NRF2 signaling is repressed in HPV-driven head and neck cancer and correlates with better prognosis: HPV-E6/E7 confers chemosensitivity by inhibiting NRF2

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Research Article

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Abstract

Background: To investigate the role of NRF2 signaling in conferring superior prognosis in patients with HPV positive (HPV^{+ve}) head & neck squamous cell carcinomas (HNSCC) compared to HPV negative (HPV^{-ve}) HNSCC and develop molecular markers for selection of HPV^{+ve} HNSCC patients for treatment de-escalation trials.

Methods: We compared NRF2 activity (*NRF2*, *KEAP1*, and NRF2-transcriptional targets), p16, and p53 levels between HPV^{+ve} HNSCC and HPV^{-ve} HNSCC in prospective and retrospective tumor samples. Cancer cells were transfected with HPV E6/E7 plasmid to elucidate if HPV infection repress NRF2 activity and sensitize to an anticancer drug.

Results: Prospective analysis revealed a marked reduction in expression of *NRF2*, and its target antioxidant genes (*NQO1, HMOX-1, GCLC,* and *GCLM)* in HPV^{+ve} tumors compared to HPV^{-ve} tumors. A retrospective analysis by IHC revealed significantly lower NQO1 in $p16^{high}$ tumors compared to $p16^{low}$ tumors and the NQO1 expression correlated negatively with p16 and positively with p53. Analysis of the TCGA database confirmed low constitutive NRF2 activity in HPV^{+ve} HNSCC compared to HPV^{-ve} HNSCC and revealed that HPV^{+ve} HNSCC patients with 'low *NQO1'* expression showed better overall survival compared to HPV^{+ve} HNSCC patients with 'high *NQO1'* expression. Ectopic expression of HPV E6/E7 plasmid in cancer cells repressed constitutive NRF2 activity, increased KEAP1, reduced total GSH levels, and enhanced cytotoxic effects of cisplatin.

Conclusions: Low constitutive NRF2 activity contributes to better prognosis in patients with HPV^{+ve} HNSCC compared to HPV^{-ve} HNSCC. Co-expression of $p16^{high}$, $NQO1^{low}$, and $p53^{low}$ could serve as a predictive biomarker for the selection of HPV^{+ve} HNSCC patients for de-escalation trials.

Introduction

In 2020, globally around 800,000 people were diagnosed with Head & neck squamous cell carcinomas (HNSCC) with an approximate mortality rate of 50% (1). Traditionally, the aetiology of HNSCC was associated with tobacco smoking and chewing. However, the current growing burden of HNSCC, specifically oropharyngeal carcinomas is attributed to Human Papillomavirus (HPV) infection (high-risk HPV-16 and HPV-18) (2). Intriguingly, HPV positive (HPV^{+ve}) HNSCC represents a distinct clinical entity with unique etiopathogenesis compared to HPV negative (HPV^{-ve}) HNSCC (3, 4). The most notable clinical feature is that patients with HPV^{+ve} HNSCC have higher rates of cure (5-year survival rate- 80% versus 50%) and lower risk of recurrence compared to HPV^{-ve} HNSCC following standard treatment modalities (3). What is concerning is that despite a better prognosis, the extent of treatment-related long-term morbidities including xerostomia and dysphagia was similar between HPV^{+ve} HNSCC patients and HPV^{-ve} HNSCC patients(3). This has prompted the evaluation of de-escalated/de-intensified treatment strategies for HPV^{+ve} HNSCC patients with a primary objective to reduce long-term complications while

maintaining the therapeutic effectiveness of the treatment modality. To date, the results of de-escalated Phase III clinical trials in patients with HPV^{+ve} HNSCC has been discouraging. The Phase III De-ESCALaTE trial and RTOG 1016 trial evaluated the strategy of combining radiation with less toxic cetuximab instead of cytotoxic cisplatin in HPV^{+ve} HNSCC patients. However, both the clinical trials reported inferior survival in the cetuximab group compared to the cisplatin group with no significant protection from long-term complications (5). Due to a lack of mechanistic understandings, there are no prognostic molecular biomarkers for patient stratification as 'good' vs. 'poor' responders to either standard or de-escalated treatment modality for HPV^{+ve} HNSCC patients.

