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Classification of TP53 Mutations and HPV Predict Survival in Advanced Larynx Cancer

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ABSTRACT

OBJECTIVE: Assess *TP53* functional mutations in the context of other biomarkers in advanced larynx cancer.

STUDY DESIGN: Prospective analysis of pretreatment tumor *TP53*, HPV, Bcl-xL and cyclin D1 status in stage III and IV larynx cancer patients in a clinical trial.

METHODS: *TP53* exons 4-9 from 58 tumors were sequenced. Mutations were grouped using three classifications based on their expected function. Each functional group was analyzed for response to induction chemotherapy, time to surgery, survival, HPV status, p16INK4a, Bcl-xl and cyclin D1 expression.

RESULTS: *TP53* Mutations were found in 22/58 (37.9%) patients with advanced larynx cancer, including missense mutations in 13/58 (22.4%) patients, nonsense mutations in 4/58 (6.9%), and deletions in 5/58 (8.6%). High risk HPV was found in 20/52 (38.5%) tumors. A classification based on crystal Evolutionary Action score of p53 (EAp53) distinguished missense mutations with high risk for decreased survival from low risk mutations (p=0.0315). A model including this *TP53* classification, HPV status, cyclin D1 and Bcl-xL staining significantly predicts survival (p=0.0017).

CONCLUSION: EAp53 functional classification of TP53 mutants and biomarkers predict survival in advanced larynx cancer. (---179 words)

INTRODUCTION

Larynx cancer is diagnosed in 12,260 patients in the United States each year, causing 3,630 deaths.[1] The high morbidity associated with the traditional surgical treatments led to the development of larynx sparing chemoradiotherapy regimens.[2,3] Furthermore, in attempts to better classify patients for different treatment modalities and increase their survival, many biomarkers have been studied.[4]

The tumor suppressor p53 (*TP53*) is mutated in up to 60% of head and neck cancers in some series.[5] It has a central role in determining cell fate in response to stressors. [6-10] The role of p53 as a prognostic biomarker in laryngeal cancer is controversial. Some studies show an association of p53 mutations with better sensitivity to chemotherapy,[11] while others associate it with poor response.[12,13] Almost 75% of the TP53 mutations reside in the DNA-binding domain, and more than 30% are in one of six "hotspot" codons. These mutations can promote cancer development. Thus, analysis based primarily on protein function is appropriate in assessing clinical outcome linked to p53 mutants, rather than only on its expression, as frequently done in the past. Functional classifications of p53 can be based on Evolutionary Action score of TP53 (EAp53),[14] yeast based transcriptional activity, [15] and published gain of function (GOF) mutations.

In larynx cancer Bcl-xl and cyclin D1 are predictors of response to therapy and survival that are linked to p53 status. [4,16] Bcl-xl is an anti-apoptotic protein member of the Bcl-2 family, [17-19] that inactivates pro-apoptotic proteins at the mitochondrial membrane. [19,20] Cyclin D1 phosphorylates and inactivates RB, allowing cell cycle progression.

Human papillomaviruses (HPV) studies in laryngeal cancer have been limited to recurrent laryngeal papillomatosis, which is caused by infection with low risk HPV types 6 and 11.[21] High-risk HPV (hrHPV), especially HPV16, has become increasingly common in head and neck cancer.[22-28] In the current study we assess the role of hrHPV, *TP53* functional classifications, Bcl-xl and cyclin D1 each as single variable factoring outcome, and as part of a multivariable model for advanced larynx cancer outcome.

METHODS

Study Population

The UMCC9520 larynx sparring cancer trial enrolled 97 patients with stage III and IV squamous cell carcinoma of the larynx. Adequate pretreatment biopsy tissue was available for DNA isolation from 58/97. A detailed description of the clinical trial was previously reported.[3]

Tissue Handling and DNA Extraction

Tumor tissue was obtained from formalin fixed paraffin embedded pre-treatment biopsies. A head and neck pathologist marked representative areas of tumor. Two cores were taken from the marked areas using a 20G needle. DNA was extracted using the QiaAmp DNA Mini Kit (Qiagen, Valencia, California, USA). DNA quantity and quality were corroborated with UV-Vis spectrophotometer using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA).

TP53 Sequencing

TP53 mutations were assessed by PCR amplification of exons 4-9 and subsequent DNA sequencing. Seven pairs of primers were used (Supplementary Table I) with AmpliTaq DNA Polymerase as indicated by manufacturer (Invitrogen, Life Technologies, NY, USA). Amplification was performed at 95°C for 10 minutes, 36 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 45 seconds and a final extension at 72°C for 10 minutes. The amplicons were examined with electrophoresis in a 1.5% agarose gel, purified using the Wizard SV Gel and PCR Clean-Up System (Promega Corporation, WI, USA) and submitted to the University of Michigan DNA Sequencing Core for bi-directional

sequencing. Mutations were confirmed with a second independent PCR reaction and sequencing.

