

XPF Expression Correlates with Clinical Outcome in Squamous Cell Carcinoma of the Head and Neck

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Abstract

Purpose: Tumor-specific biomarkers that predict resistance to DNA damaging agents may improve therapeutic outcomes by guiding the selection of effective therapies and limiting morbidity related to ineffective approaches. XPF (ERCC4) is an essential component of several DNA repair pathways and XPF-deficient cells are exquisitely sensitive to DNA damaging agents. The purpose of this study was to determine whether XPF expression levels predict clinical response to DNA damaging agents in head and neck squamous cell carcinoma (HNSCC).

Experimental Design: Quantitative immunohistochemistry was used to measure XPF expression in tumors from a cohort of 80 patients with newly diagnosed HNSCC treated with radiation therapy with or without platinum-based chemotherapy; samples were collected prospectively. Genomic DNA isolated from blood samples was analyzed for nine single nucleotide polymorphisms (SNP) in the *XPF* gene by using a custom array. The primary endpoint was progression-free survival (PFS).

Results: XPF expression was higher in tumors from the oral cavity than from the other sites ($P < 0.01$). High XPF expression correlated with early time to progression both by univariate (HR = 1.87, $P = 0.03$) and multivariate analysis (HR = 1.83, $P = 0.05$). The one year PFS for high expressers was 47% (95% CI = 31–62) compared with 72% (95% CI = 55–83) for low expressers. In addition, we identified four *XPF* SNPs that showed marginal association with treatment failure.

Conclusions: Expression level of XPF in HNSCC tumors correlates with clinical response to DNA damaging agents. XPF has potential to guide next generation personalized cancer therapy. *Clin Cancer Res*; 17(16); 5513–22. ©2011 AACR.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the eighth most frequent cancer in the United States (1). Yearly, approximately 560,000 new cases will be diagnosed worldwide, and 300,000 people will die of this disease (2).

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HNSCC is treated with surgery, chemotherapy, and radiation therapy. Frequently, concomitant chemoradiotherapy with a platinum-based DNA damaging agent (cisplatin or carboplatin) is used, either as primary treatment or as adjuvant postoperative therapy (3–4). However, resistance to chemotherapy occurs frequently, with 5 year local and distant failure rates of 50% and 15%, respectively (5). Alternative treatments that do not rely upon damaging DNA, such as surgery, taxane, 5-fluorouracil, or hydroxyurea, can also be used (3). To improve clinical outcomes, the next generation of treatment algorithms will incorporate personalized tumor analysis to identify the therapy with the greatest chance of success in an individual patient. Thus, identifying novel biomarkers that predict response to a given therapeutic approach will lead to treatment algorithms with higher success rates and lower morbidity.

Patients suffering from xeroderma pigmentosum (XP), Fanconi anemia, or severe combined immunodeficiency have mutations in genes required for nucleotide excision repair (NER) of DNA, the repair of DNA interstrand crosslinks (ICL), or nonhomologous end-joining of double-strand breaks (DSB), respectively. As a consequence, these patients are exquisitely sensitive to DNA damaging agents including ionizing radiation and crosslinking agents such

Translational Relevance

Radiation therapy and platinum-based DNA damaging therapies are the prevailing nonsurgical treatments for head and neck squamous cell carcinoma (HNSCC). However, these agents are not universally successful, and are associated with significant toxicity. Alternative nonplatinum systemic therapies are available, but it is not currently possible to predict which patients will respond best to which therapy.

Here, we show a significant association between low expression of XPF and longer progression-free survival in HNSCC treated with DNA damaging agents. XPF encodes one subunit of the DNA repair endonuclease ERCC1-XPF, which is involved in the repair of both platinum- and radiation-induced DNA damage. Measuring XPF expression in tumors by using immunohistochemistry may be useful to predict whether a patient is likely to benefit from platinum-based chemoradiation therapy. This novel biomarker may be used to improve treatment outcomes while minimizing the toxicity of ineffective therapies.

as cisplatin (6, 7). The proteins affected in these genome instability disorders repair damaged DNA and, therefore, represent potential biomarkers for predicting tumor sensitivity to genotoxic therapeutics. XPF (ERCC4) partners with ERCC1 to form a bipartite nuclease that is essential for NER and ICL repair, and participates in DSB repair (8–11). Platinum-based chemotherapeutic drugs react with DNA to induce adducts that affect 1 strand of DNA (monoadducts and intrastrand crosslinks), which are repaired by NER, as well as inducing ICLs, that are repaired by ICL repair (12–14). Because ERCC1-XPF is unique in being required for both NER and ICL repair, it is the only enzyme required for removal of all types of DNA lesions caused by cisplatin and carboplatin. In addition, it facilitates the repair of DNA lesions caused by radiation therapy (monoadducts and DSBs). Hence, it has been proposed that increased expression of ERCC1-XPF in tumors might result in resistance to chemoradiation therapy and poor clinical response. *In vivo*, ERCC1 and XPF are required to bind and stabilize one another; hence, protein levels of both are tightly linked (8). Thus, expression of either ERCC1 or XPF can be used to estimate DNA repair nuclease activity.

