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Prognostic Biomarkers for HNSCC using Quantitative Real-Time PCR and Microarray Analysis: β -Tubulin Isotypes and the p53 Interactome

Sharon Lobert^{1,*}, Mary E. Graichen¹, Robert D. Hamilton², Karen T. Pitman³, Michael R. Garrett⁴, Chindo Hicks⁵, and Tejaswi Koganti⁵

¹School of Nursing, University of Mississippi Medical Center, Jackson, Mississippi

²Division of Hematology and Oncology, School of Medicine, University of Mississippi Medical Center, Jackson, Mississippi

³Banner MD Anderson Cancer Center, Gilbert, Arizona

⁴Department of Pharmacology, University of Mississippi Medical Center, Jackson, Mississippi

⁵Department of Medicine, University of Mississippi Medical Center, Jackson, Mississippi

Abstract

In 2014, more than 40,000 people in the United States will be diagnosed with head and neck squamous cell cancer (HNSCC) and nearly 8400 people will die of the disease (www.cancer.org/acs/groups). Little is known regarding molecular targets that might lead to better therapies and improved outcomes for these patients. The incorporation of taxanes into the standard cisplatin/5-fluorouracil initial chemotherapy for HNSCC has been associated with improved response rate and survival. Taxanes target the β -subunit of the tubulin heterodimers, the major protein in microtubules, and halt cell division at G2/M phase. Both laboratory and clinical research suggest a link between β -tubulin expression and cancer patient survival, indicating that patterns of expression for β -tubulin isotypes along with activity of tumor suppressors such as p53 or micro-RNAs could be useful prognostic biomarkers and could suggest therapeutic targets.

Keywords

tubulin; tubulin isotypes; HNSCC; p53; TUBB3

In this study we used quantitative real time PCR and microarray analysis to compare HNSCC samples and associated normal tissues from 33 patients. We measured mRNA for six β -tubulin isotypes: classes I, IIA, IIB, III, IVB, and V, using quantitative real time PCR and in addition, used microarray analysis to explore the expression of >30,000 genes.

We found reduced activity of the p53 interactome is associated with progressive disease at 2 years and elevated β -tubulin class III was linked to a trend for disease free survival over the same time period. β -tubulin class III may be a prognostic biomarker for HNSCC and molecular targets within the p53 interactome may be useful for developing novel therapies.

Introduction

It is estimated that in 2014, 42,440 people will be diagnosed with head and neck squamous cell cancer (HNSCC) in the United States and nearly 8400 people will die of the disease (www.cancer.org/acs/groups). Approximately 25–60% of cancers of the oropharynx are associated with human papilloma virus (HPV) infections [Wittekindt et al., 2012]. As therapies are refined, survival for HNSCC has improved 5% over the last 10 years, with the most pronounced improvement in 2-year survival occurring for those patients who have HPV-related carcinomas. However the recognition of important tumor-related molecular biomarkers for HNSCC lags behind the discoveries for other cancers such as breast cancer (e.g. *Her2/neu*, estrogen- and progesterone-receptors), where tailored therapy accounts for a 10% improved survival in this group of patients. Growth factors, tumor suppressor proteins, cell cycle proteins, and cytoskeletal proteins have been investigated as potential molecular biomarkers for HNSCC [Haddad and Shin, 2008]. HPV proteins, E6 and E7 inactivate the tumor suppressors p53 and pRb (retinoblastoma) [Wittekindt et al., 2012] and signaling pathways related to p53 and pRb are disrupted in HPV-related cancers. Furthermore, it is estimated that p53 activity is down-regulated in 80% of HNSCCs [Rothenberg and Ellisen, 2012]. Thus HPV infection is associated with molecular changes that could alter expression of many potential biomarkers. In spite of our increasing understanding of molecular pathways in HPV-positive HNSCCs, little is known about mechanisms underlying HPV-negative HNSCCs [Rothenberg and Ellisen, 2012].

Although few strategies for HNSCC treatment target molecular markers, one significant example is the inclusion of monoclonal antibodies in chemotherapy protocols. Since epidermal growth factor signaling is implicated in HNSCCs, cetuximab, a monoclonal antibody targeting the epidermal growth factor receptor (EGFR), was tested in combination with radiation therapy in patients with HNSCC [Bonner et al., 2006]. While a significant survival benefit was realized for the group as a whole receiving cetuximab, not all patients experienced a benefit.