TP53 Mutant Classifications

Three classifications designed to classify *TP53* single base pair mutations (point mutations) were studied. The EAp53 classification that was designed and validated for head and neck cancers[14], scores *TP53* mutations as high risk mutations (H) and low risk mutations (L) obtained through a computational algorithm based on evolutionary sensitivity and amino acid conservation. Tumors without mutations were designated as wild type *TP53* (W). Insertion/deletions were not scored.

The *p53* transactivation activities (TAs) classification is based on reporter gene activity placed under the control of eight p53 response elements (p53-RE) in yeast models[15]. TAs were measured as a percentage of wild type p53 TA and classified as "supertrans"(median TA above 140%), "functional"(median TA of 76-140%), "partially functional"(median TA of 21-75%), and "nonfunctional"(median TA below or equal 20%).[15,29]

The p53 mutant gain of function (GOF) classification is compared to mutants with no evidence of gain of function (NE-GOF) and wild type (W) *TP53*. P*53* GOF mutations have been defined as those with activities independent and unrelated to wild type protein[15]. These activities were demonstrated by RNAi silencing or direct transfection of the mutant *TP53*.[30] To identify GOF mutants a literature search of PubMed without language limitation was performed using the search terms "mutant *p53*" and "gain of function".

Additionally every missense mutation in this study was used as a search term to find reports.

HPV Testing

DNA samples from the pretreatment biopsies were analyzed for the presence of high risk HPV using the multiplex HPV PCR-MassArray assay as described.[26]

Bcl-xl, Cyclin D1 and p16INK4a Immunohistochemistry

Staining intensity (scale: 1: none, 2: weak, 3: moderate and 4: strong) and proportion (scale 1: <5% staining, 2: 5-20% staining, 3: 21-50% staining and 4: 51-100% staining) were assessed by immunohistochemistry using a tissue microarray and scored by a head and neck pathologist (JBM) blinded to patient outcome as previously described [4,26].

Statistical Analysis

Associations between *TP53* groups and clinical characteristics were assessed using χ^2 statistics. Correlations with induction chemotherapy response, overall survival (OS), disease-specific survival (DSS) and time to indication for surgery (TIS) were tested by log-rank tests, and logistic and Cox proportional hazards regression models. Time to event outcomes (OS, DSS, TIS) were measured from date of diagnosis. For illustration in figures, Bcl-xl and Cyclin D1 intensity scores were grouped as "no (1)", "low (2)" and "high (3 or 4)". Multivariable analyses controlling for stage, smoking and hrHPV were performed using logistic and Cox proportional hazards models. Variables tested

included: *TP53* mutational status, presence of hrHPV DNA, Bcl-Xl and CyclinD1 expression.

RESULTS

Sample Characteristics

TP53 sequence and clinical data were obtained from a previously published study.[3] Basic clinical and tumor characteristics are shown in Table 1.

TP53 Mutants

Of the 97 eligible patients, *TP53* gene was sequenced in 58. Of these, 22 tumors contained *TP53* mutations. Five (8.6%) had deletions that were not classifieable. 53 patients classified had either wild type *TP53* 36/53 (68%) or single base changes (17/53 (32%)) corresponding to missense in 13/53 (24.5%), or non-sense mutations in 4 (7.5%). No insertions were found. A *TP53* missense mutational spectrum is presented in Supplementary Table II. Bivariate associations were tested for each p53 classification with age, T stage, N stage, smoking and HPV. The EAp53 classification[14] revealed a significant association of high risk (H) mutations and node positive tumors (p=0.03; Table 2). Seven (53.8%) of the 13 missense mutations were classified as GOF. No significant associations with clinical parameters were found for GOF or transactivation classifications, low risk mutations (L) and wild type (W).

HPV and p16INK4a

Tumor DNA from 52 patients was analyzed with the multiplex HPV PCR-MassArray assay[25]. High risk HPV was identified in 20/52 (38.5 %) tumors; 19/20 had HPV16 and one had HPV 18. Of the HPV positive tumors 15/20 (75%) had wild type *TP53* (consistent with HPV driven carcinogenesis), 3/20 (15%) had high risk *TP53* mutations and 2/20 (10%) had *TP53* deletion (questionable for HPV carcinogenesis). HPV PCR-MassArray

negative tumors presented a WT TP53 only in 53% (16/30) cases. Sixteen HPV PCR-MassArray positive samples were assessed for p16INK4a status; 31% (5/16) were p16INK4a positive. While all HPV+/p16INK4a+ tumors (5/5) had a WT TP53, only 64% (7/11) of the HPV+/p16INK4a- presented a WT TP53.

