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## Evolutionary Action Score of TP53 Coding Variants (EAp53) is Predictive of Platinum Response in Head and Neck Cancer Patients

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### Abstract

TP53 is the most frequently altered gene in head and neck squamous cell carcinoma (HNSCC) with mutations occurring in over two third of cases, however, the predictive response of these mutations to cisplatin based therapy remains elusive. In the current study, we evaluate the ability of the Evolutionary Action score of *TP53* coding variants (EAp53) to predict the impact of *TP53* mutations on response to chemotherapy. The EAp53 approach clearly identifies a subset of high risk TP53 mutations associated with decreased sensitivity to cisplatin both *in vitro* and *in vivo* in

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pre-clinical models of HNSCC. Furthermore, EAp53 can predict response to treatment and more importantly a survival benefit for a subset of head and neck cancer patients treated with platinum based therapy. Prospective evaluation of this novel scoring system should enable more precise treatment selection for patients with HNSCC.

### Keywords

Evolutionary Action; p53 mutation; cisplatin

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## INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) has an incidence of over 40,000 new cases annually in the United States, and over 500,000 worldwide with an associated disease specific mortality exceeding fifty percent (1). The treatment of locally advanced head and neck cancer has evolved over the past three decades and often requires complex, multimodality therapy, including surgical resection, and/or external beam radiation with or without neoadjuvant, concurrent, or adjuvant cisplatin-based chemotherapy (2, 3). Currently, there are no molecular biomarkers to guide selection amongst these various treatment options. *TP53* is the most frequently altered gene in human cancers and recent data from whole exome sequencing of HNSCC reveals that this gene is mutated in 60–80% of human papilloma virus negative (HPV<sup>-</sup>) cases (4, 5). The *TP53* gene has been called the “cellular gatekeeper” due to its central role in response to cell stressors such as DNA damage, hypoxia, and oncogenic stress. Cellular DNA damage often leads to stabilization and accumulation of wtp53, which in turn leads to enhanced transcription of p21 and subsequently cell cycle arrest, apoptosis and senescence. The increase in p53 stability depends critically on the phosphorylation of serine/threonine residues (6–9).

Although mutations in *TP53* have been shown to have predictive significance for response to platinum based therapy in several studies, it remains unclear how to stratify patients into response categories based on *TP53* status (10–14). Recently, we developed an algorithm termed Evolutionary Action (EAp53) that accurately stratifies patients whose tumors have *TP53* mutations associated with especially poor outcomes (high risk), from other mutations with outcomes similar to patients with wild-type *TP53* (low risk) and have validated EAp53 as a reliable prognostic marker (Neskey DM, Osman AA et al., manuscript co-submitted<sup>1</sup>). We hypothesize that high-risk p53 mutations identified by the EAp53 scoring system are associated with an abnormal functional activity that contributes to cisplatin resistance in head and neck cancer. Therefore, to determine whether EAp53 has utility as a predictive biomarker of response to cisplatin in HNSCC, we used both preclinical laboratory based models and retrospective clinical data to assess the response of tumors expressing no p53, wtp53, or a series of low and high risk p53 mutations to cisplatin. The first aspect of our preclinical model found that in clonogenic survival assays, cell lines expressing high risk

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<sup>1</sup>Neskey DM, Osman AA, et al. Evolutionary Action score of TP53 coding variants (EAp53) identifies high risk mutations associated with decreased survival and increased development of distant metastases in head and neck cancer.

p53 mutations are more resistant to cisplatin treatment than cell lines expressing low risk mutations or wild type p53.

To further characterize the preclinical response of the *TP53* mutations stratified by EA to cisplatin therapy, tumors harboring these mutations were created in an orthotopic mouse model of tongue cancer. Concordant with the differential effect of *TP53* mutations on cisplatin response observed *in vitro*, mice with tumors harboring wildtype p53 or low risk mutations showed a significant response to cisplatin therapy, while the tumors derived from cells either null for p53 expression or with high risk p53 mutations did not show any growth inhibition with cisplatin therapy. In an effort to correlate the clinical utility of the EAp53 to predict response to cisplatin in patients with HNSCC, the *TP53* mutational status of a patient cohort of 68 patients treated for locally advanced HNSCC of the oral cavity with cisplatin based induction chemotherapy followed by surgical resection was determined. Results from this analysis confirmed our preclinical findings wherein patients' tumors with high risk mutations were significantly less responsive to cisplatin based chemotherapy than tumors with low risk mutant or wild type p53. These results indicate that the *TP53* mutational status may be a useful biomarker for predicting response to cisplatin based chemotherapy in HNSCC patients.

In summary, our data clearly demonstrate that high risk *TP53* mutations are associated with decreased sensitivity to cisplatin not only in pre-clinical studies but also in an analysis of a neoadjuvant chemotherapy clinical trial. Prospective clinical studies will be necessary to confirm the utility of *TP53* status stratified by EA as a predictive biomarker of response to cisplatin based therapy for HNSCC patients which will potentially enable the personalization of therapy for patients that will most likely benefit from this treatment strategy.

## MATERIALS AND METHODS

### Cell lines

Two HNSCC cell lines—UMSCC-1 and PCI-13 were selected for their lack of p53 expression due to a splice-site in UMSCC1 (hg19:chr17:7578370C>T) and a deletion in PCI13 (hg19:chr17:7579670\_7579709del). UM-SCC-1 was provided by Dr. Thomas Carey (University of Michigan, Ann Arbor, MI) in February 2010. PCI-13 was acquired from Dr. Jennifer Grandis (University of Pittsburgh, Pittsburgh, PA) in August 2008. The naturally occurring HNSCC cell lines, HN30 (wtp53) and HN31 (mutp53) were obtained in December 2008 from the laboratory of Dr. John Ensley (Wane State University, Detroit, MI). The cell lines and their isogenic derivatives were tested and authenticated against the parental cell lines by our group using short-tandem repeat analysis (15) within 6 months of use for the current study. Details regarding cell culture, reagents, and generation of stable cell lines were previously described<sup>1</sup>.

### Classification by Evolutionary Action (EAp53) Scoring System

Missense *TP53* mutations were divided into 'high-risk' and 'low-risk' groups based on the model described previously<sup>1</sup>.

## Immunoblotting

Cells grown on 10-cm plates were treated with clinically relevant dose of cisplatin (1.5  $\mu\text{M}$ ) for 24 hours and washed with cold PBS. Western blotting was performed using standard techniques previously described (16) and primary antibodies to anti-p53 (Santa Cruz Biotechnology, sc-126), anti-phospho-p53 serine 15 (Cell Signaling, 9284), anti-p21 (Calbiochem, OP64), and anti- $\beta$ -actin (Sigma Aldrich, A1978) were used.