Studies examining ERCC1 report a positive correlation between increased ERCC1 expression and poor outcome in lung, gastric, nasopharyngeal and head and neck cancers (15–18). However, these studies used an antibody (clone 8F1) that lacks specificity to ERCC1 (19), confounding interpretation of the results (20). It remains controversial whether ERCC1 expression predicts response to genotoxic therapies. XPF, which contains the catalytic domain of the ERCC1-XPF nuclease, remains virtually unexplored as a biomarker (21, 22).

In addition to expression studies, polymorphism analysis of DNA repair genes, including XPF, can determine

whether germline allelic variants are linked to cancer susceptibility and response to DNA damaging therapy. XPF polymorphisms have been linked to the risk of developing breast, lung, melanoma, and pancreatic cancers (23–27). One polymorphism in ERCC1 correlates with HNSCC response rate or progression (28, 29). However, it is unknown whether any XPF polymorphisms predict response to DNA damaging agents.

Herein, we establish that XPF expression has the potential to be a reliable predictor of clinical outcome of HNSCC treated with genotoxic therapy, and we identify 4 single nucleotide polymorphisms (SNP) in the XPF gene that correlate marginally with treatment failure. Our results suggest that XPF could be a valuable biomarker for stratifying HNSCC patients into distinct risk categories that could help personalize treatment and improve clinical outcomes.

Materials and Methods

Immunodetection of XPF in cell and tumor lysates

Cell line origins are indicated in the Supplementary Data. Cryopreserved tumor samples for immunoblotting were collected by the University of Pittsburgh Head and Neck SPORE Tissue bank from HNSCC patients, after informed consent and Institutional Review Board (IRB) approval were obtained. Seven matched primary tumor and adjacent normal tissue pairs were randomly selected by a pathologist. Cell and tumor lysis, electrophoresis, and immunoblots were done as described elsewhere (30). For XPF immunodetection, we used the monoclonal antibody SPM228 (1:1,000; Abcam) and tubulin as a loading control (1:5,000; Sigma) followed by horseradish peroxidase- or alkaline phosphatase-conjugated secondary antibodies (1:5,000 and 1:2,500, respectively; Promega). Recombinant histidine-tagged ERCC1-XPF (Gillard, 2001) was used as a positive control (gift from Dr. Richard Wood, MD Anderson Cancer Center, Smithville, TX).

Cohort design, paraffin-embedded tumor samples, and clinical data

The study was approved by the IRB of the University of Pittsburgh and done in accordance with the Helsinki agreement. Informed consent was obtained from patients for sample collection. Biopsy-proven HNSCC cancer patients were enrolled prospectively in the context of a genomics study ($n = 522$) conducted at our institution from 2000 to 2007. Formalin-fixed paraffin-embedded pretreatment biopsies or resection specimens, blood, and clinical data were collected prospectively by the University of Pittsburgh Head and Neck SPORE Tissue bank. For this study, we retrospectively isolated a cohort within the larger genomic prospective cohort. We included patients with untreated primary HNSCC of any site, treated with curative intent with radiation with or without platinum-based chemotherapy (either in a primary or postsurgery adjuvant setting). Patients with recurrence or a previous history of HNSCC were excluded. Patients were treated from 2000 to 2006. An honest broker selected and deidentified 87

patients satisfying the eligibility criteria. Seven of the 87 samples did not contain enough tumor or were not suitable for immunohistochemistry (IHC) and were excluded from the analysis.

Immunofluorescence

Differential immunodetection of XPF and ERCC1 in cell nuclei was done as previously described (30). Briefly, WT and XP2YO cells that harbor a mutation in *XPF* that destabilizes ERCC1-XPF, were labeled with different sized latex beads and cocultured, providing an internal negative control for immunodetection of ERCC1-XPF. Immunofluorescence was done with antibodies SPM228 (1:200; Abcam) and FL297 (1:200; Santa Cruz), to detect XPF and ERCC1, respectively. Local photodamage to detect ERCC1-XPF at sites of UV-induced DNA damage was done as previously described (30).

Immunohistochemistry

Full sections of paraffin-embedded tumors were processed using standard techniques. Antigen retrieval was with 10 mmol/L sodium citrate pH 6.0 for 20 minutes at 100°C in a pressure cooker. Sections were blocked and incubated overnight with anti-XPF antibody SPM228 (1:1,200; Abcam). XPF was detected by using renaissance TSA (tyramide signal amplification) Biotin System (Perkin Elmer), with hematoxylin (Vector) counterstain.

Microscopy and quantification of immunohistochemistry

For digitally assisted quantification, IHC slides were scanned by using an automated slide scanner (Aperio). For every tumor section, 6 approximately 600- μm^2 regions were selected by a pathologist for biomarker quantification. The selected areas were analyzed by using a customized image algorithm (Aperio). XPF intensity was expressed in a 0 to 300 scale scoring system that took into account both staining intensity and the number of positive tumor nuclei. The final score represents the average of 6 regions of interest. For pathologist scoring, the pathologist was blinded to tumor samples and automated score. An H-score was determined by multiplication of the stain intensity from a I to IV scale by the percent of tumor cells stained.