A total of 46 tumors were stained for p16INK4a with 26% (12/46) been positive. From the 12 p16INK4a positive samples only 42% (5/12) were HPV PCR-MassArray positive. The p16INK4a and HPV PCR-MassArray results were not concordant in larynx cancer (Pearson correlation coefficient Rho=0.09).

TP53 Mutants and Induction Chemotherapy

Thirty-eight of the 58 patients (66%) responded to induction chemotherapy (tumor volume decrease greater than 50%). Multivariable logistic regression models with different models including T stage and N stage separately, overall stage, HPV, and smoking were run. No significant predictors of response to induction chemotherapy were found in any of the *TP53* classifications.

TP53 Mutants and Survival

Of the 58 patients with a median follow up of 95 months, the Kaplan-Meier estimates for overall survival at 1, 2, and 5 years were 90%, 83% and 67% respectively. DSS estimates were 91%, 86%, 80% respectively.

Using the EAp53 classification and univariate analysis (log-rank tests), we find a significant DSS difference between strata (p=0.026; Figure 1). Pairwise, the difference

between the H vs. L groups was significant (p=0.027). The differences between H vs W (p=0.188) and L vs W (p=0.988) groups were not statistically significant. Using a Cox model after controlling for T stage, N stage and HPV status the effect of p53 mutation was no longer significant (p=0.114). No significant differences were observed for DSS using the yeast assay based classification (p=0.603, log-rank; p=0.887 Cox) nor GOF classification (p=0.096 log-rank; p=0.130 Cox). Interestingly there was a trend for the GOF mutations to do better.

TP53 Mutants and Time to Surgery

Time to indication for surgery (TIS) was evaluated for the *TP53* classifications. No significant differences in TIS were found between groups using the EAp53, yeast transcriptional assay based nor GOF classifications (p=0.654, p=0.394 and p=0.835 respectively). After controlling for stage, smoking and HPV status the differences were still not significant (p=0.667; p=0.245; p=0.874 respectively).

HPV, p16INK4a, Bcl-xL and cyclin D1: Effects on the EAp53 Classification and Survival

The role of HPV, p16INK4a, Bcl-xL and cyclin D1 in the EAp53 classification was assessed. As a single variable, the presence of HPV did not reach significance (log-rank p=0.38) When separating the HPV positive tumors by p16INK4a status we found that the patients with HPV+/p16INK4a+ tumors tended to have a better DSS compared to the HPV+/p16INK4a- and HPV negative. This difference did not reach statistical significance (long-rank p=0.4037 and p=0.3506 respectively; Figure 2A). No patients with HPV+/p16INK4a+ tumors die due to cancer. By including only the HPV positive cases with WT *TP53* we observed that HPV positive WT *TP53* patients tended to have better

DSS than the HPV negative patients. This difference did not reach statistical significance (p=0.195, Figure 2B).

We found no evidence of an association between Bcl-xL intensity in different *TP53* groups. In multivariable analysis, Bcl-xL expression displayed a trend for poorer DSS (p=0.075, Table 3). By plotting the DSS of the *TP53* groups and sorting out the WT *TP53* based on their biomarker status, we found no significant difference across Bcl-xL staining intensities. However, the group with WT *TP53* and no expression of Bcl-xL had no deaths due to cancer (Figure 2C.).

Alone cyclin D1 was associated with poor DSS (HR 2.4, p=0.010 Cox). When sorting the wild type *TP53* by cyclin D1 staining intensity we found that high cyclin D1 staining was a predictor of poor survival compared with low or no cyclin D1 staining (p=0.051 and p=0.044 respectively; Figure 2D.) Cyclin D1 showed a trend (p=0.096) for higher intensity in the "High Risk" group of p53 mutations.

Multivariable Model for Prediction of Survival

A model with Bcl-xL, cyclin D1, EAp53 classification and hrHPV status was highly significant as a prognostic model for disease specific survival (DSS) (p=0.002, likelihood ratio test; Table 3). A full model including EAp53 classification of TP53, Bcl-xL intensity, cyclin D1 intensity, stage, smoking status and HPV status was also performed (Supplementary Table III). This model was significant for modeling DSS (p=0.011), although a backward selection algorithm suggested dropping stage and smoking for a better

model. We found no significant models for induction chemotherapy response or to time to indication for surgery.