## Transcriptional Activity of *TP53*

Transcription of p21, a canonical p53 target, was measured via luciferase reporter activity using a vector containing the 2.4 kb p21 promoter and firefly luciferase (pWWP-Luc) (Addgene, Cambridge, MA). UMSCC 1 and PCI-13 cells expressing various *TP53* constructs, HN30, and HN31 were co-transfected with pWWP-Luc and a constitutively active Renilla luciferase construct using Lipofectamine 2000. After 48 hours cells were treated with 1.5  $\mu\text{M}$  cisplatin and incubated for 24h prior to collection. Luciferase reporter activity was measured as previously described (34). The results for the p21 reporter assay are relative to the cisplatin treated wildtype (WT) which was standardized to 100 relative light units.

## Quantitative Reverse Transcription PCR (RT-qPCR) Analyses

The effect of *TP53* mutants on transcription of three downstream target genes (*p21*, *MDM2*, and *NOXA*) were determined by RT-qPCR. HNSCC (UMSCC1, PCI-13) cells stably expressing the *TP53* mutant constructs were treated with cisplatin (1.5  $\mu\text{M}$ ) for 24 hours before isolation of total RNA using RNeasy mini kit (QIAGEN). Reverse transcription was performed using the high capacity cDNA Reverse Transcription kit (Applied Biosystem) according to the manufacturer's protocol and a detailed description is included in the Supplementary Materials and Methods. The GAPDH gene was used as an internal control. Triplicate samples were examined. The expression of each target gene was normalized against GAPDH which calculated by the  $\Delta\Delta\text{CT}$  method ( $\Delta\Delta\text{CT} = [\text{CT of target gene}] - [\text{CT of internal control gene (GAPDH)}]$ ) and results were presented as fold change of expression.

**mRNA Expression Arrays**—Total RNA was isolated from cell lines by using Tri-reagent and hybridized to Affymetrix GeneChip Human Exon 1.0ST Arrays (Affymetrix) according to manufacturer's instructions and a detailed description is included in the Supplementary Materials and Methods. The expression of *TP53* target genes in pBabe and each of other groups was calculated and heat maps were generated depicting the expression patterns of these genes.

## Clonogenic Survival Assay

HNSCC cells stably expressing the *TP53* constructs were seeded in 6-well plates at various densities which allowed for approximately equal number of colonies in the control wells for each construct. The next day, cells were treated with increasing doses of cisplatin (0.01–2  $\mu\text{M}$ ) dissolved in dimethyl sulfoxide (DMSO) for 24 hours and cultured for 10 to 14 days to allow for colony formation of at least 50 cells. The cells were stained with crystal violet and analyzed as previously described (34). Each experiment was repeated more than three times

and treatments were performed in triplicates. An IC50 for each TP53 construct was calculated as the mean IC50 from each clonogenic assay.

### Orthotopic Nude Mouse Model of Oral Cavity Cancer

All animal experimentation was approved by the Animal Care and Use Committee (ACUC) of the University of Texas MD Anderson Cancer Center. Our orthotopic nude mouse model of oral cavity cancer has been previously validated and described in the literature (17). UMSCC1, and PCI 13, and cells expressing either, a high risk, low risk *TP53* mutation, a null pBabe *TP53* vector or wildtype *TP53* along with HN30 and HN31 cells were used in the study and a detailed description of the technique is included in the Supplementary Materials and Methods.

### Patient Cohort and *TP53* Sequencing

A cohort of 68 patients with oral cavity squamous cell carcinoma (SCC) treated with platinum based induction chemotherapy followed by surgery was collected from two clinical trials to investigate the predictive value of EAp53. Patient demographic, clinical data and cisplatin-based treatment regimens were previously published (12, 18). Patient data, specimens and *TP53* sequences were collected under IRB approved protocols. DNA was extracted from tissue of patients enrolled in the trials and different techniques were used to determine *TP53* sequence (19, 20). Detailed description of DNA isolation and *TP53* sequencing is included in the Supplementary Materials and Methods. Patients with either *TP53* wildtype or missense mutations were then scored by the EAp53 system into low or high risk categories as previously described<sup>1</sup>. The EA classification score was then correlated with clinicopathologic factors and patient outcome to determine associations with treatment response and survival.

### Statistical Analysis

ANOVA analysis with Student *t* tests were carried out to analyse in vitro data. For mouse studies, the two-tailed *t* test was used to compare tumor volumes between control and treatment groups. Survival was determined using the Kaplan-Meier method and compared using log rank tests. Fisher exact test or Chi square test were used to calculate the odds ratio between treatment and clinical response. *p* values <0.05 were considered significant.

## Results

### DNA damage-induced functional activity of p53 in response to cisplatin treatment is impaired in HNSCC cells expressing low and high risk *TP53* mutations

To examine in preclinical models if response to cisplatin therapy correlates with *TP53* mutational status stratified by the EA method, the p53 function of cell lines that either exogenously express various p53 constructs including wild type, low or high risk mutant isoforms or endogenously express wildtype p53 (HN30) or a high risk mutation (HN31) was assessed in these cells following treatment with cisplatin and analyzed by western blot. As expected, low basal expression levels of p53 and p21 were increased after cisplatin treatment in cells expressing wildtype *TP53* (Fig. 1 A, B and Supplementary Figure S1A). In addition to the p53 and p21 induction following cisplatin treatment in the cell lines stably

expressing wtp53, there was a similar level of p53 phosphorylation observed in the cells compared to the HN30 cell line which endogenously expressed wtp53, indicating that the stably expressed wildtype *TP53* is functionally active. In contrast, cells expressing mutant *TP53* endogenously or exogenously, had higher basal levels of p53 with minimal induction of p53 or p21 after cisplatin treatment indicating a lack of functional p53 (Fig. 1 A, B and Supplementary Figure S1A). Regardless of mutational status, phosphorylation of p53 following cisplatin was a ubiquitous finding, but p21 induction was most evident in cells expressing wtp53 and low risk mutant p53. The data suggest that while p53 phosphorylation occurs in response to DNA damage, many of the high risk mutations are not functional with respect to p21 induction, and that low risk mutations may retain partial wtp53 function.

