XPF expression correlates with clinical outcome in squamous cell carcinoma of the head and neck
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XPF is a biomarker in HNSCC

Statement of Translational Relevance

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

Here we demonstrate a significant association between low expression of XPF and longer PFS in head and neck squamous cell carcinoma 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 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.
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 in the XPF gene 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 to 72% (95% CI = 55% - 83%) for low expressers. In addition, we identified four XPF single nucleotide polymorphisms (SNPs) that demonstrated 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.
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). 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 post-operative therapy (3-4). However, resistance to chemotherapy occurs frequently, with five year local and distant failure rates of 50% and 15%, respectively (5). Alternative treatments that do not rely upon damage 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 of DNA (NER), the repair of DNA interstrand crosslinks (ICL), or nonhomologous end-joining (NHEJ) of double-strand breaks (DSBs), respectively. As a consequence, these patients are exquisitely sensitive to DNA damaging agents including ionizing radiation and crosslinking agents like 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 bi-partite nuclease that is essential for NER and ICL repair, and participates in DSB repair (8-11). Platinum-based chemotherapeutic
XPF is a biomarker in HNSCC
drugs react with DNA to induce adducts that affect one 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 virtually 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 four SNPs 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

Immuno detection of XPF in cell and tumor lysates. Cell line origins are indicated in the supplemental 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 internal 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 performed as described elsewhere (30). For XPF immunodetection we used the monoclonal antibody SPM228 (1:1000; Abcam, Cambridge, MA) and tubulin as a loading control (1:5000; Sigma, St Louis, MO) followed by horseradish peroxidase- or alkaline phosphatase-conjugated secondary antibodies (1:5000 and 1:2500, respectively; Promega, Madison, WI). 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 internal review board of the University of Pittsburgh, and performed in accordance with the Helsinki agreement. Informed consent was obtained from patients for sample collection. Biopsy proven HNSCC cancer patient were enrolled prospectively in the
XPF is a biomarker in HNSCC context of a genomics study (n=522) conducted at our institution from 2000 to 2007. Formalin-fixed paraffin-embedded pre-treatment biopsies or resection specimens, blood and clinical data were collected prospectively by the University of Pittsburgh Head and Neck SPORE Tissue bank. For the present 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 post-surgery 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 de-identified 87 patients satisfying the eligibility criteria. Seven out the 87 samples did not contain enough tumor or were not suitable for immunohistochemistry and were excluded from the analysis.

**Immunofluorescence.** Differential immunodetection of XPF and ERCC1 in cell nuclei was performed 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 co-cultured, providing an internal negative control for immunodetection of ERCC1-XPF. Immunofluorescence was performed with clones SPM228 (1:200; Abcam, Cambridge, MA) and FL297 (1:200; SantaCruz, SantaCruz, CA), to detect XPF and ERCC1 respectively. Local photodamage to detect ERCC1-XPF at sites of ultraviolet (UV)-induced DNA damage was performed as previously described (30).

**Immunohistochemistry.** Full sections of paraffin-embedded tumors were processed using standard techniques. Antigen retrieval was with 10 mM 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 (1:1200; Abcam, Cambridge, MA). XPF was detected using renaissance...
XPF is a biomarker in HNSCC

TSA™ (Tyramide Signal Amplification) Biotin System (Perkin Elmer, Waltham, MA), with hematoxylin (Vector, Burlingame, CA) counterstain.