Recently signaling associated with the transcription factor NF- κ B was shown by microarray analysis to be associated with high risk HNSCCs [Chung et al., 2006] and inhibition of this pathway has the potential to reduce HNSCC tumor invasion and metastasis [Yan et al., 2010]. However, it is clear that no single biomarker can guide treatment selection and it is essential to identify and validate patient-specific prognostic and predictive biomarker patterns for HNSCC to effect greater survival in the future [Chung et al., 2008].

β -Tubulin Classes II and III as Prognostic Biomarkers for Solid Tumors

Incorporation of taxanes into the standard cisplatin/5-fluorouracil initial chemotherapy for HNSCC has been associated with improved response rate and survival [Haddad and Shin, 2008]. Taxanes, such as docetaxel, target the β -subunit of the $\alpha\beta$ -tubulin heterodimers, the

major protein in microtubules, and halt cell division at G2/M phase. The major differences between β -tubulin isotype classes reside in the last 15–20 amino acids of the carboxyl termini [Sullivan, 1988]. These differences have been exploited in the development of isotype-specific antibodies and the characterization of seven β -tubulin classes: I, II, III, IVa, IVb, V, and VI. β -tubulin class II is coded for by two genes *TUBB2A* and *TUBB2B* located in tandem on chromosome 6p25. Thus a total of eight genes code for β -tubulins, although the functional significance of this heterogeneity remains under intense study. Mammalian microtubules in mitotic spindles are formed from a mixture of α - and β -tubulin isotype classes. In addition the distribution patterns of β -tubulin isotypes in mammalian tissues is complex and the patterns differ in normal and tumor cells [Leandro-Garcia et al., 2010].

While there are no proven predictive biomarkers for treatment response, β -tubulin class II expression was correlated with adverse outcomes for patients with HNSCC [Cullen et al., 2009], suggesting that expression of this protein may have predictive value. Published work from our lab, demonstrated that β -tubulin classes II and V mRNA expression is significantly increased in non-small cell lung cancers compared to normal lung tissue [Cucchiarelli et al., 2008]. In another study, we showed that the ratio of β -tubulin classes II to V mRNA is higher in breast cancers compared to normal breast tissue (data re-analyzed from reference [Dozier et al., 2003]). β -tubulin class III has been implicated in several studies as a biomarker for taxane resistance [Cabral, 2008]. Increases in β -tubulin class III or V were shown to induce paclitaxel resistance in CHO cells [Hari et al., 2003; Bhattacharya and Cabral, 2004, 2009]. Our work has revealed that short term drug treatment alters β -tubulin isotype expression [Lobert et al. 2011, 2013], suggesting that patient-specific drug receptor responses to chemotherapy contribute to outcomes. Thus both laboratory and clinical research suggest a link between β -tubulin expression and cancer patient survival, indicating that patterns of expression for β -tubulin isotypes along with activity of tumor suppressors such as p53 or micro-RNAs could be useful prognostic biomarkers. In this study we used quantitative real time PCR and microarray analysis to compare HNSCC samples and associated normal tissues. We found reduced activity of the p53 interactome is associated with progressive disease at two years and elevated β -tubulin class III was linked to a trend for disease free survival over the same time period.

Materials and Methods

Tissues

With the approval of the University of Mississippi Medical Center's institutional review board, participants were recruited from all patients admitted with a diagnosis of oropharyngeal, hypopharyngeal or laryngeal squamous cell carcinoma between 2009 and 2012. Based upon our experience with our previous study of NSCLCs [Cucchiarelli et al., 2008], we expected a large effect size for qRT-PCR tubulin analysis done with both matched pairs and with independent groups. Power analysis (large effect size) indicated that for Student *t*-tests or one-way ANOVA, 15 samples per group were needed for a power of 0.78 and α of 0.05. Thus we anticipated that a final sample of 30 pairs (30 normal and 30 tumor tissues in each group) would be sufficient.