Mutations in *TP53* alter the DNA binding domain conformation and disrupt the ability of p53 to bind to target gene promoters and consequently to transactivate downstream genes (21) Thus, the ability of *TP53* mutants to modulate the expression of classical wtp53 responsive target genes such as p21, MDM2, and Noxa was examined by both p21 promoter luciferase assay and reverse transcriptase quantitative PCR (RT-qPCR). Following cisplatin treatment, cells with wtp53 have an increase in promoter activity while the low risk mutations have a stable level of activity and high risk p53 have suppressed levels of p21 promoter activity relative to their basal levels and to the basal levels of the empty vector control (Fig. 1C and Supplementary Figure S1B). The mRNA levels of the target genes, p21, MDM2, and Noxa were significantly elevated in response to cisplatin treatment in HNSCC cells harboring either an exogenously expressed or endogenous wildtype p53. Cells with low risk mutations in the PCI13 cell line showed a trend toward increased target gene expression following cisplatin treatment relative to cells lacking p53, specifically the p21 level in A161S, or MDM2 and Noxa levels in Y236C (Fig. 1D). This is in contrast to cells harboring high risk mutations where the mRNA levels following cisplatin treatment were similar to cells lacking p53 (Fig. 1D). Similar observations were seen in the low risk UMSSC1 cell lines, specifically MDM2 level in A161S or MDM2 in Y236C. UMSSC1 cells harboring high risk mutations were more variable in the target gene expression following cisplatin treatment which may represent unique properties of these constructs or the cell background (Supplementary Figure S1C). The difference between p21 mRNA and protein levels observed in PCI13 and UMSSC1 cells expressing the high risk mutant (R175H) is possibly due to posttranslational modification event that resulted from less degradation of the mutant protein and therefore enhanced its stability upon cisplatin treatment. It could also be related to p63 and p73 isoforms being differentially expressed in these cells upon cisplatin addition. The p63 and p73 are well known p53-related proteins act as transcriptional activators of p21 and apoptotic inducers upon DNA damage in tumor cells (36). Taken together, these results reveal that cells with a mutated p53 have increased basal levels of protein but following cytotoxic stress there is decreased promoter activity of the canonical target, p21 and low mRNA levels of downstream target genes compared to cells expressing wildtype p53.

## **HNSCC cells bearing High risk *TP53* mutations are highly resistant to cisplatin treatment *in vitro***

To determine whether EAp53 has utility as a marker that can predict HNSCC response to cisplatin therapy, we assessed the response of HNSCC cells expressing no p53 (pBabe empty vector), wtp53, or a series of low and high risk p53 mutations to cisplatin in clonogenic survival assays. Figure 2A is representative images of clonogenic survival assay in HNSCC cell lines. As shown in Figure 2B and C, in both genetic backgrounds, the high risk mutant p53 clones were highly resistant to cisplatin, with 4 out of 5 clones having an average IC<sub>50</sub> of 0.95  $\mu$ M > 0.8  $\mu$ M, when exposed to cisplatin for a 24 h period. We have determined this *in vitro* exposure of 0.8  $\mu$ M to be equivalent to the high dose of cisplatin (i.e., 100 mg/square meter) given to patients based upon pharmacokinetic area under the curves studies in humans. Low risk mutant p53 clones were less resistant to cisplatin with an average IC<sub>50</sub> of 0.72  $\mu$ M that is statistically significant when compared to the high risk mutant p53 clones (Fig. 2B and C). Additionally, the clones expressing wildtype p53 had lower IC<sub>50</sub> values (0.15  $\mu$ M) compared to clones with null pBabe empty vector (0.44  $\mu$ M) in both PCI-13 (P <0.001) and UMSCC1 (P <0.003). These data suggest that introduction of low and high risk TP53 mutations into HNSCC cell lines resulted in a gain of function phenotype for resistance to cisplatin therapy. Furthermore, our laboratory has shown that the endogenous mutp53 of HN31 also confers a relative cisplatin resistance with an IC<sub>50</sub> of 0.60  $\mu$ M compared to its isogenic wtp53 counterpart, HN30 which has an IC<sub>50</sub> of 0.14  $\mu$ M (34). To further address loss of function versus gain of function, *TP53* was knocked down in the isogenic pair of cell lines HN30 (wtp53) and HN31 (HRmutp53) and cells were then examined for cisplatin sensitivity (Supplementary Figure S2A and S2B). The shRNAp53 HN30 cells become more resistant to cisplatin (IC<sub>50</sub>; 0.32  $\mu$ M), arguing that loss of wtp53 function can make tumors less sensitive to cisplatin. Interestingly, knockdown of mutant HR p53 in HN31 made cells more sensitive to cisplatin with an IC<sub>50</sub> value very close to the HN30 p53 knockdown (IC<sub>50</sub> of 0.30  $\mu$ M versus 0.32  $\mu$ M), indicating a gain of function associated with the HR mutation. Collectively, the data argue that *in vitro* both loss of wtp53 and a HR-associated gain of function contribute to increased cisplatin resistance.

## **Expression profile of high risk *TP53* mutations shows a lack of p53 transcriptional activity while low risk mutations retain some residual function**

Given the apparent gain of function phenotype seen in our *in vitro* studies of the p53 mutants, we performed mRNA expression profiling in an effort to identify genes and pathways specific to the high risk mutp53 that could explain their relative resistance to cisplatin. The principle component analysis (PCA) of the gene expression profiles for cisplatin treated UMSCC1 cell lines, wtp53, pBabe, low risk mutation (A161S), or high risk mutation (C238F), revealed that the high risk mutation expression profiles were more similar to the pBabe cell line which lacks p53 expression. This is apparent from component 1 (x-axis) which accounted for 40% of variance in expression. Furthermore, the low risk mutation profile had smaller variances (20%) in expression from the pBabe and high risk mutations as seen by the large component 2 (y-axis) contribution. In contrast, the wtp53 had the largest variances from the high risk mutation and pBabe and was also distinct from the low risk mutation profiles (Fig. 3A and B).

Ordinal logistic regression models were performed to identify genes that contribute to either a gain of function (GOF) or loss of function (LOF) phenotype where the magnitude of expression from highest to lowest is either high risk, low risk, wtp53, and pBabe or wtp53, low risk, high risk, and pBabe respectively. Surprisingly based on our *in vitro* data, the number of significant genes with the false discovery rate set at < 10%, that contribute to a LOF phenotype was dramatically higher than the number of genes associated with a GOF phenotype, 1190 and 0 respectively (Fig. 3B and C). Furthermore, the BUM (beta-uniform mixture) plot analyses for the two potential phenotypes reveal an enrichment of genes with low *p* values leading to non-uniform distribution of genes in LOF analysis compared to the more uniform distribution in the GOF analysis (Fig. 3D and E). These results validate the higher *p* value cutoff used in the ordinal logistic regression analyses for LOF compared to GOF (Fig. 3B and C). As expected, the primary pathways that were driving these expression patterns were regulated by p53. Overall, the results from the mRNA expression profile reveal that the introduction of a high risk p53 mutation leads to a reduction in the wildtype p53 function to levels similar to pBabe cells that lack p53 expression. Additionally, introduction of a low risk mutation results in an expression pattern suggestive of residual wildtype p53 function as seen by the intermediate level of expression of *TP53* target genes (Fig. 3F and Supplementary Table 4).