*Microscopy and quantification of immunohistochemistry.* For digitally assisted quantification, immunohistochemistry slides were scanned using an automated slide scanner (Aperio, Vista, CA). For every tumor section, six ~600 μm² regions were selected by a pathologist for biomarker quantification. The selected areas were analyzed using a customized image algorithm (Aperio, Vista, CA). XPF intensity was expressed in a 0-300 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-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 using DNA isolation kits (Gentra Systems Inc., Minneapolis, MN). A custom 384-SNP panel was screened using the Illumina® GoldenGate technology (Illumina, SanDiego, CA). 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 six features: 1) haplotype tag SNPs for both Caucasians and Africans defined by predicted LD scores R² ≥ 0.8; 2) functional SNPs characterized by amino acid substitutions; 3) potential for regulatory changes; 4) SNPs that alter protein stability; 5) evolutionary conservation across species and 6) published epidemiological data. To ensure high quality genotype results, a number of routinely followed quality control procedures were performed including: 1) quality control
XPF is a biomarker in HNSCC

and quantification of incoming DNA samples; 2) multiple internal controls built into each
genotyping assay including the screening of each SNP allele ~30 times; 3) bar-coded labeling of
sample plates; and 4) 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 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 died from other causes were censored at their last date
of record. The association of XPF and progression-free survival 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 one year, an
alternate binary endpoint of progression (treatment failure) at one year was also analyzed. A
Wilcoxon test compared XPF expression between patients who progressed within one year
versus those who survived at least one 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
progression-free survival 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 minor 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 immunohistochemistry between machine-based
XPF is a biomarker in HNSCC

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 immunohistochemistry on paraffin-embedded sections. We first determined that the commercially available monoclonal antibody clone SPM228 is specific for XPF using biochemical methods and immunolocalization with positive and negative controls. On immunoblot, the antibody detected a band of the appropriate molecular weight (~120 kD) in lysates of wild-type (WT) cells but not XP-F 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 if the antibody can distinguish between cells with high and low XPF expression, WT and XP2YO cells labeled with latex beads of different sizes were co-cultured 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, based on the 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’-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 performed immunofluorescence with SPM228 and a specific ERCC1 antibody (19) to determine if the signals co-localized. Indeed, both antibodies
detected identical subnuclear domains of irradiated cells, which are sites of NER of UV-induced DNA damage (32) (Fig. 1C). These data provide strong experimental evidence that SPM228 is specific for the DNA repair protein XPF.

To determine if the antibody recognizes XPF in paraffin-embedded tissue, WT and XP2YO human fibroblasts were fixed in formalin and paraffin-embedded in parallel with non-cancerous human oropharyngeal tissue, and immunohistochemistry performed. 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 immunohistochemistry 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 if that assumption was valid, we measured XPF expression in lysates of seven randomly selected HNSCC cell lines by immunoblot (Fig. 2A). Expression of XPF ranged from 64%-177% relative to WT human fibroblasts (100%) and XP2YO (0%). To determine if this variability in XPF expression was an artifact of *in vitro* culture, XPF levels were also measured by immunoblot in seven 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 3-40% of that detected in WT human fibroblasts; XPF expression in tumors ranged from 7-97% of that detected in WT human fibroblasts. Interestingly, in six of seven cases, the levels of XPF expression in tumors were increased compared with levels in normal adjacent tissue. These data demonstrate that the level of XPF protein varies between HNSCCs, encouraging pursuit of the
XPF is a biomarker in HNSCC

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 epidemiological 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 chemoradiation. 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 (stage 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).

XPF protein expression was measured in each of the tumors using both digital image guided quantitative immunohistochemistry (Supplemental Figure 1) and pathologist read (H-score). Digital image guided quantitative immunohistochemistry and pathologist scores were in agreement (Spearman rank correlation=0.78, p<0.001), with a tendency for the pathologist to underestimate density (Supplemental Figure 2). Digital analysis score was used for further analysis. To determine whether XPF expression varied with tumor site, we analyzed differences based on 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 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 one year. Thirty-two out of 37 failures occurred within one 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 two groups, based on high and low XPF expression, using the median XPF expression to define the groups. Kaplan-Meier survival estimates were performed. The two groups had distinct survival curves, with the lowest XPF expression group showing longer PFS (Fig. 3C). The probability of one year PFS for the low XPF expressing group was 72% (95% CI = 55% - 83%) compared to 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 since 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 thirteen 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 two allelic variants were rare, these patients were grouped with carriers of a single allelic variant for analysis. Among the remaining nine SNPs, proportional hazards regression analysis identified four SNPs with marginally significant association with disease progression (Table 3). Interestingly, these
four allelic variants were linked to more rapid treatment failure: rs3136155(CT/T) (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 four 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 four 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 five 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 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 four 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 due to 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 non-coding RNA such as microRNA. The four 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, 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
XPF is a biomarker in HNSCC (markers for response to EGFR antagonist in colorectal cancer) (42), the anti-apoptotic 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 non-specific antibody (clone 8F1) was used in these studies (19).