Many human cancers, including HNSCC, are associated with aberrant constitutive hyperactivation of transcription factor nuclear erythroid 2 related factor 2 (NRF2) (6). NRF2 regulates the transcription of genes encoding a battery of cytoprotective proteins such as antioxidant enzymes (NADPH: quinone oxidoreductase 1 (NQO1), heme oxygenase-1 (HMOX1)), GSH biosynthesizing enzymes (glutamate-cysteine ligase), phase II enzymes (Glutathione *S*-transferases), and multidrug-resistance proteins by binding to 'antioxidant response elements' present in their promoter regions (7). Intrinsically high NRF2 activity maintains higher antioxidant capacity in cancer cells that supports tumorigenesis and confers resistance to cytotoxic chemotherapy and or radiotherapy, mainly by reducing the intracellular build-up of toxic reactive oxygen species (8). In contrast, NRF2 inhibition in cancer cells by knocking down *NRF2* using siRNA (9) or pharmacological inhibitor depletes antioxidant capacity, and sensitizes to anticancer drugs and ionizing radiation (10). Several studies have demonstrated that constitutive hyperactivation of the NRF2 pathway (indicated by higher expression of NRF2-target genes) inversely correlates with the prognosis and overall survival of patients with various cancers including HNSCC and lung (11, 12). Mutagenic and non-mutagenic mechanisms are implicated in aberrant constitutive hyperactivation of NRF2 in cancers (8). Most frequently, the hyperactivation phenotype of NRF2 signaling is caused by somatic gain-of-function mutations in *NRF2* or loss-of-function mutations in *KEAP1* and or *CULLIN-3* (*Cul3*) (8). In normal cells, actin-bound KEAP1 sequesters NRF2 in the cytoplasm and serves as an adapter molecule to Cul3-containing E3 ubiquitin ligase, which mediates NRF2 ubiquitination and proteasomal degradation. Loss of function mutations in *KEAP1* or *CUL3* hampers NRF2 ubiquitination and proteasomal degradation that results in aberrant nuclear accumulation of NRF2 leading to heightened NRF2 transcriptional activity in cancer cells(8). Three independent lines of evidence prompted us to hypothesize that constitutively low NRF2 activity contributes to better prognosis in HPV^{+ve} HNSCC compared to HPV^{-ve} HNSCC. First, the comprehensive genomic analysis by the Cancer Genome Atlas (TCGA) consortium (13) and other investigators (4, 14, 15) have revealed that HPV^{-ve} HNSCC tumors are more frequently associated with activating mutations in the NRF2 pathway (*NRF2, KEAP1*, or *CUL3).* By contrast, in HPV^{+ve} HNSCC tumors, the mutations in the same genes were very rare. Second, the endogenous levels of ROS in HPV^{+ve} head and neck (H&N) cancer cell lines are found to be two-times higher than HPV^{-ve} H&N cancer cell lines (16). Third, intracellular oxidative stress milieu supports HPV replication (ref) and there are several instances where viruses such as Hepatitis C and enterovirus down-regulate NRF2 signaling (17, 18) to induce an oxidative stress and support the viral replication process. In

this study, we report that HPV^{+ve} HNSCC tumors are associated with low constitutive NRF2 activity than HPV^{-ve} HNSCC tumors and low constitutive NRF2 activity among HPV^{+ve} HNSCC patients positively correlates with better prognosis. We also demonstrate that ectopic expression of HPV E6/E7 plasmid in cancer cells featuring high constitutive NRF2 activity represses NRF2 signalling, lowers GSH levels and sensitizes to anti-cancer drug cisplatin. Lastly, we postulate an algorithm based on co-expression levels of p16 (HPV status), NQO1 (NRF2 activity) and p53 to predict prognosis and for patient selection (better responder vs poor responders) for de-escalation trials among HPV^{+ve} HPV HNSCC.