Single nucleotide polymorphism genotyping analyses

Genomic DNA was isolated from whole blood samples from the same 80 HNSCC cases by using DNA Isolation Kits (Gentra Systems Inc.). A custom 384-SNP panel was screened by using the Illumina GoldenGate technology (Illumina). This 384-SNP panel was designed to determine the genotype for polymorphisms in DNA repair genes, including *XPF*, and cell-cycle control genes. For *XPF*, a total of 13 SNPs were evaluated.

SNP chip design and quality control

The SNP selection strategy incorporated 6 features: (i) haplotype tag SNPs for both Caucasians and Africans defined by predicted LD scores $R^2 \geq 0.8$; (ii) functional

SNPs characterized by amino acid substitutions; (iii) potential for regulatory changes; (iv) SNPs that alter protein stability; (v) evolutionary conservation across species, and (vi) published epidemiologic data. To ensure high quality genotype results, a number of routinely followed quality control procedures were carried out, including (i) quality control and quantification of incoming DNA samples; (ii) multiple internal controls built into each genotyping assay including the screening of each SNP allele approximately 30 times; (iii) bar-coded labeling of sample plates; and (iv) statistical measures of success for assay development and genotyping confidence scores. A conservative minimum GenTrain score of 0.45 and Cluster Sep score of 0.25 must be achieved for each SNP reported. Minimum SNP and sample call rates were set at 95%.

Statistical analyses

Progression-free survival (PFS) was defined as the elapsed time between the initiation of DNA damaging therapy and first recorded date of disease progression. Patients without progression, lost to follow-up or who died from other causes, were censored at their last date of record. The association of XPF and PFS was conducted with proportional hazards regression. Covariates considered for the adjustment of the effect of XPF included age, gender, T stage, N stage, site of disease, whether the primary therapy included surgery and whether the patient was treated as part of a University of Pittsburgh Cancer Institute therapeutic protocol. Because it was observed that most disease progressions occurred within 1 year, an alternate binary endpoint of progression (treatment failure) at 1 year was also analyzed. A Wilcoxon test compared XPF expression between patients who progressed within 1 year versus those who survived at least 1 year without progression. To illustrate the results of the proportional hazards model in a figure, XPF expression was split at the median and the resulting PFS was described with Kaplan-Meier plots. A log rank test was used to test difference in subgroups. SNPs were examined by proportional hazards regression contrasting the homozygous variant allele to the common allele. No prior probabilities were used for this analysis and therefore *P* values were adjusted by the method of Benjamini and Hochberg (31) to estimate the false discovery rate. The agreement in XPF IHC between machine-based quantitative image analysis and pathologists H-score was analyzed by correlation and linear regression.

Results

Specificity of XPF detection

The first critical step in testing hypotheses about whether a biomarker predicts clinical outcome is to develop a reliable method to measure that biomarker in clinical specimens (19, 30). The most readily available specimen is formalin-fixed, paraffin-embedded tumors. Thus, our goal was to identify an antibody that can be used to quantitatively measure XPF protein levels by IHC on paraffin-embedded sections. We first determined that the

commercially available monoclonal antibody clone SPM228 is specific for XPF by using biochemical methods and immunolocalization with positive and negative controls. On immunoblot, the antibody detected a band of the appropriate molecular weight (~120 kDa) in lysates of wild-type (WT) cells but not XPF cells (XP2YO; from a patient with virtually undetectable *XPF* levels; Fig. 1A). As expected, the WT band migrated slightly ahead of recombinant His-tagged XPF. To determine whether the antibody can distinguish between cells with high and low XPF expression, WT and XP2YO cells labeled with latex beads

of different sizes were cocultured to create test samples with an internal negative control. Brightfield imaging was used to identify fields with WT and XPF-deficient cells adjacent to one another on the basis of different bead size (Fig. 1B). Immunofluorescence with SPM228 revealed bright nuclear staining in WT cells, but no nuclear signal in neighboring mutant XPF-deficient XP2YO cells (Fig. 1B' and B''). To confirm that the nuclear staining is specific for XPF, we irradiated cells with UV through a filter, to create patches of DNA damage and conducted immunofluorescence with SPM228 and a specific ERCC1 antibody (19) to determine