The tissues were placed in RNeasy[®] (Qiagen, Valencia, CA) immediately after surgical removal. Eighty-two paired normal and tumor tissue samples were obtained prior to treatment (164 samples total). The normal tissue sample was taken at the same time as the tumor sample but from a site distant to the tumor. Of these, 33 pairs met our criteria for RNA quality: concentration sufficient for qRT-PCR analysis, A260/280>1.8 and RIN>6.0. The RNA Integrity Number (RIN) limit was set empirically from Agilent Lab Chip electropherograms of tissue sample RNA. Although other individual samples in the pool of 164 samples met our criteria, we only included samples where both the normal and tumor sample were acceptable.

Real Time Quantitative RT-PCR

β -tubulin isotypes and HPV16 E6 were measured by quantitative real time PCR. Our primers for β -tubulin isotypes have been previously reported [Hiser et al., 2006]. We designed and validated HPV primers using HPV infected cells (SiHa cervical carcinoma cells, from ATCC). The primers for HPV16 E6 were forward: AAG CAA CAG TTA CTG CGA CG; reverse: GGA CAC AGT GGC TTT TGA CA. For all other primers, we used the Qiagen Primer Assays (Qiagen, Valencia, CA). Our method for extracting total RNA and performing quantitative real time RT-PCR has been previously described [Hiser et al., 2006; Cucchiarelli et al., 2008]. We used quantitative real time PCR with a standard curve on each plate to measure β -tubulin isotypes. The standard curve is made with a known amount of cDNA purified from an agarose gel and run as a template in 10-fold serial dilutions. Sybr Green 1 was used as the fluorescent dye for double stranded product. Data were normalized as mRNA copies/ μ g of total RNA. Comparative real time PCR was used to validate the expression of genes in the p53 interactome that changed in the microarray analysis comparing tumor samples from patients with no evidence of disease (NED) at 2 years with those who experienced disease progression, recurrence or death (PRD). The pooled NED samples were used as the calibrator with the Ct method [Lobert et al., 2010]. GAPDH expression was used to normalize the data.

Microarrays

RNA was isolated from 15 paired normal and HNSCC tumor tissues (a subset of the total 33 paired tissues) and evaluated for quality and integrity (Bio-Rad Experion[™] System) as done previously [Westbrook et al., 2014]. Whole genome transcript analysis was performed using Affymetrix 3000 7G System platform using Human GeneChip 1.0ST arrays. The Human GeneChip 1.0ST covers >30,000 coding transcripts, along with a small number of lincRNA transcripts. RNA samples were processed per manufacturers' protocols using Ambion[®] Whole-Transcript (WT) Expression kit, and Affymetrix[®] GeneChip WT Terminal Labeling and Controls Reagent kit on Affymetrix equipment (Scanner 3000 7G System). Hybridized chips were automatically washed, stained and scanned at the UMMC Institutional Molecular and Genomics Core. Data obtained from these gene expression studies are deposited in the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>) with the GEO accession number GSE58911.

Microarray Data Analysis

We performed supervised analysis comparing gene expression levels between groups. In the first step we compared gene expression levels between cases and controls to identify significantly differentially expressed genes distinguishing the normal and tumor groups. In the next step we compared gene expression levels between patients with disease progression (PRD) and patients with no evidence of disease (NED). For both analyses, we used a Student *t*-test to identify significantly differentially expressed genes distinguishing the two groups. Permutation test was applied to reliably estimate the *P*-values. We used a false discovery rate (FDR) [Benjamini and Hochberg, 1995] to correct for multiple hypothesis testing. Due to small sample sizes (9 PRD tumor samples and 5 NED tumor samples), we did not partition the data into test and validation sets, but instead we used an out of sample (leave-one-out) validation procedure to identify genes with predictive power [Reich et al., 2006]. Supervised analyses were performed using the Pomello II software package [Morrissey and Diaz-Uriarte, 2009]. To determine whether the identified genes are functionally related and have similar patterns of expression, we performed unsupervised analysis using hierarchical clustering. Under this approach, we computed the Pearson correlation coefficients between all possible pairs of significantly differentially expressed genes. Using the Pearson correlation coefficient as the distance measure and the complete linkage method, the genes were subjected to hierarchical clustering using GenePattern [Reich et al., 2006]. Prior to clustering, gene expression data were normalized using the median normalization and were standardized and centered [Eisen et al., 1998]. To further investigate whether the identified genes are involved in the same molecular functions, biological processes and cellular components we used gene ontology (GO) analysis. To gain insights about the broader context in which significantly differentially expressed genes operate, we performed network and pathway analyses and visualization using the Ingenuity™ Pathway Analysis (IPA) program (Qiagen, Valencia, CA).