Materials & Methods

Patients and specimen collection

For the prospective study, we collected tumor biopsies from a total of 50 randomly selected patients with untreated resectable, non-metastatic clinical-stage I-IV squamous cell carcinoma (SCC) of the oropharynx, oral cavity, hypopharynx, larynx, and the upper esophagus who visited hospitals for treatment in and around Mysore region between June 2017 and February 2020. Patients who had undergone therapy or patients with SCC of the salivary glands and with recurrent cancers were excluded. The demographic details were recorded by administering a questionnaire. For the retrospective study, the hospital databases from 2015–2018 were searched for patients who were histopathologically confirmed with SCC of the head and neck region. A total of 121 archived formalin-fixed paraffin-embedded (FFPE) tissue blocks of tumors from untreated HNSCC subjects were retrieved from the pathology laboratory archives. Demographic and clinical profiles of the study subjects including age, sex, site of the tumor with histology, stage, grade, and nodal involvement were collected by reviewing the medical records with the help of the Pathologist. For both the prospective and retrospective cohort samples, histological diagnosis was re-evaluated by our in-house pathologist (N. Manoli) and clinico-pathological features such as cancer stage and grade were assigned as per the 8th edition of American Joint Committee on Cancer (AJCC) guidelines. The study was conducted in accordance with the declaration of Helsinki and the protocol was approved by the Institutional Ethics Committee of JSS Medical College, Mysore (JSSMC/IEC/25/1991/2017–2018). The current study fulfils recommendations of the REMARK guidelines for tumor biomarker prognostic studies (19).

Immunohistochemical (Ihc) Analysis And Scoring

Each FFPE tissue block was assigned a specific patient ID and the tissues embedded in paraffin wax were sectioned (5µm) using a microtome (Leica Biosystems, RM2125 RTS) and stained with hematoxylin and eosin (H&E). Tissue morphology and architecture were reviewed by our in-house pathologist. Additional sections of 5µm were taken on Poly-L-Lysine coated slides for immunohistochemical (IHC) analysis and were processed as described previously (20, 21). The primary antibodies p16 (1:400, PathnSitu Biotechnologies, Cat# PM143), NQO1 (1:200, Cell Signaling Technology, Cat# 62262), and p53 (1:300, Cell Signaling Technology, Cat# 9282) were used in the indicated dilutions. The slides stained

with H&E as well as processed for IHC were observed and representative photographs were captured at different magnifications using a bright field microscope (Olympus BX-53). The scoring for each biomarker was performed by three methods. i) Manual scoring by the pathologist in a blinded fashion. First, the slides were scored for staining intensity (no staining = 0, weak staining = 1, moderate staining = 2 and strong staining = 3). Then the extent of cells stained were counted (0%= 0, 1–10%= 1, 11–50%=3 and 51–100%= 3). The final score was determined by multiplying both the scores with a maximum score of 9 and the least score of 0. The score above 4 was considered high as it represents ≥ 60% strong diffuse staining. ii) Scoring using Image J software by following the method as described by Varghese et al. (22). An additional plug-in called IHC profiler, which is freely available (https://sourceforge.net/projects/ihcprofiler/) was installed along with the routine software. Images captured at 20x magnification were used for analysis and scoring of each IHC marker. The output file gives information on the percentage contribution of high positive, positive, low positive and negative cells. iii) Semi-quantitative H-score assessment by multiplying the staining intensity (0, non-staining; 1, weak staining; 2, moderate staining; 3, strongly staining) and the percentage of stained cell (0-100%) [H-score = (1 × % weakly stained cells)+(2 × % moderately stained cells)+(3 × % strongly stained cells)]. The H-score comprises values ranging from 0 to 300 and any score above 100 was considered high for that specific marker. The tissue sections were considered “high” for a particular marker if 2 out of 3 three scoring methods showed high expression. The scores of individual patients for each marker by different scoring methods are presented in **Additional file 3.** The assays and scoring were performed in a blinded manner.