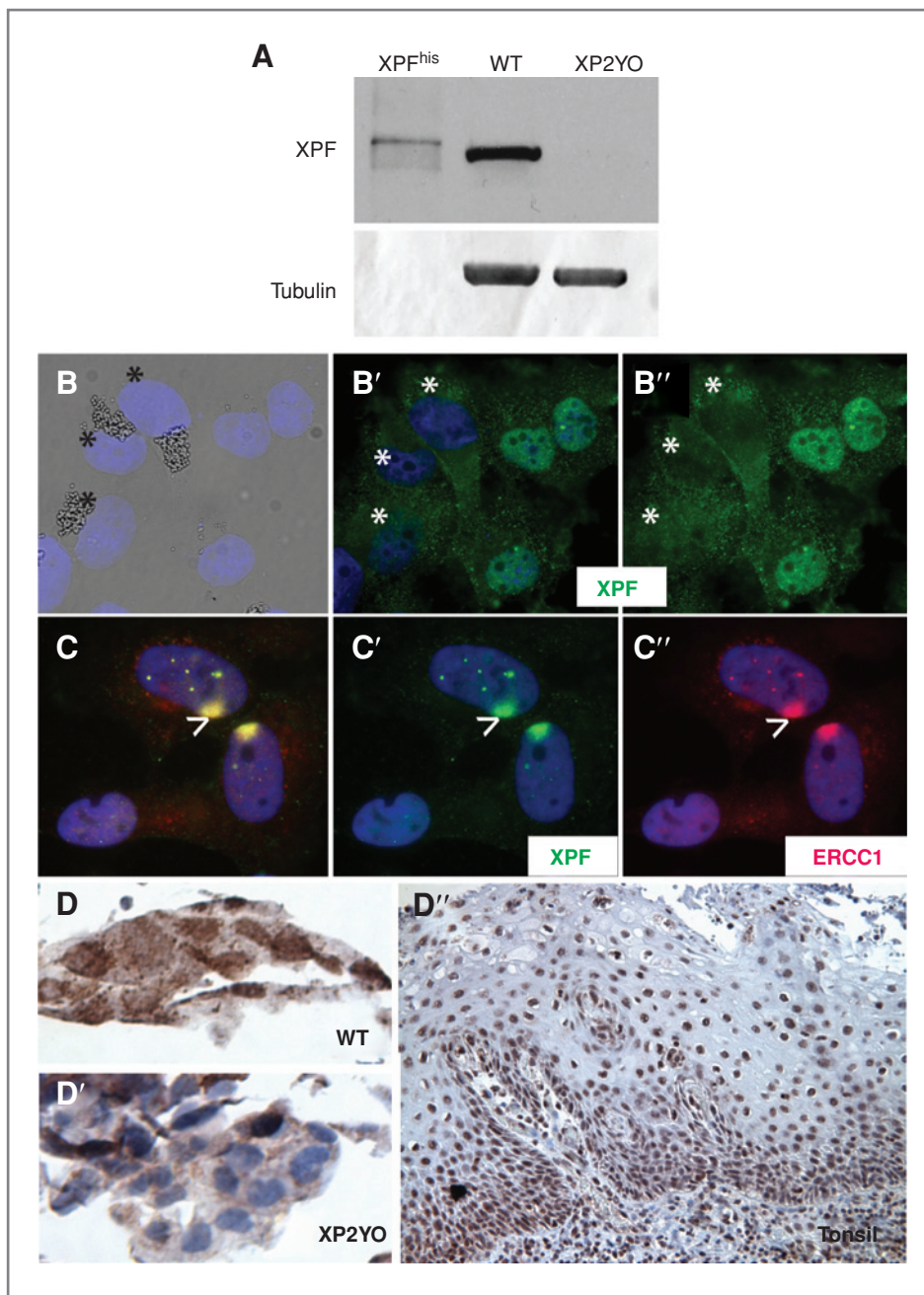


Figure 1. Antibody SPM228 is specific for XPF. **A**, immunoblot of whole cell lysates of immortalized human fibroblasts from a normal individual (WT) and an XP-F patient with virtually undetectable XPF level (XP2YO). The SPM228 antibody detects a 120-kDa band specifically in the WT and not in XP2YO negative control cells. Native XPF runs slightly faster than recombinant his-tagged XPF protein (XPF^{his}). Tubulin was used as loading control. **B–B''**, testing the specificity of SPM228 in immunofluorescence. **B**, XP2YO cells were labeled with large latex beads and cocultured with WT cells to create a test sample containing both XPF-positive and -negative cells. The different cell types were distinguished by brightfield imaging to visualize which cells contained beads in their cytoplasm. * indicates XPF-deficient XP2YO cells labeled with beads. [4',6'-diamidino-2-phenylindole (DAPI), blue]; **B'** and **B''**, immunodetection of XPF with SPM228. The WT cells have a strong nuclear signal, compared with neighboring XP2YO cells (XPF green, DAPI blue). **C–C''**, immunodetection of XPF (green) and ERCC1 (red) after UV irradiation of cells through a filter with 8- μ m pores. XPF and ERCC1 colocalize at sites of DNA damage (arrowhead). **D–D''**, specificity of SPM228 in IHC. WT and XP2YO cell pellets were fixed, paraffin embedded and sectioned. IHC reveals strong nuclear signal in WT cells (**D**) compared with the XPF mutant cells (**D'**). In oropharyngeal tissue, the antibody gives a strong nuclear signal with low cytoplasmic background (**D''**).

whether the signals colocalized. Indeed, both antibodies detected identical subnuclear domains of irradiated cells, which are sites of NER of UV-induced DNA damage (ref. 32; Fig. 1C). These data provide strong experimental evidence that SPM228 is specific for the DNA repair protein XPF.

To determine whether the antibody recognizes XPF in paraffin-embedded tissue, WT and XP2YO human fibroblasts were fixed in formalin and paraffin-embedded in parallel with noncancerous human oropharyngeal tissue and IHC was done. Nuclear staining was detected in WT, but not XPF mutant cells, and in the nuclei of human tissue (Fig. 1D). Thus, the monoclonal antibody clone SPM228 is specific for XPF and can be used for IHC to detect XPF expression.