Survival Analysis

Patient survival data for 30 of the patients at 12, 18, and 24 months were assigned to one of two groups: those who showed no evidence of disease (NED) and those who had progressive disease, recurrent disease or those who died (PRD). Data were plotted against β -tubulin isotype groups: (1) samples where isotype levels in tumors were 10-fold greater compared to normal tissues and (2) those with less than a 10-fold difference between normal and tumor tissues. Log rank (Mantel–Cox test) was applied and 95% confidence intervals were determined. Data were analyzed using GraphPad™ Prism 6.

Results

Increased β -Tubulin Class III Expression in HNSCC

We wanted to know whether β -tubulin isotypes are markers for HNSCC. In normal and tumor tissue samples from 33 patients diagnosed with HNSCC prior to treatment (Table I), we measured β -tubulin isotype classes I, IIA, IIB, III, IVB, and V mRNA using real time qRT-PCR. β -Tubulin isotype classes I and V were found to be the most abundant and β -tubulin class III was the least abundant (i.e. Class I=V>IIB>IIA=IVB>III). We set 10-fold as the level for significant differences between normal and tumor tissues based upon our

previous work with breast tumor tissues and non-small cell lung cancer tissues [Dozier et al., 2003; Cucchiarelli et al., 2008]. In the HNSCC and normal tissues, we found that there was a >10-fold increase in β -tubulin class III in nearly 50% ($n=15$) of the 33 tumor samples compared to the paired normal sample (Fig. 1A). For other β -tubulin isotypes a >10-fold was found in fewer tissues: for class I (six samples), class IIA (three samples), class IIB (six samples) class IVB (three samples), and class V (one sample). Furthermore, when mRNA copies/ μ g total RNA were summed for all β -tubulin isotypes for each patient sample, there were two samples that had 11-fold increase in total tubulin in tumor tissue compared to normal. The average fold-difference between normal and tumor samples was 2.0 (± 2.0) for all for total tubulin mRNA (Fig. 1B). Thus an increase in a single β -tubulin isotype is compensated by a decrease in another.

Because the β -tubulin class III is implicated in both drug resistance and improved outcomes in specific cells lines and tissues, [Cabral, 2008] we wanted to know whether the large increases in β -tubulin class III found here in specific tumor samples were associated with better or worse patient outcomes. We obtained 2-year survival data for 30 of the 33 patients in this study; three of the initially enrolled patients were lost to follow-up. Patients were assigned to one of two groups: those who had no evidence of disease at the end of 2 years from the time of diagnosis (NED) and those who experienced progressive disease, recurrent disease or death (PRD). Figure 2 shows that survival with NED is improved for patients who had >10-fold increases in β -tubulin class III mRNA ($n=13$), although not statistically significant ($P=0.28$), compared to those who had less than a 10-fold increase ($n=17$). We did similar analyses for β -tubulin class I and for β -tubulin classes IIA plus IIB; however, we did not find a survival benefit.

Because HPV infection is associated with better outcomes for patients with HNSCC, we measured HPV16 E6 mRNA in all tumor samples (Table I). Nine of the 33 tumor samples were HPV-positive and seven of these nine patients showed NED at 2 years from diagnosis (one was lost to follow-up at six months and one had metastatic disease at six months). Seven of the remaining 24 patients, who were HPV-negative, showed NED at 2 years. Only four of the 15 patients where β -tubulin class III increased >10-fold in tumor samples were HPV-positive. Thus, HPV infection alone does not account for the trend for better outcomes for patients with increased β -tubulin class III.