DISCUSSION

Three classifications for *TP53* mutations were analyzed. Only the EAp53 classification[14] based on mutant *TP53* transcriptional activity showed clinical significance. This classification predicted DSS (p=0.026). In this clinical trial, patient selection through induction chemotherapy resulted in larynx preservation in 70% of patients and DSS of 87%.[3] Although patient survival was improved, there was still a subset of patients with poor outcome. The EAp53 classification has potential to identify this subset of patients.

Bivariate associations were tested for EAp53 classification with age, T stage, N stage, smoking status, hrHPV, Bcl-xL and cyclin D1 staining. Of interest, high risk mutations found by EAp53 classification were associated with lymphatic metastasis at presentation (p=0.03) and a trend for high expression of cyclin D1 (p=0.096). It is known that node positive patients have poorer survival.[31] Therefore, EAp53 classification could potentially guide therapeutic approaches such as neck dissection, neck irradiation or the addition of chemotherapy to radiation treatment. More aggressive treatment to the neck of patients with high risk *TP53* mutations could improve patient outcome. We also found a trend for high risk *TP53* mutants to have high expression of cyclin D1. Cyclin D1 was previously found to be associated with overall and disease specific survival.[4] We propose a mechanism in which cyclin D1 overexpression is unchecked by defective p53. High risk *TP53* mutations have a lower expression of BTG2 (B-cell Translocation Gene 2) protein compared to their WT and low risk counterparts.[14] BTG2 inhibits cyclin D1 transcription.[32,33] Thus, its low expression is a likely mechanism for cyclin D1 overexpression. Furthermore, cyclin D1 itself could be constitutively overexpressed by dysregulation in the β-catenin[34,35],

RAS/MAPK or IP3K pathways[36]. The EAp53 classification and cyclin D1 have proven to be powerful predictors of survival in advanced laryngeal cancer patients, but further studies are needed to characterize the cellular mechanisms by which these markers interact and affect survival in this type of cancer.

GOF mutations affect important cellular activities like proliferation, apoptosis, chemoresistance, metabolism, cell to cell interactions or cytoskeleton conformation.[30] Only (7/58) 12% of the sequenced patients had a *TP53* GOF mutation. These showed no impact on response to induction chemotherapy, time to surgery or survival. We grouped all GOF mutations together. More specific classification of GOF mutations based on the cellular functions altered could offer a better understanding of how these mutations affect patient outcome. Larger studies and a more comprehensive GOF classification are needed for assessment of their impact in laryngeal cancer.

The proportion of tumors with mutant *TP53* 22/58, (37.9%) was lower than expected [37] *TP53* mutations are uncommon in HPV-positive tumors[38]. The low rate of *TP53* mutations in this study is consistent with the high proportion of hrHPV positive larynx tumors 20/52 (38.5%) we found. This is also consistent with previous reports of HPV in up to 25% of laryngeal carcinomas[39,40] and the increasing frequency of HPV-related head and neck cancers generally.[41,42] A recent report from the Tissue Cancer Genome Atlas (TCGA) head and neck cancer group included 72 larynx cancer cases. From these 66/72 (91.6%) have mutated TP53 and only 5/72 (7%) are HPV PCR-MassArray positive.[43] Patient selection could be causative of the discrepancy found between the studies. While in the TCGA study samples were collected without regard to neither disease stage nor patient functional status,

in our study patients where part of a clinical trial and were selected with a good functional status and an advanced stage of disease (Stage III and IV).[3,43] Differences in geographical distribution of the patients could be another factor affecting the HPV detection rates, as geographic variability of HPV related HNSCC have been reported.[44,45]

The biology of the HPV positive tumors was assessed using p16INK4a Immunohistochemistry. P16INK4a status did not correlate with HPV status (Rho=0.09), showing a dichotomy of HPV related larynx cancer. All HPV positive tumors with p16INK4a positive staining presented a WT TP53 and showed a better DSS with cero deaths caused by cancer. These HPV related tumors show characteristics of the typical HPV E6 and E7 driven cancers. A different subset of HPV positive tumors with p16INK4a negative staining did not show a clear correlation with TP53 mutational status and patients presented a DSS similar to the HPV negative patients (Figure 2A). Further studies addressing other mechanisms of HPV carcinogenesis like viral DNA integration, methylation profiling, miRNA profiling and characterization of other HPV related cellular pathways will determine the role of HPV in this subset of HPV positive larynx tumors.