Gene Expression Analysis Using Tcga Database

We downloaded *KEAP1, NRF2, TP53*, and NRF2-target genes expression data of HPV^{+ve} (n = 36) and HPV^{-ve} HNSCC (n = 243) patients from The Cancer Genome Atlas (TCGA) database (13) using the online resource cBioPortal for Cancer Genomics and performed the statistical analysis. We also correlated the expression levels of *NRF2*, *NQO1*, *HMOX1*, *KEAP1*, or *TP53* with overall survival rates among HPV^{+ve} HNSCC patients (data was available for n = 16).

General Methods

Method details related to HPV detection by PCR, quantitative real-time PCR, cell culture and transfection, measurement of NQO1 enzyme activity and total GSH levels, immunoblotting, cytotoxicity assay, and colony-forming assay are available as **Additional file 1**.

Statistical analysis

All the cell culture experiments were performed two or three times and the results were expressed as mean ± SEM. Differences in expression levels of genes between HPV^{+ve} and HPV^{-ve} groups were

analysed using unpaired t-tests with Welch's correction and Tukey's post hoc test was used to determine the significance. Kruskal-Wallis one-way ANOVA, followed by non-parametric Mann-Whitney U-test was used to analyze the data of transfection experiment with three groups. Pearson correlation coefficient test was used to correlate the histoscores of various markers. A non-linear regression curve fit was used to determine the IC_{50} values. Kaplan-Meier survival curves with log-rank statistics were used to compare the overall survival between the high expression and low expression groups. The “p” value of < 0.05 was considered significant unless otherwise stated. All the statistical analysis was performed using GraphPad Prism version 5.0 software for Windows.

Results

A prospective cohort of HNSCC patients and HPV detection

The primary tumor bio-specimen were obtained from HNSCC patients (n = 50), who underwent biopsy or surgical resection. The demographic profile and clinicopathological characteristics are provided in Table 1. Among the 50 HNSCC patients in the study, 16% (n = 8) had poorly differentiated tumors, 46% (n = 23) had moderately differentiated tumors and 38% (n = 19) had well-differentiated tumors. The pathological evaluation indicated 36% (n = 18) had stage I tumors, 40% (n = 20) had stage II tumors, 20% (n = 10) had stage III tumors and 4% (n = 2) had stage IV tumors of the head and neck. Except for alcohol consumption, there was no significant correlation between age, tobacco chewing, and HPV positivity in our cohort. All the tumors were analysed for HPV infection using PCR targeting the consensus L1 region of HPV and were grouped into HPV^{+ve} (n = 15, 30%) and HPV^{-ve} (n = 35, 70%) (**Supplementary Fig. 1A**). The HPV^{+ve} DNA was further analysed to identify the high-risk subtype of HPV and it was found that 12 subjects harboured HPV-16 DNA and the remaining 3 had HPV-18 DNA (**Supplementary Fig. 1B and 1C**). In addition to HPV DNA, overexpression of p16INK4a (henceforth referred to as p16) is considered as a marker of active high-risk HPV infection and viral oncoprotein activity (23, 24). Of the 50 HNSCC patients, we were able to collect 30 FFPE tissue blocks of the respective biopsy specimens. IHC analysis of p16 was performed to evaluate the concordance between HPV DNA and p16 expression. A total of 15 (50%) patients were positive for HPV by PCR testing whereas 17 patients (56.6%) were positive for HPV by p16 IHC with a discordance rate of < 10% (**Supplementary Table 2**). All patients who were HPV-PCR positive were also positive for p16 by IHC (**Supplementary Table 3** for individual patient data). Chi-square test of independence revealed a strong correlation in HPV positivity between PCR testing and p16 IHC (p = 0.000012). With this concordance, the p16 IHC positive tumor samples were considered as HPV^{+ve} in the further experiments.