Significant Reduction in Activity of p53 Interactome in PRD Tumor Samples Compared to NED

Because patterns of biomarkers could be useful as predictors of patients' outcomes or in selection of optimal chemotherapy, we used microarrays to evaluate the expression of >30,000 genes in a subset of our samples (15 pairs of normal and tumor tissues). Only 12 of the initial 15 pairs of normal and tumor samples were used in this analysis because the initial heat map comparison of all pairs of tissue samples showed that three were outliers (i.e., Three patient tumor samples had gene expression patterns that were similar to their normal tissue sample). Of these three patients, two experienced PRD at two years (patients 17 and 57) and one showed NED (patient 21). Comparing 12 pairs of normal and tumor samples, we found that the expression of 336 genes changed significantly ($P=0.005$)

(Supporting Information Fig. 1 and Table SI). We found no significant change in β -tubulin isotypes in this analysis. This is likely due to the lower sensitivity of microarray analysis compared to qRT-PCR. The largest and most frequent changes we found by qRT-PCR in tumor samples compared to normal were in β -tubulin class III, the isotype that is in lowest abundance.

When we compared tumor samples from patients who experienced NED at 2 years from diagnosis ($n=4$) with those who experienced PRD ($n=7$), we found significant changes in the expression of 250 genes ($P=0.01$) and, using IngenuityTM IPA software (Supporting information Table SII), we identified significant changes in cellular and molecular functions and signaling pathways associated with cancer, cell development, and cell morphology (Supporting Information Table SIII) (Note that one patient of the initial 12 was lost to follow-up, so we only did this comparison for 11 tumor samples). The same analysis at $P=0.005$ yielded 40 genes with significant differences in expression between the two groups (Fig. 3 and Table II). These data were used for further analysis and validation.

Only β -tubulin class V was significantly reduced in this comparison of 11 tumor samples (Supporting Information Table SII, *TUBB6*, 0.92 fold PRD/NED). However, we could not confirm this by qRT-PCR (PRD/ NED mean 1.25 ± 1.35 fold difference for the 11 tumor samples). There were no differences in the other β -tubulin isotypes comparing PRD and NED samples using microarray analysis. When the data for these 11 patients were analyzed for correlation with HPV status, we noted three of the four patients who were NED at 2 years from diagnosis, were HPV-positive and only one of the seven patients who experienced PRD at 2 years was HPV-positive. Thus, as expected, HPV status was linked to better outcomes. Further detailed analysis with IngenuityTM IPA software showed that of the 40 genes identified, six clustered in the p53 interactome (Fig. 4), although the expression of p53 was not altered. Two genes showed increased expression in patients who experienced PRD compared to NED (*CUL9* and *PTPRH*) and four showed decreased expression (*AKAP12*, *LYPLA1*, *PRDX3*, and *ELK3*). A brief description of each gene is shown in Table III. We used the five NED samples as the calibrators in comparative qRT-PCR and validated the up-regulation of *CUL9* and *PTPRH* and the down-regulation of *AKAP12* and *PRDX3* (Figs. 5A and 5B, Table III). *ELK3* was decreased on average but not significantly. We could not validate the down-regulation of *LYPLA1*. In addition, we confirmed that there was no significant change in p53 mRNA levels. These data together suggest reduced signaling associated with the p53 interactome in the PRD samples compared to NED.

Discussion

Our goal in this study was to identify potential biomarkers in addition to HPV that would suggest better outcomes, thus helping to guide treatment decisions. In our study of 33 patients, 9 had tumors that were HPV-positive and 24 patients had tumors that were HPV-negative. Seven of the nine patients (78%) with tumors that were HPV-positive and seven of the 24 patients (29%) who had HPV-negative tumors showed no evidence of disease at 2 years from diagnosis. Thus, although the patients with tumors that were HPV-positive predictably had better outcomes, it remains unclear why a subset of HPV-negative patients also showed no evidence of disease at two years from diagnosis. It is possible that by

identifying patterns of biomarkers that are associated with disease progression and also involved in important signaling pathways, novel therapeutic targets can be found.

Reduced activity of the tumor suppressor p53 is common in HNSCCs [Rothenberg and Ellisen, 2012 ; Wittekindt et al., 2012]. In these tumors, HPV infection is linked to reduced p53 activity through the action of E6 protein [Wittekindt et al., 2012]. The fact that HPV-associated HNSCCs respond more readily to treatment [Gillison, 2004] suggests that the effect of E6 can be overcome through p53-dependent or –independent mechanisms. Additional signaling pathways are implicated including Rb/ INK4/ARF and Notch [Rothenberg and Ellisen, 2012].