### High risk *TP53* mutations are associated with decreased response to cisplatin therapy and overall survival in an orthotopic mouse model of tongue cancer

To further characterize the ability of the EAp53 to predict mutations' response to cisplatin therapy, tumors harboring these mutations were created in an orthotopic nude mouse model of tongue cancer. Animals underwent a 4 week course of cisplatin therapy during which the tumor volumes and overall survival were monitored. Consistent with the differential effect of *TP53* status on cisplatin response observed *in vitro*, tumors in mice injected with tumor cells expressing wildtype p53 showed a significant response to cisplatin therapy, while the tumors derived from cells null for p53 expression harboring the pBabe vector control or high risk p53 mutations did not show any growth inhibition with cisplatin therapy (Fig. 4 Panel A–C). Interestingly, the response of tumors with low risk mutations was more similar to the response of wtp53-bearing cells, which in agreement with the mRNA levels and expression array data in that the low risk mutations appear to retain some wildtype p53 function. To compare relative tumor response between tumors to cisplatin, the area under the tumor growth curve was calculated for each animal and the mean AUC was plotted for each treated tumor and their corresponding control (Supplementary Figure S3). Mice with tumors that harbor endogenous or exogenous wildtype p53 or low risk mutations have a significant response ( $p < 0.0001$ ) to cisplatin therapy while mice harboring pBabe (null) or high risk p53 mutations show minimal growth inhibition with cisplatin therapy. This gradient of response corroborates the expression array data and once again implies a partial wildtype p53 function for the low risk mutations and lack of p53 function for the high risk mutations. The resistance to cisplatin seen in the high risk mutations was also associated with decreased survival in animals bearing tumors with high risk *TP53* mutations (Fig. 4 Panel D–F). These results demonstrate that the EA method can predict the p53 mutations that are least likely to respond to platinum based therapy *in vivo* in an orthotopic murine model of oral cancer.



## EAp53 classification predicts response to platinum-based chemotherapy in patients with locally advanced oral cavity cancer

To determine the reliability of EAp53 to predict response to treatment in patients with oral cavity cancer, we identified a cohort of patients with locally advanced oral cavity cancer who received platinum based induction chemotherapy in the context of prospective clinical trials. This cohort consisted of 68 patients of which 26 of the tumor samples (38%) had missense mutations of *TP53* while 42 tumor samples (62%) had wildtype p53 (Table 1). We have shown that the EAp53 system identified three groups independently, low EAp53 score, high EAp53 score, and wildtype p53<sup>1</sup>. Univariate analysis in the training set revealed that the low EAp53 score mutations, i.e. low risk, and wild type were not statistically different whereas the high EAp53 score mutations, termed high risk mutations, appeared to be distinct from the other two groups<sup>1</sup>. Given the similar outcomes, patients with tumors having low risk mutations were combined with wildtype p53 (wtp53). Therefore, the *TP53* status was further classified by EAp53 into low risk group (42 wildtype *TP53* and 12 low risk *TP53* mutations), and a high risk group consists of 14 high risk *TP53* mutations (Table 1 and Supplementary Table S1). Review of the pathological findings revealed that 13 of the patients (93%) with high risk *TP53* mutations had residual disease while only one patient showed complete response to cisplatin-based therapy. In contrast, 24 of the 54 patients (44%) with wildtype p53 and low risk *TP53* mutations achieved complete pathologic response while 30 patients (56%) had residual disease. These data demonstrate that relative to low risk *TP53*, high risk mutations are greater than 10 fold more likely to have residual disease following cisplatin based chemotherapy,  $p=0.029$ , (Table 1). EAp53 status was also found to be better predictor of cisplatin response than the previous classification system developed by Poeta et al (11), which showed no statistically significant association with neoadjuvant response ( $p=0.248$ , Table 1) in our cohort. The other clinicopathologic data analyzed were not associated with a response to cisplatin therapy (Table 1). Additionally, patients with tumors having high risk *TP53* mutations appear to have decreased overall survival relative to patients with low risk *TP53* status,  $p=0.04$ , (Fig. 5A) in a Kaplan Mier analysis. There was also a trend toward decreased disease free survival in patients with high risk *TP53* mutations but it did not reach statistical significance,  $p=0.08$  (Fig. 5B). On univariate and multivariate analyses the survival benefit of low risk p53 status in the log rank tests was not observed in Cox Proportional Hazard Ratio Model (Supplementary Table S2 and S3). Overall, these data provide evidence that EAp53 can predict a subset of patient with high risk *TP53* mutations that have a decreased response to platinum based chemotherapy and a poorer overall survival.

## Discussion

Currently there are not any established molecular biomarkers to predict response to chemotherapeutic agents in HNSCC. Recent whole exome analysis has confirmed that *TP53* is the most frequently mutated gene in HNSCC occurring in 60–80% of cases but the challenge remains to identify the mutations associated with resistance to current cytotoxic therapies and therefore decreased survival outcomes (4, 5). In the current study, we implemented a novel classification system, EAp53, which has the ability to predict response to cisplatin based therapy not only in preclinical models of HNSCC but also in patients with

locally advanced oral cavity squamous cell carcinoma. We utilized a previously described collection of isogenic head and neck squamous cell carcinoma cell lines harboring a series of *TP53* mutations or wildtype *TP53* which allowed us to specifically examine the impact of *TP53* alterations on response to cisplatin as the genetic backgrounds of these cell lines are otherwise identical<sup>1</sup>. To confirm that the exogenous expression of p53 in these cell lines accurately represents the function of both wtp53 and mutant p53 (mutp53), cells endogenously expressing either wildtype or mutant p53 were also used for comparison.

The results from this study reveal that relative to wildtype *TP53*, both high and low risk mutations show a decrease in cisplatin-mediated p21 induction, a known transcriptional target of p53 in response to cisplatin treatment. In low risk mutant *TP53* cells, this diminished p21 induction appears to be associated with a reduction in the p21 promoter activity and an intermediate level of mRNA expression relative to wtp53 expressing cells. In contrast, the cell lines with either exogenous high risk *TP53* mutations or endogenous mutp53 had decreased p21 promoter activity following cisplatin treatment as previously described (22, 23). The functional activity of high *TP53* mutations was partially corroborated by the RT-qPCR results with the high risk mutations having a decreased level of target gene expression following cisplatin treatment. It has been suggested that loss of upregulation of key p53 target genes may contribute to the gain of function phenotype of some mutp53 (22–25). Although mutated p53 is unable to bind to sequence specific DNA of target gene promoters secondary to alterations in the DNA binding domain, it has been suggested that p53 either recognizes target promoters independent of this region or binds the promoters at regions unique from p53-binding sites (23, 26). Furthermore, the loss of upregulation of p53 target genes may be enhanced by the constitutive overexpression of mutp53 due to their inability to effectively activate MDM2, a negative regulator of p53 abundance (27, 28). This latter notion could be supported by our finding that cisplatin treatment reduced the mRNA level of MDM2 in cells expressing low and high risk *TP53* mutants.

To further assess the effect of suppression of p21 on response to chemotherapy in the EA-p53 high risk mutations, we show in a clonogenic survival assay that HNSCC cells expressing low risk mutations have an intermediate level of resistance while high risk *TP53* mutations have a high level of resistance to platinum-based therapy relative to the pBabe vector control, confirming a GOF phenotype with regard to cisplatin sensitivity. The differences in cisplatin sensitivity between the wtp53 and mutp53 may be due in part to the inhibition of accelerated cellular senescence by mutated p53 (29).