Table 1: Demographic profile, risk factors and baseline clinico-pathological characteristics of the prospective cohort of HNSCC subjects (n=50) stratified based on HPV status

Baseline characteristics	Total (n=50)	HPV^{+ve} (n=15)	HPV^{-ve} (n=35)
Age (mean±SD)	57.2±11.1	56.2±12.7	57.3±10.5
Gender			
Male	39	11	28
Female	11	04	07
Primary tumor site			
Oropharynx	33	10	23
Esophagus	08	01	07
Others (Larynx, Hypopharynx, Pyriform fossa & Palate)	09	04	05
Smoker			
Yes	21	10	11
No	29	05	24
Tobacco chewing			
Yes	23	07	16
No	27	08	19
Alcohol			
Yes	23	09	14
No	27	06	21
Oral sex			
Yes	16	06	10
No	24	07	17
Not disclosed	10	02	08
Tumor grade			
1	19	05	14
2	23	08	15
3	08	02	06
Tumor stage			
I	22	06	16
II	20	06	14

III	07	03	04
IV	01	00	01

Prospective Analysis: Hpv Hnscc Exhibit Low Constitutive Nrf2 Activity Than Hpv Hnscc

To test our hypothesis, as an indicator of NRF2 activation, we analysed mRNA expression of *NRF2* and its downstream target genes, *NQO1, HMOX-1, GCLC*, and *GCLM* in HPV^{+ve} and HPV^{-ve} HNSCC tumors by qPCR analysis. We observed that *NRF2* and its target genes were significantly lower in HPV^{+ve} HNSCC tumors compared to HPV^{-ve} HNSCC tumors (Fig. 1A). Next, we correlated the expression of *NRF2* and its target genes in HNSCC tumors to p16 expression. We found that p16 positive ($p16^{high}$) HNSCC tumors were associated with significantly lower expression of *NRF2* gene and its target genes (*NQO1, HMOX-1, GCLC, GCLM*) as compared to p16 negative ($p16^{low}$) HNSCC tumors (**Supplementary Fig. 2**). Both PCR and the p16 IHC testing method for HPV positivity confirmed that HPV^{+ve} HNSCC is associated with low NRF2 activity as compared to HPV^{-ve} HNSCC tumors.

To further illustrate NRF2 activity status in HPV^{+ve} and HPV^{-ve} HNSCC tumors, we assessed the expression of NQO1 protein, a surrogate for NRF2 activation by IHC and correlated with p16 status (representative microscopy images are presented in Fig. 1B). The histoscores of NQO1 in $p16^{low}$ HNSCC tumors were significantly higher than in $p16^{high}$ HNSCC tumors (Fig. 1C). Further, the histoscores of NQO1 showed a strong negative correlation ($p = 0.0008$, $r^2 = -0.58$) with the histoscores of p16 (Fig. 1D). These results suggested that NRF2 activity is constitutively lower in HPV^{+ve} HNSCC tumors than HPV^{-ve} HNSCC tumors.

Next, we analysed the publically available TCGA dataset (13) to evaluate the status of the NRF2 pathway in HPV^{+ve} HNSCC (n = 36) and HPV^{-ve} HNSCC (n = 243). We found significantly lower expression of NRF2-target genes- *NQO1* ($p = 0.0001$), *GCLC* ($p = 0.0001$), and *HMOX1* ($p = 0.01$) in HPV^{+ve} HNSCC than HPV^{-ve} HNSCC (Fig. 1E). Although not significant, mRNA levels of *NRF2* ($p = 0.64$) and *GCLM* ($p = 0.25$) also showed a trend of lower expression in HPV^{+ve} HNSCC as compared to HPV^{-ve} HNSCC. The data from the TCGA database provide supporting evidence to our prospective cohort data that suggest HPV^{+ve} HNSCC tumors are associated with lower constitutive NRF2 activity than HPV^{-ve} HNSCC.