Because changes in patterns of β -tubulin isotypes suggest dysregulated cellular functions, [Lobert et al., 2013] we wanted to know whether the changes in β -tubulin comparing HNSCC tissue and normal tissue from a site distant to the tumor might be implicated in tumor development and/or disease progression. In our study, we found that total tubulin mRNA does not change significantly in any individual pair of normal and tumor tissues, indicating the changes in β -tubulin isotypes compensate for each other and keeping the overall amount of β -tubulin mRNA relatively constant. We found that β -tubulin class V mRNA is the only β -tubulin isotype that does not change significantly comparing all pairs of normal and tumor tissues (Tumor/Normal 1.62 ± 1.09). This result supports the work of Cabral and colleagues that showed that β -tubulin class V protein is critical for cell proliferation [Bhattacharya and Cabral, 2004]. In their work, small changes (15% or more) in β -tubulin class V protein, a minor fraction of all tubulin in CHO cells, resulted in a significantly reduced microtubule network.

We found that nearly half of the tumor samples ($n=15$) had large increases in the least abundant β -tubulin isotype, β -tubulin class III, and that these increases were associated with a trend for no evidence of disease (NED) at 2 years following diagnosis. Because this trend may be one of many changes in mRNA expression associated with better outcomes for patients, we used microarray analysis to identify significant patterns in gene expression. Survival data were available for 13 of the 15 patients with tumor samples showing high levels of β -tubulin class III. Four of the 13 were HPV-positive and NED at 2 years; four were HPV-negative, and NED at 2 years; five were HPV-negative and PRD at 2 years. Thus for 8 of 13 patients (61%), β -tubulin class III levels were linked to better outcomes regardless of HPV status.

Analysis of the microarray data using Ingenuity™ Pathway Analysis (IPA) for the 40 genes found to be differentially expressed ($P=0.005$) in PRD samples compared to NED samples and validation using qRT-PCR indicate reduced signaling activity of the p53 interactome for patients whose tumors progressed. This is evident in the reduction in *AKAP12* and *PRDX3* in PRD samples compared to NED samples. *AKAP12* localizes protein kinase A and C at discrete areas within the cell serving as a scaffolding protein for cell signaling [Goeppert et al., 2013]. It is reported to be a tumor suppressor acting through several mechanisms and has both anti-angiogenic and antimigratory functions [Goeppert et al., 2013]. Furthermore, it is reported to be down-regulated in some tumors, such as hepatocarcinomas and glioblastomas. *PRDX3* acts as an antioxidant and is thought to protect mitochondria from oxidative stress

[Safeian et al., 2012]. Its up-regulation has been associated with cervical cancers [Kim et al., 2009]. *PTPRH* is a receptor type member of the family of protein tyrosine phosphatases (PTPs) that regulate cell to cell adhesion through interaction with cadherins or other signaling pathways [Sallee et al., 2006]. Thus the increase in *PTPRH* also suggests disruption of intracellular signaling. Together, the reduced amounts of both *AKAP12* and *PRDX3* and increased *PTPRH* in PRD samples indicate persistent altered signaling patterns linked to poor outcomes. Because we only were able to measure mRNA changes, it is possible that these differences do not describe protein levels. However, the differences in mRNA described here by microarray and qRT-PCR indicate dysregulated signaling patterns consistent with worse disease in patients whose tumors recurred or progressed over 2 years. Initial and persistent patterns like these could be useful for treatment selection (e.g. more aggressive or less aggressive treatment for disease where the aberrant signaling persists). In addition, such patterns could suggest targets for novel treatments. These data together contribute to our understanding of how reduced activities of specific genes in the p53 interactome are associated with dysregulated metabolism and intracellular signaling and worse prognosis. Future cell culture work comparing targets of *AKAP12*, *PRDX3*, and *PTPRH* when these genes are upregulated or down-regulated will shed light on the mechanism underlying the signaling changes found in our study.