XPF expression in HNSCC cell lines and tumors

The hypothesis that XPF could be a useful biomarker to predict clinical outcome relies on the supposition that level of XPF expression varies in HNSCC tumors. To determine whether that assumption was valid, we measured XPF expression in lysates of 7 randomly selected HNSCC cell lines by immunoblot (Fig. 2A). Expression of XPF ranged from 64% to 177% relative to WT human fibroblasts (100%) and XP2YO (0%). To determine whether this variability in XPF expression was an artifact of *in vitro* culture, XPF levels were also measured by immunoblot in 7 HNSCC tumors matched with normal adjacent tissue from the same patients. The tumors showed remarkable variability in the level of XPF expression (Fig. 2B). XPF

expression in normal tissue ranged from 3% to 40% of that detected in WT human fibroblasts; XPF expression in tumors ranged from 7% to 97% of that detected in WT human fibroblasts. Interestingly, in 6 of 7 cases, the levels of XPF expression in tumors were increased compared with levels in normal adjacent tissue. These data show that the level of XPF protein varies between HNSCCs, encouraging pursuit of the hypothesis that XPF levels might predict clinical outcome in HNSCC. Furthermore, the data suggest that XPF overexpression may be common in HNSCC, relative to normal tissue.

Association of XPF expression with clinical outcome in a cohort of patients with HNSCC

To test whether XPF levels predict tumor sensitivity to therapy, we collected data from 80 patients treated with curative intent for HNSCC with DNA damaging agents. The epidemiologic data, tumor site, and stage for this cohort are described in Table 1. Forty-two patients were treated with surgery and adjuvant therapy, the remaining 38 were treated with definitive radio- or chemoradiotherapy. Most tumors originated in the oropharynx (39%), oral cavity (34%), and larynx (16%). As expected from patients treated with radiation and chemotherapy, the majority of the tumors (77%) were of advanced clinical stage (stages III and IV), with predominance of stage IV tumors (56%). All patients received radiation therapy; 88% also received adjuvant chemotherapy with a platinum-based compound (cisplatin or carboplatin). The primary endpoint measured in this patient cohort was PFS (33).

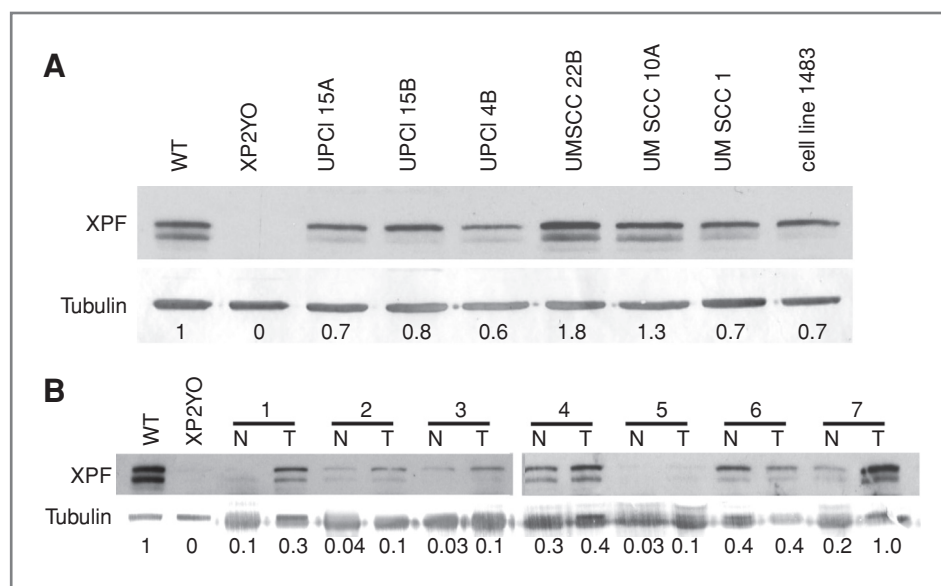


Figure 2. XPF expression varies in HNSCC cell lines and tumor lysates. A, immunoblot of 7 HNSCC cell lines for XPF. There is a variable amount of XPF detected in the different HNSCC tumor cell lines. Note that the XPF band appears as a 120 kDa doublet which may represent posttranslational modifications. Bands were quantified by densitometry. B, immunoblot of lysates from 7 tumors (T) and matched adjacent tissue (N). The amount of XPF protein varies greatly between tumors. Interestingly, there is also variation in XPF level between tumors and adjacent tissue in most paired samples. Six of 7 tumors display high XPF expression compared with normal adjacent tissue. Band intensity ratios, normalized to controls, are included below immunoblots. Tubulin was used as a loading control.