Kumar et al.[16] analyzing the Department of Veteran's Affairs Laryngeal Cancer Trial,[2] found that low Bcl-xL and high p53 staining were predictors of larynx preservation in patients treated with induction chemotherapy followed by definitive radiation. Here we found no significant effect of Bcl-xL on clinical outcome although in multivariable analysis, a trend was noted for poorer disease specific survival to be associated with Bcl-xL expression. The association with organ preservation was not explored in this analysis. It is interesting to note that among WTp53 patients with no Bcl-xL expression, no tumor

failures were seen. We observed similar trend to improved survival with Bcl-xL expression in the VA Study. Both studies gave induction chemotherapy(cisplatin 100 mg/m2 on day 1 and fluorouracil 1,000 mg/m2/d for 5 days). In the VA study tumor response was assessed after two cycles of induction and responders went on to an additional cycle of chemotherapy followed by radiotherapy alone[2]. In contrast, in UMCC9520 a single round of the same induction therapy was followed by response assessment and either immediate surgery/RT (non-responders) or radiotherapy with concurrent cisplatin (100 mg/m2) given on day 1, 22, 43.[3] The addition of concurrent cisplatin to radiation in UMCC9520, could change the association of Bcl-xL expression as a biomarker of outcome. Recent studies suggest that cisplatin can activate extrinsic apoptotic pathways, induce necrosis,[46] or cell senescence.[47] In these scenarios cell death is no longer completely dependent on the intrinsic mitochondrial apoptotic machinery,[48] which could explain loss of power for Bcl-xL as a predictive biomarker. Nonetheless, the importance of a functional intrinsic apoptotic pathway in the response to chemoradiotherapy is evidenced by the fact that in our study, patients whose tumor did not stain for the apoptotic inhibitor Bcl-xL and had WT *TP53* did not die due to cancer (Figure. 3C.).

We constructed a model for the prediction of disease specific survival based on a combination of biomarkers and clinical characteristics. The model including classification of *TP53,* Bcl-xL intensity, cyclin D1 intensity, stage, smoking status and HPV status was able to predict survival (p=0.011). By including only variables with a strong predictive value (i.e. Bcl-xL, cyclin D1, p53 mutation classification and HPV status) we obtained a selected model that was highly significant for the prediction of disease specific survival (p=0.0017, Likelihood Ratio Test Table 4). In this model we can distinguish two types of

patients: high risk *TP53* mutation was predictive of poor DSS, and low risk *TP53* mutation was predictive of good DSS (p=0.026; Figure 1.). In patients with WT *TP53* high staining of cyclin D1 was predictive of poor DSS, while no/low staining for cyclin D1, no staining for Bcl-xL and HPV positivity were predictors of good DSS (Figure 2; Supplementary table IV). Thus, treatment modalities targeting p53 and cyclin D1 could increase survival in advanced larynx cancer patients that fail to respond to current treatment. Novel targeted therapies like mutant p53 reactivating small molecules, mdm2 inhibitors, cyclin dependent kinase inhibitors or inhibitors of β-catenin, RAS/MAPK or IP3K pathways could potentially counteract these markers and offer better outcome. The model should be tested in larger cohorts.

CONCLUSIONS

A functional classification of TP53 mutants based on EAp53 predicts DSS in advanced larynx cancer patients with mutant TP53. A high proportion of hrHPV related larynx cancers in this cohort reflects the epidemic of HPV related head and neck cancers. A dichotomy in the biology of hrHPV is observed in larynx cancer. A model including Bcl-xL staining, cyclin D1 staining, p53 mutation classification and HPV status was highly significant for predicting disease specific survival. High cyclin D1 expression and high risk *TP53* mutations were associated with poor DSS. New therapies targeting these pathways could benefit patients that fail to respond to current treatment modalities.