Increases or decreases in β -tubulin isotypes have been reported as potential biomarkers for tumor prognosis [Cabral, 2008; Cucchiarelli et al., 2008; Cullen et al., 2009]. Most specifically, β -tubulin class III has been implicated in taxane resistance [Cabral, 2008]. Our work was motivated by these reports suggesting that levels of β -tubulin class III could impact outcomes for cancer patients and therefore might be a biomarker for patients with HNSCC. We found that in nearly half of the patient HNSCC tumor samples β -tubulin class III levels were increased 10-fold or more compared to paired normal tissues. We also found that there is a trend for better 2 year survival outcomes for these patients. Thus, for this group of patients, β -tubulin class III was a possible biomarker for better outcomes and, at a minimum, is not mechanistically associated with resistance to treatment. We also found that decreased activity of the p53 interactome, as evidenced by altered mRNA levels for specific interacting proteins (*AKAP12*, *PRDX3*, and *PTPRH*), was associated with worse 2-year survival outcomes, suggesting that these target genes are candidates for exploration of signaling pathways that could be novel therapeutic targets.

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Fig. 1. Relative β -tubulin mRNA in 33 pairs of HNSCC tissues and associated normal tissue
A horizontal line on each plot indicates a 10-fold increase in mRNA levels in the tumor sample compared to normal tissues. (A) qRT-PCR was used to measure β -tubulin isoforms. The amount of mRNA was normalized per μ g total RNA and then the tumor amount was divided by the amount in the associated normal tissue. (B) The total amount of mRNA was summed for each sample and then the tumor amount was divided by the amount in the associated normal tissue.

Fig. 2. Two year survival plot for patients with tumor tissues that expressed 10-fold greater amounts of β -tubulin class III compared to those whose tumor tissues had lower amounts. Closed squares ■ samples with >10-fold increase in β -tubulin class III ($n=13$) and closed circles ● samples with less than and 10-fold increase in β -tubulin class III ($n=17$). The error bars represent the 95% confidence interval (CI). The log rank (Mantel-Cox) test $P=0.28$.

Fig. 3.
Heat map comparing tumor samples from patients who showed no evidence of disease at two years (NED) and those who showed progression, recurrence or death (PRD), $P=0.005$.

Fig. 4. Ingenuity IPA analysis was used to evaluate the microarray data for tissue samples from patients who showed no evidence of disease at 2 years (NED) and those who showed progression, recurrence or death (PRD)

Changes in gene expression associated with the p53 interactome are shown. Blue indicates genes that were down-regulated and red indicates genes that were up-regulated in PRD compared to NED.

Fig. 5. PCR validation of gene up-regulation and down-regulation shown in Fig 4
 Statistical data are shown in Table III. (A) *CUL9*, *PTPRH*, and *p53*. (B) *AKAP12*, *LYPLA1*, *ELK3*, and *PRDX3*.

Table 1

Study Participants

ID	Gender	Age	Stage ^d	CA site	HPV status
4	M	46	T2,N2c,M0 SCCa	Left soft palate	Negative
7	M	58	T4b,N3,M0 SCCa	Supraglottic	Negative
8	M	45	T4a,N2c,M0 SCCa	Supraglottic	Negative
9	F	45	T3,N0,M0 SCCa	Right false vocal cord	Negative
10	M	61	T4b,N0,M0 SCCa	Right pharyngeal	Negative
11	F	72	T1,N0,M0 SCCa	Left tonsil & right Pharyngeal wall	Negative
13	M	75	T3,N0,M0 SCCa	Right tonsil	Negative
14	M	47	T4a,N3,M0 SCCa	Tongue base	Negative
17	M	70	T3,N2b,M0 SCCa	Supraglottic	Negative
18	M	62	T4a,N2c,M0 SCCa	Supraglottic	Negative
21	M	58	T2,N3,M0 SCCa	Left base of tongue	Negative
22	M	64	T4a,N1,M0 SCCa	Left hypopharyngeal	Negative
24	M	55	T3,N2c,M0 SCCa	Soft palate	Positive
26	M	61	T4a,N2b,M0 SCCa	Supraglottic	Negative
27	M	51	T3,N2c,M0 SCCa	Left tonsil	Negative
29	M	65	T3,N1,M0 SCCa	Epiglottic	Positive
33	M	68	T2,Nx,M0 SCCa	Right AE fold	Negative
34	M	58	T2,N1,M0 SCCa	Supraglottic	Negative
36	M	63	T4a,N0,M0 SCCa	Left false cord	Positive
43	M	73	T3,N0,M0 SCCa	Left AE fold	Positive
48	M	47	T2,N2b,M0 SCCa	Right tonsil	Positive
54	M	52	T4a,N2c,M0 SCCa	Left piriform sinus	Positive
55	M	48	T4,N2,M0 SCCa	Nasopharynx	Positive
57	F	64	T4a,N2c,M0 SCCa	Left tongue base	Unknown
64	F	66	T4a,N2b,M0 SSCa	Left tonsil	Negative
66	M	48	T4a,N3,M0 SCCa	Supraglottic	Positive
67	M	42	T4a,N2c,M0 SSCa	Left supraglottis	Negative
68	M	53	T4a,N0,M0 SSCa	Aryepiglottic fold	Negative