Retrospective Analysis Of P16, Nqo1, And P53 Reveals Lower Nrf2 Activity In Hpv Hnscc

To further validate our hypothesis, we retrospectively analysed HNSCC tumors by IHC analysis for NQO1 and p16 expression. We collected archived FFPE blocks from 121 HNSCC patients who had visited the medical centres for biopsy or surgery resection. Of the total 121 HNSCC patient FFPE blocks, 3 FFPE

blocks were excluded after re-evaluation by the in-house pathologist as they were not squamous cell carcinoma. The clinicopathological characteristics and HPV status of 118 patients are summarized in Table 2. HPV infection was evaluated by p16 IHC and 42 of 118 HNSCC patients (36%) were HPV positive. Next, HNSCC tumor tissue was analysed for NQO1 and p53 expression by IHC (representative microscopy images are presented in Fig. 2A) employing both manual and digital scoring. The agreement between manual and digital scoring was measured by Pearson's correlation (**Supplementary Fig. 3**) and the expression of p16, NQO1 and p53 was divided into high and low based on the histoscores. Representative microscopy images of IHC staining of p16, NQO1, and p53 with scores assigned as per the staining intensity is presented in **Supplementary Fig. 4**. The scores for individual patients are listed in **Additional file 3**.

Table 2: Demographic profile and baseline clinico-pathological characteristics of the retrospective cohort of HNSCC subjects (n=118) stratified based on HPV status

Baseline characteristics	**Total (n=118)**	**HPV^{+ve} (n=42)**	**HPV^{-ve} (n=76)**
Age (mean±SD)	58.6±11.2	59.3±11.07	58.2±11.2
Gender			
Male	78	28	50
Female	40	14	26
Primary tumor site			
Oropharynx	41	21	20
Esophagus	54	16	38
Others (Larynx, Hypopharynx, Pyriform fossa & Palate)	23	05	18
Tumor grade			
1	39	11	28
2	63	25	38
3	16	06	10
Tumor stage			
I	59	18	41
II	42	18	24
III	13	05	08
IV	04	01	03

In agreement with our prospective cohort findings, the retrospective analysis also revealed a significantly lower expression of NQO1 in HPV^{+ve} HNSCC tumors as compared to HVP^{-ve} HNSCC tumors (Fig. 2B) suggesting a low basal NRF2 activity in the former group. There was an inverse correlation between p16 and NQO1 expression in HNSCC tumors samples (Fig. 2D). We also observed few double-positive cases ($p16^{high}$/ $NQO1^{high}$; 07 cases) and double-negative cases ($p16^{low}$/ $NQO1^{low}$, 21 cases).

Next, we evaluated the NRF2 pathway status in HPV^{+ve} and HPV^{-ve} HNSCC in relation to p53 status. HPV E6 protein binds to p53 and directs it to ubiquitination and proteasomal degradation (25). Therefore, most of the HPV^{+ve} HNSCC tumors exhibit loss of wildtype p53 whereas HPV^{-ve} HNSCC patients are predominantly associated with loss of function p53 mutation (26). In our data, we observed a significantly lower expression of p53 protein in $p16^{high}$ HNSCC as compared to $p16^{low}$ HNSCC (Fig. 2C). Pearson correlation coefficient test showed an inverse correlation between p53 with p16 expression in HNSCC tumor samples (Fig. 2E). Next, we correlated the expression of p16, NQO1, and p53 proteins in HNSCC tumors. Nearly 75% of $p16^{high}$ HNSCC tumors (32/43) harboured low co-expression of NQO1 and p53, which further confirmed low constitutive activation of NRF2 pathway in HPV^{+ve} HNSCC. A positive correlation was observed between NQO1 and p53 protein expression in HNSCC cases (Fig. 2F). Based on the expression levels of NQO1, p53 and p16 in HPV^{+ve} HNSCC patients, we identified four distinct subgroups: $p16^{high}/NQO1^{low}/p53^{low}$ (n = 32/43), $p16^{high}/NQO1^{high}/p53^{low}$ (n = 04/43), $p16^{high}/NQO1^{low}/p53^{high}$ (n = 04/43), $p16^{high}/NQO1^{high}/p53^{high}$ (n = 02/43).

Ectopic expression of HPV E6/E7 markedly repressed the constitutive NRF2 activation in cancer cells and decreased total GSH levels.