Although our initial *in vitro* experiments suggested a gain of function phenotype for high risk *TP53* mutations, our attempt to identify pathways driving this characteristic through expression array analysis revealed, that the introduction of high risk mutations actually produces a largely nonfunctional p53 (i.e. suppressed levels of expression of *TP53* target genes) that is most similar to a complete absence of the protein. Furthermore, the low risk mutations have distinct expression variances from cells either lacking p53 or containing a high risk mutation which implies this mutation may retain some wildtype functions. The discrepancy between the expression array data and the *in vitro* experiments could be due to different post translational modifications of the low and high risk mutations that would not

be detected on an mRNA expression array or an altered protein-protein interactome through which p53 gains function through functionally significant interactions with important cellular protein targets (30, 31). In support of this latter mechanism, we have recently reported that GOF p53 mutations can bind to and inactivate, the master metabolic regulatory protein, AMPK, which leads to gains of oncogenic functions (32).

Furthermore, evidence is now accumulating to indicate that different p53 mutations possess different functions in different tissues, potentially reflecting differences in the expression of their cellular targets (35). Therefore, understanding the consequences of each p53 mutation in relationship to disease progression and response to therapy promises to be an extremely complex undertaking and thus highlights the importance of our current study.

We further characterized not only the functional spectrum of p53 mutations but also the ability of EAp53 to predict response to cisplatin *in vivo* with an orthotopic nude mouse model of tongue cancer. These results confirmed that tumors bearing high risk mutations are more resistant to cisplatin compared to tumors expressing either wtp53 or low risk mutant p53. The lack of tumor response to treatment in mice with high risk p53 bearing tumors was similar to tumors lacking p53 expression which corroborates the expression array data. In addition to having an improved tumor response, animals with tumors harboring wtp53 and low risk mutations treated with cisplatin had an improved survival compared to both their untreated controls and the animals with high risk p53 tumors.

Finally, in an effort to evaluate the predictive ability of *TP53* mutational status stratified by the EA method, we analyzed a cohort of patients with locally advanced oral cavity squamous cell carcinoma who had been treated with platinum based neoadjuvant chemotherapy followed by surgery (12, 18). This analysis revealed that EAp53 can identify a population of patients with high risk p53 mutations that do not respond to platinum based treatment and had decreased overall survival. Although these results are encouraging, there are limitations to this study. The percentage of patients with wtp53, 62%, is higher than expected which could be attributed to either the sensitivity of sequencing *TP53* from paraffin tissues or sequencing exons 5–8 since only ~80% of mutations occur within the DNA binding domain (33). Therefore, it is possible that the portion of the wildtype p53 patients' tumors that did not have a clinical response actually harbored *TP53* mutations. Taken together, these explanations may partly account for not only the high percentage of wtp53 but also the large number of wtp53 nonresponders. Nonetheless, EAp53 was predictive of lack of response to platinum based chemotherapy in patients with high risk mutant p53, and identified patients with oral cavity squamous cell carcinoma that have decreased survival. To further validate these clinical findings, additional studies of p53 mutational status in larger cohorts of HNSCC patients that have received neoadjuvant platinum based induction therapy are ongoing.

In summary, the EAp53 model clearly identifies a subset of high risk *TP53* mutations associated with decreased sensitivity to cisplatin in pre-clinical models. Additionally, EAp53 can predict response to treatment and more importantly a survival benefit for a subset of patients treated with platinum based therapy. In order to fully evaluate the role of EAp53 as a predictive biomarker of platinum response, prospective clinical trials in which