Table 1. Patient and tumor characteristics

Characteristic	Subcategory	n (%)
Patients		80
Mean age		56.8
	<60 y	48 (60)
	>60 y	32 (40)
Gender	M	63 (79)
	F	17 (21)
Smoker	Yes	56 (70)
	No	24 (30)
Tumor site	Oral cavity	27 (34)
	Oropharynx	31 (39)
	Larynx	13 (16)
	Hypopharynx	5 (6)
	Sinonasal cavity	3 (4)
	Unknown primary	1 (1)
Stage	I	3 (4)
	II	14 (18)
	III	17 (21)
	IV	45 (56)
	n/a	1 (1)
T stage	T1	11 (12)
	T2	31 (32)
	T3	25 (26)
	T4	29 (27)
	n/a	4 (3)
N stage	N0	28 (35)
	N1	20 (25)
	N2	25 (31)
	N3	5 (6)
	n/a	2 (3)
Treatment	XRT + platinum	70 (88)
	XRT alone	10 (13)
	Primary CRT or XRT	38 (47)
	Surgery + CRT or XRT	42 (53)
Mean follow-up (d)	1,129 (910–1,348) ^a	

^a95% CI.
Abbreviation: XRT, x-ray therapy; CRT, chemoradiation therapy.

XPF protein expression was measured in each of the tumors by using both digital image guided quantitative IHC (Supplementary Fig. S1) and pathologist read (H-score). Digital image guided quantitative IHC and pathologist scores were in agreement (Spearman rank correlation = 0.78, $P < 0.001$), with a tendency for the pathologist to underestimate density (Supplementary Fig. S2). Digital analysis score was used for further analysis. To determine whether XPF expression varied with tumor site, we analyzed differences on the basis of tumor location. Significant differences were found; tumors of the oral cavity expressed higher levels of XPF than tumors from other sites (Fig. 3A).

To evaluate whether XPF expression level predicted clinical outcome, we used a proportional hazards model. By both univariate and multivariate analysis, adjusting for

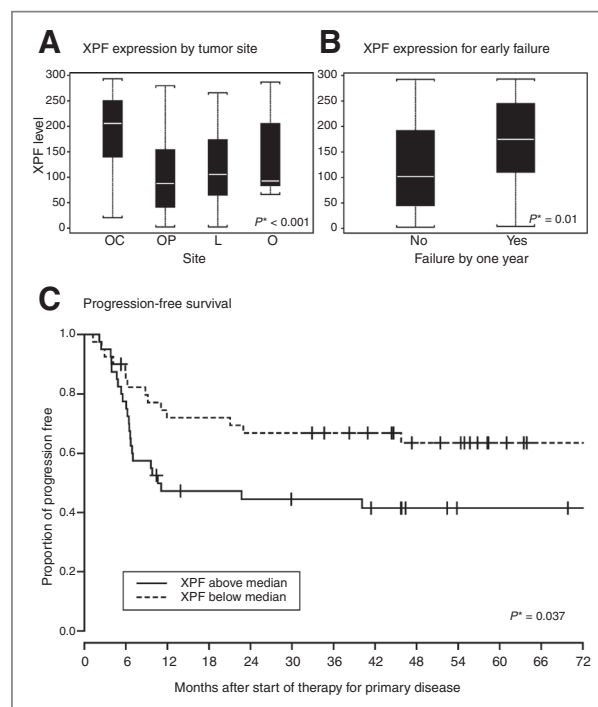


Figure 3. Low XPF expression significantly associates with improved PFS. A and B, graphical representation of XPF expression in tumors ($n = 80$), quantified by digitally assisted quantitative IHC and reported in box and whisker plots. A, XPF expression varies depending on the tumor site with the highest level found in the oral cavity. A Kruskal–Wallis test for equality among the 4 groups was significant ($P < 0.001$); oral cavity (OC), oropharynx (OP), larynx (L), and other (O). B, XPF expression is significantly higher in tumors from patients who fail treatment within 1 year ($n = 32$) compared with patients who are cured or fail treatment at a later time point ($n = 46$). XPF differed by group (Wilcoxon $P = 0.01$). C, PFS for low and high XPF expressing tumors was evaluated with Kaplan–Meier estimates. The cohort ($n = 80$) was split at the median XPF expression level, and PFS was plotted. Low vertical tick marks denote censoring times. Patients with XPF below the median had a 1 year probability of failure of 28% compared with 53% for those above the median (log rank $P = 0.037$); * denotes statistical significance.

significant covariates, high XPF expression was associated with clinical failure (HR = 1.87, $P = 0.03$ and HR = 1.83, $P = 0.05$, respectively; Table 2). This was particularly apparent for aggressive tumors that failed treatment within 1 year. Thirty-two of 37 failures occurred within 1 year of treatment and XPF was higher in these tumors ($P = 0.010$; Fig. 3B). To further illustrate the association between XPF expression and PFS, we split the cohort into 2 groups, on the basis of high and low XPF expression, by using the median XPF expression to define the groups. Kaplan–Meier survival estimates were carried out. The 2 groups had distinct survival curves, with the lowest XPF expression group showing longer PFS (Fig. 3C). The probability of 1 year PFS for the low XPF expressing group was 72% (95% CI = 55–83) compared with 47% (95% CI = 31–62) for the high XPF expressing group. Taken together, this data provides compelling evidence that XPF levels associate with treatment failure in HNSCC.