Abstract to conclusion 3066 words

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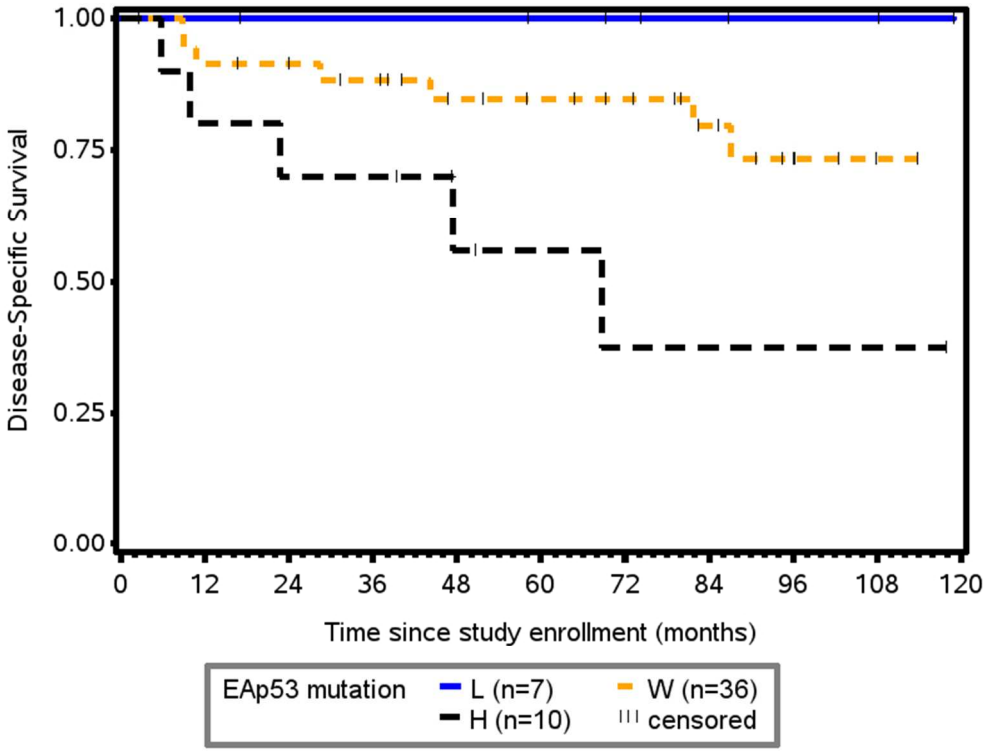
FIGURE LEGENDS

Fig. 1. Disease-Specific Survival for patients with advanced laryngeal cancer classified by *TP53* mutation type based on EAp53 as low risk *mutations* (L), high risk mutations (H) and wild type (W).

Fig. 2. Disease-specific survival by (A) HPV status with HPV positives sorted by p16INK4a status, (B) by EAp53 classification with TP53 wild types sorted by HPV status, (C) by EAp53 classification with TP53 wild types sorted by Bcl-xL intensity staining, (D) by EAp53 classification with TP53 wild types sorted by cyclin D1 intensity staining and (WT, wild type; mut, mutation. Intensity staining scores: 1=no, 2=low, 3 or 4=high).



67x50mm (300 x 300 DPI)

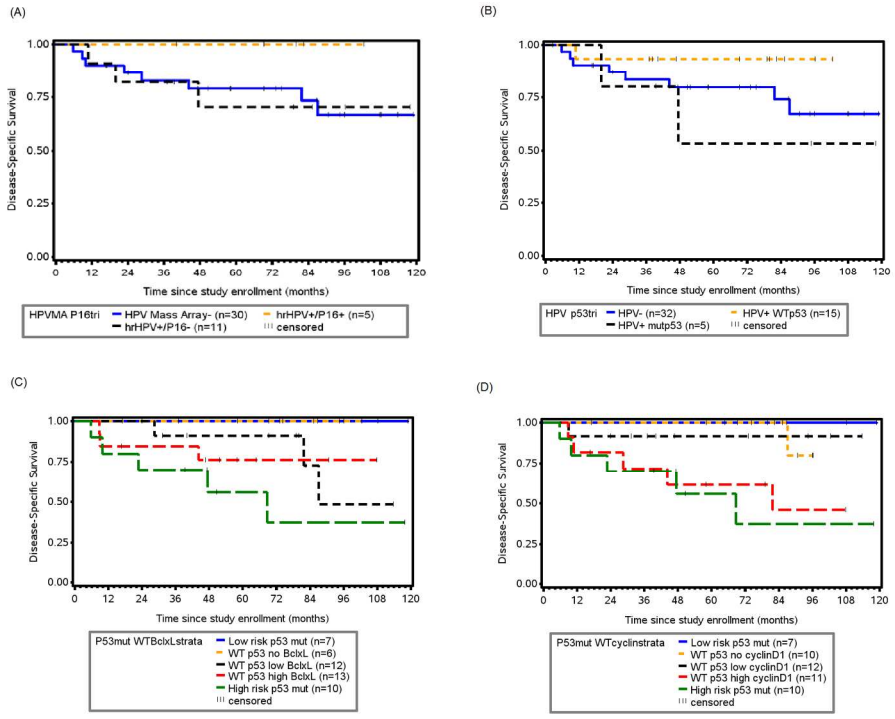


Fig. 2. Disease-specific survival by (A) HPV status with HPV positives sorted by p16INK4a status, (B) by EAp53 classification with TP53 wild types sorted by HPV status, (C) by EAp53 classification with TP53 wild types sorted by Bcl-xL intensity staining, (D) by EAp53 classification with TP53 wild types sorted by cyclin D1 intensity staining and (WT, wild type; mut, mutation. Intensity staining scores: 1=no, 2=low, 3 or 4=high).
529x423mm (96 x 96 DPI)

