytoplasmic staining strong, suggesting cross-reactivity in tissues fixed for IHC which might account for the varying results observed with FL297 (41, 42).

Accurate and reproducible methods are essential when assaying clinical biomarkers. The recent report by Fibroulet and colleagues (20) in lung cancer emphasizes the importance of integrating careful controls into the performance of IHC-based biomarker studies, particularly when interrogating ERCC1. Their report is consistent with our conclusion that currently available lots of 8F1 should not be used in future biomarker studies. Although Fibroulet and colleagues conclusively showed this, they did not reevaluate the lung tumors in their cohort with additional antibodies against ERCC1. On the basis of Western blot analysis, our data indicate that HPA029773 is a reasonable replacement for the early, "specific" batches of 8F1 in IHC/AQUA applications for research applications. In addition, Fibroulet and colleagues noted that all commercially available antibodies to ERCC1 react with multiple isoforms, some of which are catalytically inactive, which may obscure interpretations. However, there are limited data regarding the role of ERCC1 isoforms in DNA repair (24), and our own Western blot analyses (Fig. 4) typically detected only a single species of correct molecular weight in analyzed specimens, reducing this concern.

ERCC1 association with XPF (ERCC4) is essential for both NER and DSB repair, and the expression levels of these enzymes are closely linked (15, 32, 43). Fibroblasts deficient in the ERCC1–XPF (ERCC4) complex are sensitive to radiation, with coincident defects in DSB repair (15). Decreased ERCC1–XPF activity has also been implicated in increased hypoxic radiosensitivity, which is of particular interest in SCCHN (44, 45). Thus, improved survival among ERCC1-low patients treated with radiotherapy has a sound biologic rationale. Furthermore, a retrospective series reported by Vaezi and colleagues (46) used a quantitative IHC platform to measure XPF expression in 80 SCCHN tumors. Their finding that low XPF expression is statistically associated with a prolonged PFS, thus, corroborates our findings (46). In that study, the primary site of disease was heterogeneous, and treatments consisting of chemoradiation, radiotherapy alone, or surgery plus adjuvant radiotherapy, were not considered separately. Despite this, the authors showed a statistically significant increase in PFS among tumors with low XPF expression. Nonetheless, the DNA repair mechanisms associated with radioresistance are complex, and will warrant evaluation of interactions between ERCC1, XPF, and other DNA repair pathways.

In view of the magnitude of the survival disadvantage associated with high ERCC1 expression in resected patients requiring radiotherapy or chemoradiation, future studies of adjuvant therapy, especially among HPV-negative patients, may benefit from incorporation of ERCC1 status as a stratification variable to ensure balance across treatment arms. Currently, there are limited nonplatinum chemotherapy options for patients with SCCHN. Future work should focus on developing optimal therapies for HPV-negative, ERCC1-high patients, and might also explore methods to target and inhibit ERCC1-XPF to restore sensitivity to cisplatin and radiation.

In conclusion, although limited, the results of our retrospective review are intriguing. Although the lack of randomized data hinders our ability to make definite conclusions as to whether ERCC1 represents a predictive biomarker for treatment selection, the data presented here are strongly suggestive. A prospective, randomized trial design will be necessary to define this with complete confidence. The current work additionally supports the significance of such a study and illustrates the importance of selecting the appropriate antibody and assay conditions for all such endeavors. Much of the debate surrounding the utility of ERCC1 IHC would be addressed by the availability of a clinical-grade reagent. We are now working under the auspices of the NCI Clinical Assessment Development Program to develop a standard clinical assay that uses an antibody adequate for this purpose.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: R. Mehra, M.N. Lango, B. Burtness


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.-H. Yang, K.Q. Cai, J. Weaver, M.K. Singh, A. Nikonova, B. Flieder, J.A. Ridge

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Mehra, F. Zhu, D.-H. Yang, K.Q. Cai, E.A. Golemis, H. Cooper, B. Burtness

Writing, review, and/or revision of the manuscript: R. Mehra, D.-H. Yang, K.Q. Cai, E.A. Golemis, M.N. Lango, J.A. Ridge, B. Burtness

Administrative/technical, or other support (i.e., reporting or organizing data, constructing databases): R. Mehra

Study supervision: R. Mehra

References


Acknowledgments

The authors thank Eric Ross (Director, Biostatistics and Bioinformatics Facility), Emmanuelle Nicolas (Genomics Facility), and Mary Donovan (Data Manager, Biosample Repository) for their assistance with this work.

Grant Support

This work was financially supported by the CA06927 NIH core grant and the Pew Charitable Trust (to the Fox Chase Cancer Center) and CA63666 (to E.A. Golemis), and support from the Fox Chase Cancer Center Head and Neck Keystone.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 28, 2013; revised September 25, 2013; accepted September 26, 2013; published OnlineFirst October 2, 2013.


Quantification of Excision Repair Cross-Complementing Group 1 and Survival in p16-Negative Squamous Cell Head and Neck Cancers

Ranee Mehra, Fang Zhu, Dong-Hua Yang, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-13-0152

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2013/10/02/1078-0432.CCR-13-0152.DC1

Cited articles
This article cites 42 articles, 20 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/19/23/6633.full.html#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
/content/19/23/6633.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.