Herein, we sought to determine if XPF, the essential binding partner of ERCC1, could be 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 significant association between XPF expression and PFS after adjusting 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 for confounding clinical and pathological factors.

Robust mechanistic data explains how expression of XPF could influence clinical outcome in tumors treated with DNA damaging agents. XPF, with its partner ERCC1, play 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 demonstrate 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.

Biochemical analysis of tissue lysates revealed that XPF levels were higher in tumors than in adjacent normal tissue, in six out of seven 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 to other sites is interesting. While 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, since XPF expression was higher in oral cavity tumors, we asked if 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 over-representation 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 report, in any cancer, demonstrating 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.
XPF is a biomarker in HNSCC

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Presentation at meetings

This work was presented at the 2010 ASCO annual meeting in Chicago, on June 4 2010.

Disclosure of Potential Conflict of Interest


X.W., D.T.W. are employees and/or stockholders of On-Q-ity, Inc.
XPF is a biomarker in HNSCC

References

XPF is a biomarker in HNSCC

XPF is a biomarker in HNSCC


Figure legends

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 kD band specifically in the WT and not in XP2YO negative control cells. Native XPF runs slightly faster than recombinant his-tagged XPF protein (XPF\textsuperscript{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 co-cultured 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. (DAPI, blue); (B’-
XPF is a biomarker in HNSCC

B””) Immunodetection of XPF with SPM228. The WT cells have a strong nuclear signal, compared to 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 μm pores. XPF and ERCC1 co-localize at sites of DNA damage (arrow head). (D-D””) Specificity of SMP228 in immunohistochemistry. WT and XP2YO cell pellets were fixed, paraffin-embedded and sectioned. IHC reveals strong nuclear signal in WT cells (D) compared to the XPF mutant cells (D’). In oropharyngeal tissue, the antibody gives a strong nuclear signal with low cytoplasmic background (D”).

Figure 2. XPF expression varies in HNSCC cell lines and tumor lysates.

(A) Immunoblot of seven HNSCC cell lines for XPF. There is a variable amount of XPF detected in the different HNSCC tumor cell lines. Note that XPF band appears as a 120 kD doublet which may represent posttranslational modifications. Bands were quantified by densitometry. (B) Immunoblot of lysates from seven 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 seven tumors display high XPF expression compared to normal adjacent tissue. Band intensity ratios, normalized to controls, are included below immunoblots. Tubulin was used as a loading control.

Figure 3. Low XPF expression significantly associates with improved progression-free survival.

(A-B) Graphical representation of XPF expression in tumors (n=80), quantified by digitally assisted quantitative immunohistochemistry and reported in box and whisker plots. (A) XPF
XPF is a biomarker in HNSCC

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 one year (n=32) compared to patients who are cured or fail treatment at a later time point (n=46). XPF differed by group (Wilcoxon p = 0.01). (C) Progression-free survival 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 progression-free survival was plotted. Low vertical tick marks denote censoring times. Patients with XPF below the median had a one year probability of failure of 28% compared to 53% for those above the median (log rank p = 0.037); * denotes statistical significance.
Table 1. Patient and tumor characteristics