TABLES

Table 1: Patient and Tumor Characteristics

Characteristic	N		No. of Patients	%[a]
Gender	58	Male	48	83%
		Female	10	17%
T stage	58	T2	5	9%
		T3	30	52%
		T4	23	40%
N stage	58	N0	29	50%
		N1	12	21%
		N2	15	26%
		N3	2	3%
AJCC Stage	58	Stage III	24	41%
		Stage IV	34	59%
Smoking Status	58	Never	2	4%
		Former	12	21%
		Current	42	75%
HPV	52[b]	Positive	20	39%
		Negative	32	62%
EAp53 Risk Classification	58	Wild Type P53	36	62%
		Deletion	5	9%
		High (H)	10	17%
		Low (L)	7	12%
Yeast Assay Based Classification	58	Functional	38	66%
		Deletion	5	9%
		Partially functional	1	2%
		Nonfunctional	14	24%
Gain of Function (GOF) Classification	58	Wild type	37	64%
		Deletion	5	9%
		GOF Mutation	7	12%
		No GOF Mutation	9	16%
			Mean	**(std)**
Age (years)			57.2	(11.2)

a). % may not add to 100 due to rounding.
b). n=52 for HPV due to tissue availability.

Table 2: Nodal status, HPV and EAp53 Classification

Nodal Status	Deletion	High Risk	Low Risk	Wild Type	Total
N0	3	2	6	18	29
	60%	(20%)	(85.7%)	(50%)	
N+	2	8	1	18	29
	40%	(80%)	(14.2%)	(50%)	
Total	5	10	7	36	58

HPV Status	Deletion	High Risk	Low Risk	Wild Type	Total
HPV-	2	6	6	18	32
	(50%)	(66.7%)	(100%)	(54.6%)	
HPV+	2	3	0	15	20
	(50%)	(33.3%)	(0%)	(45.5%)	
Total	4	9	6	33	52[a]

a). n=52 for HPV due to tissue availability.

Table 3: Cox Proportional Hazards Model Results from Selected Model for Prediction of Disease-Specific Survival

Parameter	Value	p-value	Hazard Ratio	95% Hazard Ratio Confidence Limits	
Cyclin D1		0.0325	3.782	1.117	12.803
Bcl-xL		0.0751	0.307	0.084	1.127
EAp53 Classification	H vs. WT	0.0186	7.591	1.404	41.043
EAp53 Classification	L vs. WT	ie.	ie	ie	ie
HPV	Neg vs. Pos	0.0262	58.123	1.619	2086.187

H=High risk mutation; L=Low risk mutation; WT=Wild type TP53; ie=inestimable because zero events in the L group.

SUPPLEMENTARY TABLES

SUPPLEMENTARY TABLE I
TP53 Sequencing Primers

Primer	Primer Sequence
p53 Exon 4.1 F	5′-CCCATCTACAGTCCCCCTTG -3′
p53 Exon 4.1 R	5’-GGTGTAGGAGCTGCTGGTG-3’
p53 Exon 4.2 F	5′- CTGAAGACCCAGGTCCAGATGAA -3′
p53 Exon 4.2 R	5′- AACTGACCGTGCAAGTCACA -3′
p53 Exon 5.1 F	5′- CTTGTGCCCTGACTTTCAACTCTGTCTC -3′
p53 Exon 5.1 R	5′- CAGCGCCTCACAACCTCCGTCAT -3′
p53 Exon 5.2 F	5′- CTGTGCAGCTGTGGGTTGATTCCACACC -3′
p53 Exon 5.2 R	5′- TGGGCAACCAGCCCTGTCGTCTCTCCA -3′
p53 Exon 6 F	5′-CCAGGCCTCTGATTCCTCACTGATTGCTC-3′
p53 Exon 6 R	5′-GCCACTGACAACCACCCTTAACCCCTC-3′
p53 Exon 7 F	5′-GCCTCATCTTGGGCCTGTGTTATCTCC-3
p53 Exon 7 R	5′-GGCCAGTGTGCAGGGTGGCAAGTGGCTC-3′
p53 Exon 8 F	5′-GTAGGACCTGATTTCCTTACTGCCTCTTGC-3′
p53 Exon 8 R	5′-ATAACTGCACCCTTGGTCTCCTCCACCGC-3′
p53 Exon 9 F	5′-CACTTTTATCACCTTTCCTTGCCTCTTTCC-3′
p53 Exon 9 R	5′-AACTTTCCACTTGATAAGAGGTCCCAAGAC-3′

Abbreviations: F, Forward; R, Reverse.