patients' tumors are stratified based on their p53 mutational status and correlated with the response to treatment and survival are necessary.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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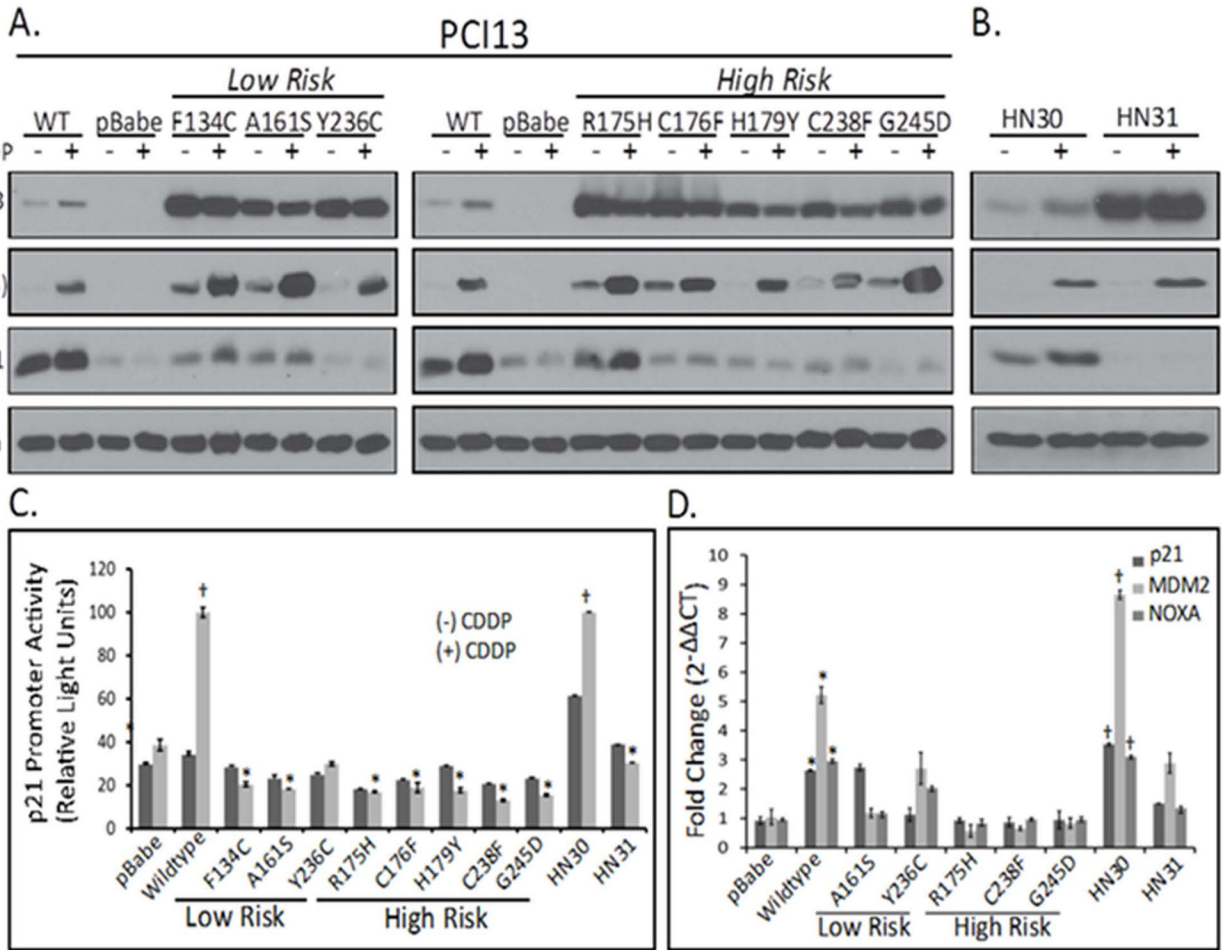
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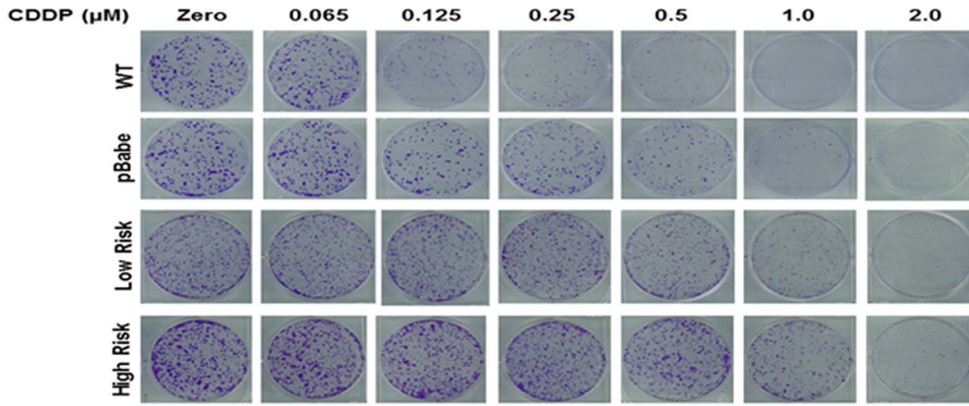
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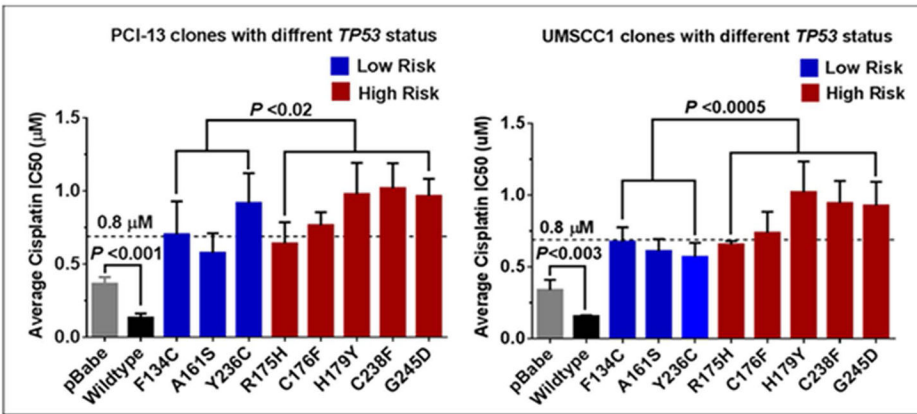
**Figure 1. Functional activity of TP53 induced by DNA damage is impaired in HNSCC expressing low and high risk TP53 mutations**

Western blot of isogenic HNSCC cell line, PCI-13, that stably expresses wildtype or mutated p53 constructs or HN30 and HN31 HNSCC cell lines that endogenously express wildtype TP53 and mutated TP53 respectively (Panel A and B). p21 reporter luciferase activity of the same PCI13 isogenic cells lines along with HN30 and HN31, (Panel C). Quantitative RT-PCR of p21, MDM2 and Noxa in PCI-13 isogenic cells lines and HN30 and HN31 HNSCC cell lines (Panel D). CDDP is abbreviation for cisplatin. \* defined as a significant change,  $p < 0.05$ , from cisplatin treated wtp53. † defined as significant increase in activity or expression above basal levels.