Association between XPF SNPs and clinical outcome

We postulated that because the level of XPF expression associated with clinical outcome in HNSCC, XPF SNPs, which potentially alter XPF expression or function, may also have predictive value. We analyzed blood collected prior to treatment ($n = 80$) to determine whether 13 different XPF single nucleotide allelic variants correlated with PFS in our cohort. Four SNPs were detected in too few carriers to be statistically useful and were excluded from the analysis. Because homozygote carriers of 2 allelic variants were rare, these patients were grouped with carriers of a single allelic variant for analysis. Among the remaining 9 SNPs, proportional hazards regression analysis identified 4 SNPs with marginally significant association with disease progression (Table 3). Interestingly, these 4 allelic variants were linked to more rapid treatment failure: rs3136155 (CT/TT; HR = 2.0, raw $P = 0.053$), rs1799799 (TC/CC; HR = 1.94, raw $P = 0.065$), rs3136202 (GA/AA; HR = 1.94, raw $P = 0.065$), and rs31336166 (TG/GG; HR = 1.94, raw $P = 0.065$). After adjustment for multiple testing, the expected false discovery rate of these associations was 48%. These SNPs are all located in introns and are not predicted to alter splicing; therefore, they are unlikely to modulate XPF activity. To understand better the mechanism by which the SNPs may affect clinical outcome, we assessed whether any of the 4 variant alleles correlated with XPF protein expression. Unexpectedly, the presence of SNP variants did not correlate with XPF protein expression in this cohort. Therefore, we identified 4 SNPs that are potential candidates to predict disease progression in HNSCC, independent of modulation of XPF protein expression, but these results need to be confirmed in a larger cohort.

Discussion

DNA damaging agents such as platinum-based compounds and radiation therapy constitute an essential part of the armamentarium against HNSCC. However, there is a high frequency of tumor resistance to these therapies, with approximately 50% local treatment failure at 5 years (5). These therapies cause substantial morbidity, with up to 82% of patients experiencing severe side effects (34). Patients that are likely to fail DNA damaging therapy could be better served by alternative treatment regimens, such as surgery or a taxane-based regimen. As new therapeutic options emerge, it is increasingly important for clinicians to be able to predict in advance whether a tumor is likely to respond to a particular type of treatment. Thus, a biomarker or panel of biomarkers predicting a high chance of failing DNA damaging therapy would be extremely valuable.

SNPs are attractive for use as biomarkers, as they can be reliably detected by using a simple blood test, and the technique does not require tumor tissue or sophisticated histochemical techniques. SNPs in XPF could mark altered DNA repair capacity by modifying XPF expression or function. For this reason, SNPs in XPF have previously been evaluated for their association with cancer risk (35–39). To our knowledge, no report has yet found SNPs in XPF to be a

biomarker for clinical outcome. We identified 4 allelic variants of XPF that associate marginally with worse clinical prognosis. This is consistent with previous reports that polymorphisms in ERCC1 predict worse outcome in HNSCC (28, 29). However, in the absence of external validation, it is possible that these results are because of type I error resulting from multiple testing. The mechanistic implications of these SNP findings remains incompletely understood. One might hypothesize that SNPs in XPF might correlate with a change in protein expression, activity level, or function, which in turn could impact response to treatment. However, none of the SNPs we analyzed correlated with XPF protein expression. However, it is also possible that the SNPs mark a more complex haplotype, which predict disease progression through an unrelated mechanism, for example, perhaps through distant regulation of other gene products near the XPF genetic locus, or through noncoding RNA such as microRNA. The 4 SNPs that we identified may prove useful in the development of prognostic blood tests for HNSCC patients and deserve further clinical investigation in a prospective setting after validation in a second study population.

In contrast to SNPs, several tumor protein biomarkers have previously been identified that predict tumor response in HNSCC. For instance, epidermal growth factor receptor (EGFR) overexpression correlates with shorter disease-free survival (40, 41). In addition, cell–cell adhesion receptor E-cadherin and its partner beta-catenin (which regulate epithelial–mesenchymal transition), amphiregulin and epiregulin (markers for response to EGFR antagonist in colorectal cancer; ref. 42), the antiapoptotic gene BCL-XL (43) and TP53 (43) have all been recommended for further investigation as biomarkers in the design of clinical trials on HNSCC (44, 45). In DNA repair pathways, Ku80, which participates in DSB repair, has recently been identified as a marker of outcome in HNSCC treated with radiotherapy (46). ERCC1 has been previously identified as a biomarker that predicted outcome in HNSCC, but recent reports challenge the earlier conclusion (15, 16, 18, 20), possibly because a nonspecific antibody (clone 8F1) was used in these studies (19).