ID	Gender	Age	Stage ^a	CA site	HPV status
70	M	63	T4b N2c M0 SCCa	Right piriform sinus	Positive
72	M	62	T3 N2c MX SCCa	Supraglottic	Negative
76	M	43	T4a N2x M0 Seca	Supraglottis	Negative
77	M	65	T4b N2c M0 SCCa	Supraglottis	Negative
81	M	61	T2 N2b M0 SCCa	Left soft palate	Negative

^a TNM staging based upon T, tumor size; N, lymph node involvement; M, metastases.

Table II

Comparison of Change in Expression of 40 Genes for NED and PRD HNSCC Samples from Heat Map in Fig 3

Gene name	P-value	RATIO PRD/NED
ACER3	0.0031	0.917479
ACOT9	0.0031	0.930677
ACVR1B	0.0031	1.050331
ALDOA	0.0031	0.963257
APBB3	0.0031	1.070442
APLP2	0.0031	0.964317
C15orf26	0.0031	0.953576
C21orf49	0.0031	1.050354
CAP2	0.0031	0.866817
CCNYL1	0.0031	0.935505
CDS2	0.0031	0.944214
CEACAM19	0.0031	0.9584
CLGN	0.0031	1.161298
CTSC	0.0031	0.91541
CUL9	0.0031	1.088211
EIF4A1P2	0.0031	0.936355
ELK3	0.0031	0.934826
GPR34	0.0031	0.831776
KLHDC10	0.0031	0.939163
MIR190A	0.0031	1.073495
MIR200A	0.0031	1.139085
MS4A6A	0.0031	0.895003
MSI2	0.0031	1.094563
NACA2	0.0031	0.927218
POPDC3	0.0031	0.866562
PRDX3	0.0031	0.950822
PTPRH	0.0031	1.103867
RPUSD1	0.0031	0.975695
SDCCAG8	0.0031	0.933109
SNX7	0.0031	0.871213
VSIG4	0.0031	0.885805
ZAK	0.0031	0.878411
ZNF417	0.0031	1.053604
LPHN1	0.005819	1.075826
ZBTB10	0.005959	1.063267
AKAP12	0.005989	0.877236
LYPLA1	0.005989	0.93487
MPP1	0.005989	0.896424

Gene name	<i>P</i> -value	RATIO PRD/NED
RANBP17	0.005989	0.809589
ACSL4	0.005994	0.9205

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Table III

Relative Changes in mRNA

Gene	Function (www.genecards.org)	Mean change PRD compared to NED	STD	95% CI
<i>P53CUL9</i>	Tumor suppressor protein	2.709	2.003	1.035–4.384
	Cytoplasmic anchor protein in p53-associated protein complex; may be part of ubiquitin protein ligase complex	2.543	1.229	1.515–3.570 ^a
<i>PTPRH</i>	Receptor type tyrosine protein phosphatase H; found in cancer cells but not in associated normal tissue	4.672	3.789	1.504–7.804 ^a
<i>AKAP12</i>	A-kinase anchor protein; functions in localizing protein kinase A and C within the cell; scaffolding protein for cell signaling.	0.5391	0.3569	0.2407–0.8375 ^a
<i>LYPLA1</i>	Acyl-protein thioesterase 1; regulates lysophospholipids	1.339	0.8611	0.6191–2.059
<i>ELK3</i>	ETS transcription inhibitor; wild type p53 prevents its phosphorylation by MAP kinases	0.8913	0.6702	0.3309–1.452
<i>PRDX3</i>	Thioredoxin-dependent peroxide reductase	0.5028	0.3496	0.2105–0.7950 ^a

^a significantly up- or down- regulated.