The above findings from our prospective and retrospective cohort analysis as well as in silico analysis of TCGA database, evidently suggested that HPV^{+ve} HNSCC tumors are associated with lower constitutive NRF2 activation as compared to HPV^{-ve} HNSCC. However, whether HPV infection directly influences NRF2 signaling in cancer cells remained unclear. A growing body of evidence suggests that viral infection in epithelial and immune cells may cause activation or repression of NRF2 signaling (17, 18, 27–29). Thus, we sought to test a hypothesis that HPV infection represses NRF2 signaling in cancer cells. To test our hypothesis, we selected the non-small cell lung cancer cell line, A549 as it exhibits strikingly high constitutive activation of NRF2 due to loss of KEAP1 function (30). To mimic HPV infection, we ectopically expressed HPV-16 E6/E7 in A549 cells. The presence of HPV-16 E6/E7 was confirmed in the transfected cells by amplifying an 113bp segment using conventional PCR (**Supplementary Fig. 5A**) and qPCR analysis of *E6/E7* mRNA (**Supplementary Fig. 5B**) expression levels. As compared to mock control and plasmid control, the mRNA levels of *NRF2* and its downstream effector genes, *NQO1*, *GCMC*, *GCLC*, *HMOX-1*, were dramatically reduced in A549 cells transfected with HPV-16 E6/E7 plasmid (Fig. 3A). In concordance with gene expression levels, protein expression of NRF2, NQO1, and HMOX-1 in A549 cells transfected with HPV-16 E6/E7 plasmid was also significantly reduced as compared to mock or plasmid control transfected cells (Fig. 3C). To validate our hypothesis, we attempted to replicate the above findings in other cancer cell types and selected colorectal cancer cell line HT-29, which also harbours

aberrant constitutive NRF2 activation mainly caused by hypermethylation of the *KEAP1* gene (31). Similar to the findings from A549 cells experiments, the mRNA expression levels of *NRF2* and its downstream target genes were markedly decreased in HT-29 cells transfected with HPV-16 E6/E7 plasmid as compared to mock or plasmid control transfected cells (Fig. 3B). Similarly, the protein expression of NRF2, NQO1, and HMOX1 were also markedly reduced in HT-29 cells transfected with HPV-16 E6/E7 plasmid as compared to mock or plasmid control (Fig. 3C).

Next, we correlated the abundance of NQO1 protein with its functional activity by measuring NQO1 enzyme activity. In concordance with the NQO1 protein levels, NQO1 enzyme activity was also significantly reduced in cell lysates of both A549 and HT-29 cells transfected with HPV-16 E6/E7 as compared to mock and plasmid control (Fig. 3D). NRF2 maintains intracellular GSH levels by regulating de novo synthesis through the expression of *GCLC* and *GCLM* (7) and redox cycling by increasing glutathione reductase. We found that in both A549 and HT-29 cells, transfection with HPV-16 E6/E7 caused a marked reduction in total GSH levels as compared to mock or plasmid control transfected cells (Fig. 3E). Due to lack of access to HNSCC cell lines, we could not perform the above experiments in the context of HPV-associated HNSCC. However, the above findings emanating from two different cancer-type cell lines suggest that HPV-16 infection in cancer cells down-regulates NRF2 signaling and thereby reduces antioxidant defences including intracellular GSH levels.

HPV E6/E7 expression sensitizes cancer cells to chemotherapeutic drug

Given that ectopic expression of HPV E6/E7 significantly ablated NRF2 regulated antioxidant defences including GSH levels, we asked whether HPV E6/E7 expression sensitizes cancer cells to anticancer drug, cisplatin. Cisplatin treatment induced greater cytotoxicity in A549 cells transfected with HPV E6/E7 plasmid as compared to plasmid control or mock-transfected cells (Fig. 4A**)**. The IC_{50} dose of cisplatin was significantly lower in A549 cells transfected with HPV E6/E7 (8.3μM) as compared to A549 cells transfected with plasmid control (26μM) (Fig. 4B). The IC_{50} dose of cisplatin was similar between plasmid control and mock-transfected A549 cells.