Herein, we sought to determine if XPF, the essential binding partner of ERCC1, could be reliably measured in tumors and used as a biomarker to predict clinical outcome. Importantly, our study, like most, has several limitations. First, there was a limited quantity of tumor tissue available from individual patients, preventing a comparison of XPF expression by multiple methods (e.g., immunohistochemistry and immunoblot), in normal tissue vs. tumor, and in tumors before and after chemoradiation therapy. Second, the cohort included HNSCC tumors from several mucosal sites including the oral cavity, pharynx and larynx. Third, despite strict inclusion criteria the study is a retrospective analysis of prospectively collected samples in which the initial cohort was not prospectively accrued to test XPF level specifically. Finally, patients were not enrolled in a single clinical trial, so treatment regimens varied. However, despite these limitations, we detected a

Table 2. Proportional hazards models for disease progression

Covariate	Reference	Univariate		Multivariate	
		HR (95% CI)	P	HR (95% CI)	P
Age	Nonlinear		0.014		0.012
Gender	Female	0.77 (0.36–1.63)	0.487		
T stage 3 or 4	T stage 1 or 2	2.05 (1.01–4.20)	0.048	2.01 (0.97–4.14)	0.060
Site oropharynx	Oral cavity	0.52 (0.25–1.07)	0.076		
Site larynx	Oral cavity	0.35 (0.12–1.05)	0.061		
Site other	Oral cavity	0.58 (0.19–1.75)	0.330		
Chemoradiation surgery	Radiation	0.39 (0.18–0.86)	0.020	0.33 (0.14–0.76)	0.009
	None	1.18 (0.62–2.25)	0.624		
XPF	67–218 ^a	1.87 (1.07–3.25)	0.027	1.83 (0.99–3.38)	0.053

^aAcross the inter-quartile range.

significant association between XPF expression and progression free survival after adjusting for confounding clinical and pathological factors.

Robust mechanistic data explain how expression of XPF could influence clinical outcome in tumors treated with DNA damaging agents. XPF, with its partner ERCC1, plays important roles in NER, ICL, and DSB repair pathways used to correct the genotoxicity of platinum compounds and radiation. Experiments in cells from XPF-deficient patients and animal models of their disease show that low XPF expression is associated with an exquisite sensitivity to DNA damaging agents (8, 9, 47). Similarly, tumors expressing a low level of XPF are more likely to be sensitive to genotoxic agents such as cisplatin and radiation. Our result that tumors with low levels of XPF have a better clinical outcome is consistent with this mechanism.

Table 3. Omnibus test for association between SNPs and disease progression

SNP	Common allele	Variant allele	HR ^a	Raw P ^b
rs1799799	T	C	1.94	0.065 ^c
rs1799801	T	C	1.46	0.265
rs3136105	T	C	1.41	0.415
rs3136146	G	A	1.69	0.191
rs3136152	G	A	2.30	0.240
rs3136155	C	T	2.00	0.053 ^c
rs3136166	T	G	1.94	0.065 ^c
rs3136189	T	C	1.44	0.285
rs3136202	G	A	1.95	0.065 ^c

^aHR for common/variant genotype.

^bLog rank test.

^cMarginal statistical significance based on unadjusted P value.

Biochemical analysis of tissue lysates revealed that XPF levels were higher in tumors than in adjacent normal tissue, in 6 of 7 paired specimens. This raises the possibility that XPF expression might be induced during tumorigenesis. Cisplatin induces increased expression of ERCC1 in some ovarian cancer cell lines (48). This is a particularly important line of investigation because it argues for the need for repeated measurement of XPF levels in tumors during the course of treatment to predict changes in treatment response and to minimize unnecessary side effects if drug resistance emerges.

The observation that XPF expression is higher in tumors originating in the oral cavity compared with other sites is interesting. Although the reason for this difference is unclear, it may explain why cancers from the oral cavity are typically treated primarily with surgery, reserving chemoradiation for adjuvant therapy. Importantly, because XPF expression was higher in oral cavity tumors, we asked whether our analysis was biased. Theoretically, it is possible that the patients with low XPF expression could have a longer PFS simply because this group has an overrepresentation of oropharyngeal and laryngeal tumors, which may have a better prognosis, irrespective of XPF expression. Two arguments make this possibility unlikely. First, univariate analysis showed that PFS did not strongly associate with tumor sites ($P = 0.157$). Second, multivariate analysis revealed that in this cohort, XPF is an independent factor associated with PFS, irrespective of tumor site. XPF is therefore a bona fide predictor of clinical outcome.

In summary, this is the first article, in any cancer, showing that the expression level of XPF associates with clinical outcome and that XPF may represent a biomarker predicting success of DNA damaging therapy. These findings will inform the design of prospective clinical trials providing personalized treatment for HNSCC as a function of the anticipated susceptibility of the tumor to DNA damaging agents, in hopes of minimizing unnecessary treatment-related toxicities and improving outcomes.

Disclosure of Potential Conflicts of Interest

X. Wang and D.T. Weaver were employees and/or stockholders of On-Qity, Inc. No other potential conflicts of interest were disclosed.

Authors' Contributions

A. Vaezi, X. Wang, S. Buch, L. Wang, and R.R. Seethala performed the experiments. A. Vaezi and W. Gooding analyzed the data. A. Argiris, D.T. Weaver, A.D. D'Andrea, M. Romkes, L.J. Niedernhofer, and J.R. Grandis planned the study. A. Vaezi, L.J. Niedernhofer, and J.R. Grandis wrote the manuscript.

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