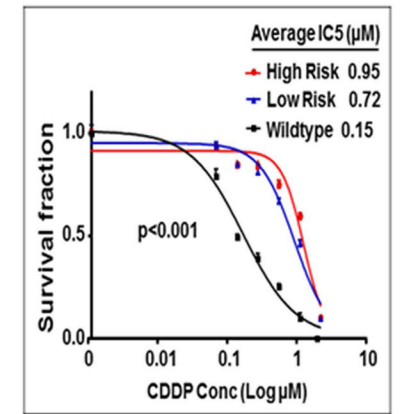
**A. PCI-13 cells**



**B.**



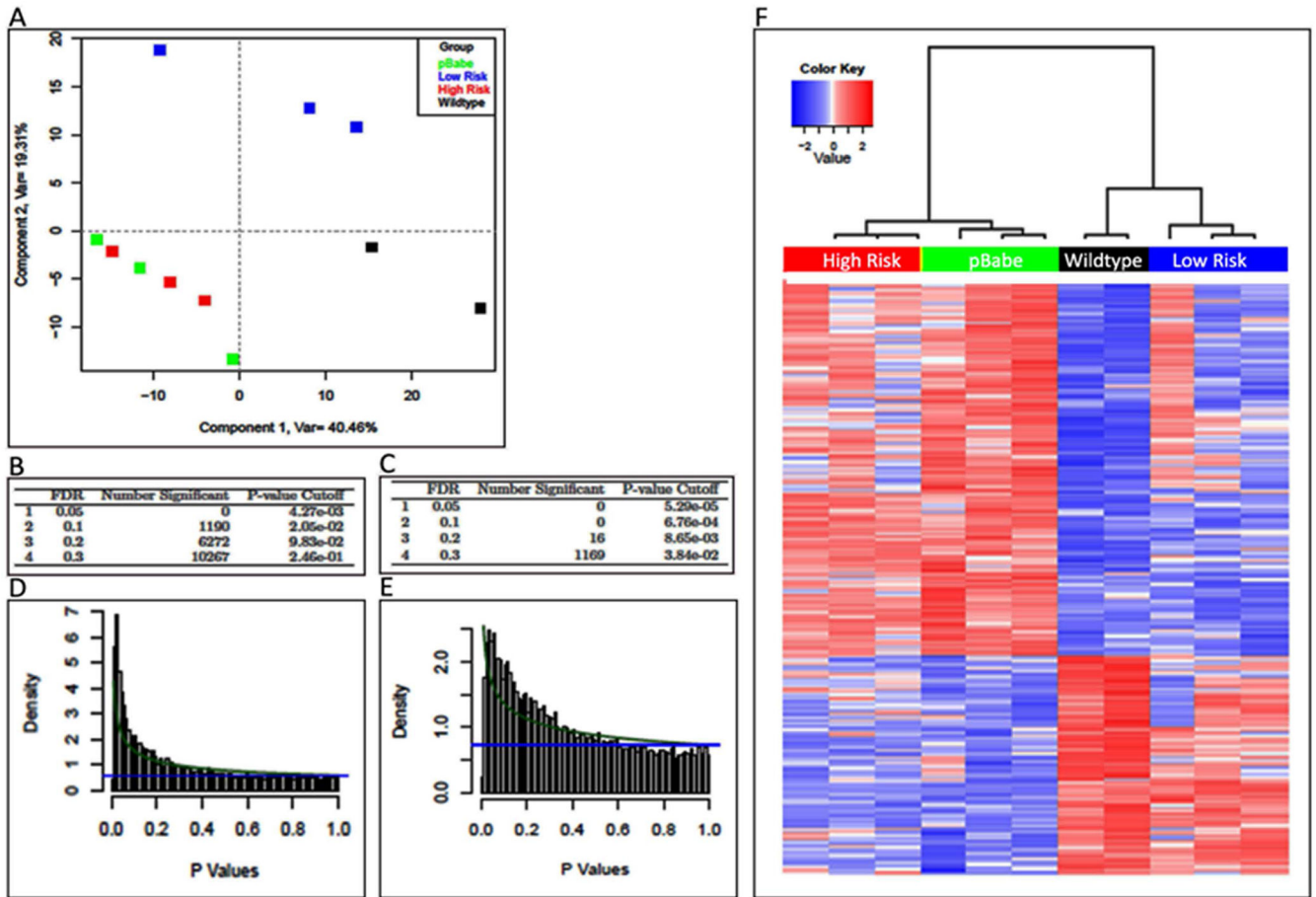
**C.**



**Figure 2. High risk TP53 mutations are resistant to cisplatin *in vitro***

Cisplatin sensitivity was determined by the clonogenic survival assay in HNSCC isogenic cell lines (PCI-13 and UMSCC1) harboring wildtype and TP53 mutant constructs. Representative images of colony formation assay in HNSCC cell lines (Panel A). Calculated IC50 of cisplatin for individual TP53 constructs in both UMSCC1 and PCI-13 isogenic cell lines (Panel B). Horizontal dashed line delineates the increase in cisplatin IC50 over an *in vitro* dose (0.8 μM) that is equivalent to the high dose of cisplatin (i.e., 100 mg/square meter) given to patients based upon pharmacokinetic area under the curves studies in humans. The average dose response curve of cisplatin for low and high risk mutations and wildtype TP53 (Panel C).  $P < 0.001$ , low and high risk mutation vs. wildtype TP53.





**Figure 3. Gene expression array analysis reveals that introduction of high risk mutations results primarily in a loss of function phenotype**

Principle component analysis of the variance in gene expression between cisplatin treated HNSCC clones with different p53 status revealed that wtp53 had much larger variance in expression than pBabe and high risk p53 mutations (component 1). The low risk p53 mutations also had more variance in expression than pBabe and high risk p53 mutations (component 2); whereas, wtp53 and low risk p53 mutations had less variance compared to each other, indicating that high risk p53 mutations resemble a loss of function (Panel A). Ordinal regression analysis for a loss of function (LOF) and gain of function (GOF) phenotypes (panel B and C). LOF phenotype altered gene expression in the order of WT>Low Risk>High Risk>pBabe, where WT had the largest change and High risk and pBabe were similar (panel B). A GOF phenotype identified genes with altered expression in the order of High risk>Low risk>Wildtype>pBabe (panel C). Results showed that at each given statistical cutoff more genes were statistically significant in the LOF order than in the GOF order. BUM plots of loss and gain function ordinal regression analyses (Panel D&E). The BUM plots further indicate a stronger bias toward low p-values in the loss of function (LOF) order. Heat map of 1190 significant genes from the LOF ordinal logistic (Panel F). Since the FDR indicates the rate of false-positives among the -positives, many analyses use a cutoff close or equal to 0.1 for the FDR rather than the 0.05 cutoff traditionally used for p-

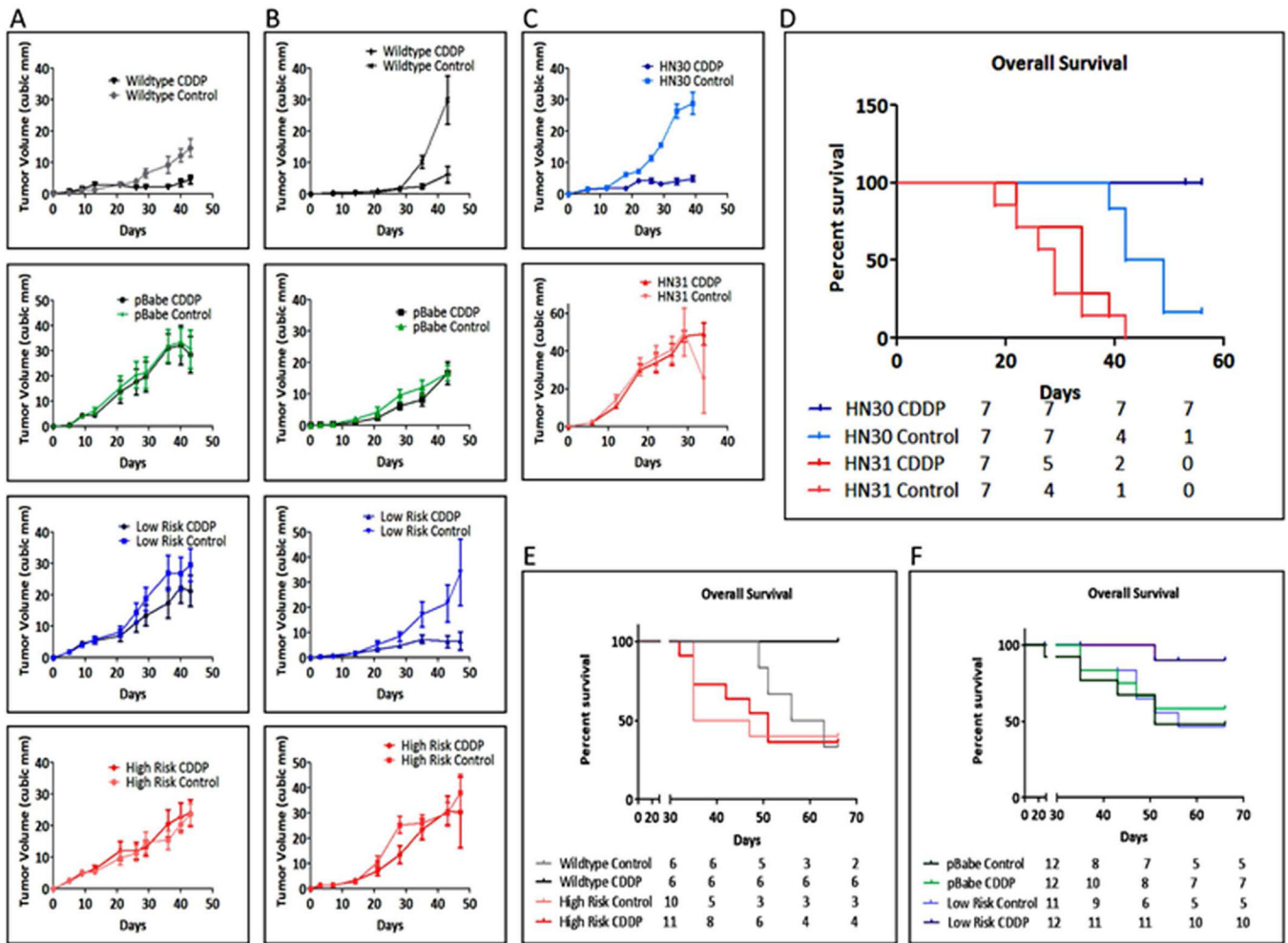
values. Therefore, a lowest statistical cutoff correspond to a false discovery rate (FDR) of a 0.1 was chosen to differentiate between the loss of function (LOF) and gain of function (GOF) phenotypes.