SUPPLEMENTARY TABLE II

Mutational Spectrum of *TP53* Point Mutations in Advanced Larynx Cancer

Codon	Amino Acid Substitution
157	Valine to phenylalanine
158	Arginine to cysteine
163	Tyrosine to cysteine
167	Glutamine to stop codon
180	Glutamic acid to stop codon
193	Histidine to leucine
205	Tyrosine to cysteine
236	Tyrosine to cysteine
244	Glycine to cysteine
245	Glycine to valine
245	Glycine to alanine
249	Arginine to serine
282	Arginine to tryptophan
282	Arginine to tryptophan
293	Glutamic acid to stop codon
306	Arginine to stop codon
307	Alanine to threonine

SUPPLEMENTARY TABLE III
Hazards Ratio Estimates of a Full Model for Prediction of Disease-Specific Survival

Parameter	Value	p-value	Hazard Ratio	95% Hazard Ratio Confidence Limits	
Cyclin D1		0.0486	3.650	1.008	13.218
Bcl-xL		0.1297	0.364	0.099	1.345
EAp53 Classification	H vs. WT	0.0247	9.109	1.324	62.653
EAp53 Classification	L vs. WT	ie.	ie	ie	ie
Stage	3 vs. 4	0.8209	1.237	0.196	7.825
Smoking Status	N vs. C	ie.	ie	ie	ie
Smoking Status	F vs. C	0.6986	0.552	0.027	11.152
HPV	Neg vs. Pos	0.0367	51.163	1.277	2050.548

H=High risk mutation; L=Low risk mutation; WT=Wild type TP53; N=Nonsmoker; C=Current smoker; F=Former smoker; ie= inestimable because zero events in the L group.

SUPPLEMENTARY TABLE IV

Pairwaise Comparison of Disease Specific Survival of Patients with WT *TP53* *based on Cyclin D1 Staining, Bcl-xL Staining and HPV Status*

Strata Comparison		Chi-Square	p-Values	
			Raw	Tukey-Kramer
High risk p53 mut	WT p53 high cyclinD1	0.0481	0.8264	0.9995
High risk p53 mut	WT p53 low cyclinD1	5.1372	**0.0234**	0.1558
High risk p53 mut	WT p53 no cyclinD1	5.4547	**0.0195**	0.1337
Low risk p53 mut	WT p53 high cyclinD1	4.6986	**0.0302**	0.192
Low risk p53 mut	WT p53 low cyclinD1	0.000165	0.9898	1
Low risk p53 mut	WT p53 no cyclinD1	0.0128	0.9098	1
WT p53 high cyclinD1	WT p53 low cyclinD1	3.8075	0.051	0.2902
WT p53 high cyclinD1	WT p53 no cyclinD1	4.0616	**0.0439**	0.2585
WT p53 low cyclinD1	WT p53 no cyclinD1	0.00816	0.928	1
High risk p53 mut	WT p53 HPVneg	0.3689	0.5436	0.9298
High risk p53 mut	WT p53 HPVpos	5.0895	**0.0241**	0.1085
Low risk p53 mut	WT p53 HPVneg	1.3761	0.2408	0.6439
Low risk p53 mut	WT p53 HPVpos	0.2105	0.6464	0.9679
WT p53 HPVneg	WT p53 HPVpos	1.6797	0.195	0.5654
High risk p53 mut	WT p53 high BclxL	1.9906	0.1583	0.6206
High risk p53 mut	WT p53 low BclxL	1.9683	0.1606	0.6257
High risk p53 mut	WT p53 no BclxL	6.7551	**0.0093**	0.0705
Low risk p53 mut	WT p53 high BclxL	0.7854	0.3755	0.9021
Low risk p53 mut	WT p53 low BclxL	0.8046	0.3697	0.8981
Low risk p53 mut	WT p53 no BclxL	0.000206	0.9886	1
WT p53 high BclxL	WT p53 low BclxL	0.000088	0.9925	1
WT p53 high BclxL	WT p53 no BclxL	0.7683	0.3808	0.9056
WT p53 low BclxL	WT p53 no BclxL	0.7898	0.3742	0.9012