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<td>(25)</td>
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<td>(88)</td>
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<td></td>
<td>Primary CRT or XRT</td>
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<td>(47)</td>
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<tr>
<td></td>
<td>Surgery + CRT or XRT</td>
<td>42</td>
<td>(53)</td>
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<tr>
<td>Mean follow-up</td>
<td>(days)</td>
<td>1129</td>
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</table>

*95% CI  
XRT = x-ray therapy; CRT = chemoradiation therapy
Table 2. Proportional hazards models for disease progression

<table>
<thead>
<tr>
<th>Covariate</th>
<th>Reference</th>
<th>Hazard Ratio</th>
<th>95% CI</th>
<th>p</th>
<th>Hazard Ratio</th>
<th>95% CI</th>
<th>p</th>
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<td>0.36-1.63</td>
<td>0.014</td>
<td>2.05</td>
<td>0.97-4.14</td>
<td>0.012</td>
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<tr>
<td>gender</td>
<td>female</td>
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<td>1.01-4.20</td>
<td>0.048</td>
<td>2.01</td>
<td>0.97-4.14</td>
<td>0.060</td>
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<tr>
<td>T stage 3 or 4</td>
<td>T stage 1 or 2</td>
<td>2.05</td>
<td>1.01-4.20</td>
<td>0.048</td>
<td>2.01</td>
<td>0.97-4.14</td>
<td>0.060</td>
</tr>
<tr>
<td>site oropharynx</td>
<td>oral cavity</td>
<td>0.52</td>
<td>0.25-1.07</td>
<td>0.076</td>
<td>0.58</td>
<td>0.19-1.75</td>
<td>0.330</td>
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<tr>
<td>site larynx</td>
<td>oral cavity</td>
<td>0.35</td>
<td>0.12-1.05</td>
<td>0.061</td>
<td>0.35</td>
<td>0.12-1.05</td>
<td>0.061</td>
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<td>oral cavity</td>
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<td>0.19-1.75</td>
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<td>0.58</td>
<td>0.19-1.75</td>
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<td>0.39</td>
<td>0.18-0.86</td>
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<td>0.62-2.25</td>
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<td>XPF</td>
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<td>1.07-3.25</td>
<td>0.027</td>
<td>1.83</td>
<td>0.99-3.38</td>
<td>0.053</td>
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</table>

*Across the inter-quartile range
Table 3. Omnibus test for association between SNPs and disease progression

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<th>SNP</th>
<th>Common allele</th>
<th>Variant allele</th>
<th>Hazard ratio *</th>
<th>Raw p * *</th>
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<tr>
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<td>T</td>
<td>C</td>
<td>1.94</td>
<td>0.065#</td>
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<td>C</td>
<td>1.46</td>
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<td>C</td>
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<td>rs3136146</td>
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<td>A</td>
<td>1.69</td>
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* Hazard ratio for common/variant genotype
** Log rank test
# Marginal statistical significance based on unadjusted p value
Figure 2

A

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<th></th>
<th>WT</th>
<th>XP2YO</th>
<th>UPCI 15A</th>
<th>UPCI 15B</th>
<th>UPCI 4B</th>
<th>UMSCC 22B</th>
<th>UM SCC 10A</th>
<th>UM SCC 1</th>
<th>cell line 1483</th>
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<td>0.1</td>
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</table>

B

<table>
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<td>0.1</td>
<td>0.3</td>
<td>0.04</td>
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<td>0.1</td>
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<tr>
<td>Tubulin</td>
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Figure 3

A  XPF expression by tumor site

B  XPF expression for early failure

C  Progression free survival

- XPF above median
- XPF below median

p* < 0.001

p* = 0.01

p* = 0.037
Clinical Cancer Research

XPF expression correlates with clinical outcome in squamous cell carcinoma of the head and neck

Alec Vaezi, XiaoZhe Wang, Shama C Buch, et al.

Clin Cancer Res  Published OnlineFirst July 7, 2011.

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