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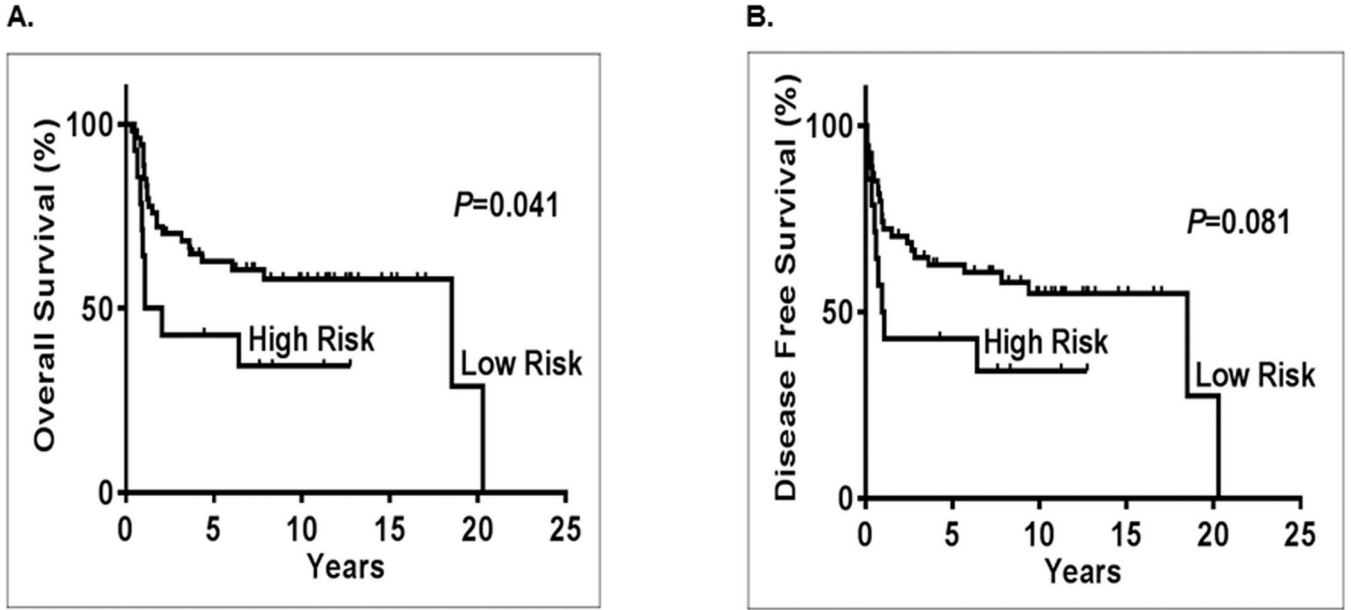
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**Figure 4. High risk *TP53* mutations are resistant to cisplatin in an orthotopic nude mouse model** HNSCC cell lines exogenously expressing, PCI 13 and UMSCC1 (Panel A and B), and endogenously HN30 and HN31 (Panel C), expressing p53 were injected into the tongue of nude mice. Kaplan Meir curves of the orthotopic tongue model created with either cell lines HN30 or HN31 (Panel D) or *TP53* isogenic cell lines PCI-13 or UMSCC1 (Panel E and F).



**Figure 5. EAp53 classification predicts response to platinum-based chemotherapy in patient with oral cavity squamous cell carcinoma**

Log rank tests of Kaplan Meir survival plots for a cohort of patients treated with platinum based induction chemotherapy followed by surgery revealed that EAp53 can predict patients with high risk mutations have a decreased overall survival,  $p=0.041$  (A). There was a trend toward decreased disease free survival in patients with high risk *TP53* mutations that almost reached statistical significance,  $p=0.08$  (B).

**Table 1**

Odds ratio of response to platinum based therapy for various clinicopathologic features.

Characteristic	Total	Clinical Response		OR	95% CI	p-value	Global p-value
		Yes	No				
All Patients	68	25	43				
Age							
	<59	19	27				
	59	6	16	1.877	(0.620, 5.675)	0.265	-
Gender							
	male	19	31				
	female	6	12	0.816	(0.262, 2.536)	0.725	-
T stage							
	2	12	9				
	3	9	23	3.407	(1.070, 10.847)	0.038	
	4	4	11	3.667	(0.874, 15.384)	0.076	0.073
N Stage							
	0	14	25				
	1	5	7	0.784	(0.209, 2.938)	0.718	
	2b	4	4	0.56	(0.121, 2.593)	0.458	
	2c	2	2	0.56	(0.071, 4.421)	0.582	
	3	0	5	6.255	(0.322, 121.429)	0.226	0.404
N Stage							
	<2b	19	32				
	>=2b	6	11	1.089	(0.346, 3.422)	0.885	
TP53 Status							
	Wildtype	20	22				
	Mutant	5	21	3.818	(1.211, 12.034)	0.022	
EA Status							

Characteristic	Total	Clinical Response		OR	95% CI	p-value	Global p-value
		Yes	No				
Low Risk	54	24	30				
High Risk	14	1	13	10.4	(1.269, 85.233)	0.029	
Poeta classification							
Non-disruptive*	61	24	37				
Disruptive	7	1	6	3.9	(0.440,34.387)	0.248	

Abbreviations: OR - odds ratio, CI - confidence interval, EA - Evolutionary Action Score.

a: Used Fisher's Exact test to calculate *p*-values.

b: If contingency table is larger than 2x2, then global *p*-value is calculated using either ChiSq test or Fisher's exact test and *p*-value is calculated for each 2x2 sub-table. For comparison with the EAp53 system, patient tumors were also classified as disruptive and non-disruptive according to Poeta et al.

\* Patients with wildtype TP53 or silent mutations were classified by as non-disruptive; however, the association was still not significant even when patients with wildtype TP53 or silent mutations were removed.