Next, colony-formation assay revealed that HPV E6/E7 plasmid transfected cells treated with cisplatin formed fewer colonies compared to cisplatin-treated plasmid control or mock-transfected cells. In the vehicle-treated groups, the clonogenecity of A549 cells transfected with HPV E6/E7 was modestly low but not statistically significant when compared to cells transfected with plasmid control. The clonogenecity was comparable between the mock and plasmid control transfected groups (Fig. 4C and 4D). These results suggest that ectopic expression of HPV E6/E7 sensitized cancer cells to cisplatin toxicity and significantly reduced the cell proliferation which is because of the repression of NRF2 pathway by HPV E6/E7.

Increased constitutive expression of KEAP1 in HPV^{+ve} HNSCC tumors and in HPV E6/E7 transfected cancer cells

In normal and cancer cells, NRF2 signaling is predominantly influenced by the constitutive expression of KEAP1 and high expression of *KEAP1* in cancers is associated with low constitutive NRF2 activity (32, 33). To explore the potential mechanism underlying diminished NRF2 activity in HPV^{+ve} HNSCC tumors, we evaluated *KEAP1* gene expression levels in our prospective cohort and TCGA cohort. We found that the *KEAP1* mRNA expression is significantly higher in HPV^{+ve} HNSCC tumors compared to HPV^{-ve} HNSCC (Fig. 5A). We also evaluated the mRNA expression of *KEAP1* in HNSCC tumors in relation to p16 expression by IHC. The *KEAP1* mRNA expression was significantly higher in p16^{high} tumors compared p16^{low} tumors (Fig. 5A). In agreement with our study cohort findings, the TCGA dataset also revealed significantly higher mRNA expression of *KEAP1* in HPV^{+ve} HNSCC tumors compared to HPV^{-ve} HNSCC (Fig. 5B). These findings prompted us to explore if the ectopic expression of HPV E6/E7 influences KEAP1 expression in cancer cells. Ectopic expression of HPV E6/E7 significantly increased *KEAP1* mRNA in A549 cells and HT29 cells as compared to respective mock or plasmid control transfected cells (Fig. 5C). Immunoblot analysis also confirmed a significant increase in KEAP1 protein expression in HPV E6/E7 plasmid transfected cells compared to mock or plasmid transfected cells (Fig. 5D). Taken together, evidence from tumor samples and in-vitro experiments suggest that HPV infection might suppress NRF2 activity potentially by increasing KEAP1 expression.

Low Nrf2, Nqo1, And P53 Expression Correlates With Increased Overall Survival In Hpv Hnscc Patients

Lastly, we assessed whether *NRF2* and *NQO1* expression are prognostic in HPV^{+ve} HNSCC using TCGA database. A previous study has reported low NRF2 activity was associated with better overall survival in HPV^{-ve} HNSCC patients (11). Therefore, in this study, we correlated NRF2 activity and overall survival in HPV^{+ve} HNSCC patient cohort using the TCGA database. The data relating to overall survival was available only for 16 out of 36 patients with HPV^{+ve} HNSCC in the TCGA database. Although our analysis of the TCGA dataset showed no significant differences in the levels of *NRF2* expression between HPV^{+ve} HNSCC and HPV^{-ve} HNSCC (Fig. 1C), we first evaluated the relationship between *NRF2* and the disease-specific survival. Based on the median value of *NRF2* gene expression levels, we segregated the HPV^{+ve} HNSCC patients into top quartile and bottom quartile and correlated with overall survival. We observed that HPV^{+ve} HNSCC patients with low *NRF2* showed better overall survival as compared to HPV^{+ve} HNSCC patients with high *NRF2* expression (Fig. 6A). Next, we analysed the correlation between *NQO1* expression and overall survival among HPV^{+ve} HNSCC. In agreement with *NRF2* expression, low *NQO1* expression was associated with better survival compared to high *NQO1* expression among HPV^{+ve} HNSCC (Fig. 6B). We also examined if *KEAP1* expression influences overall survival among HPV^{+ve} HNSCC patients. HPV^{+ve} HNSCC patients with high *KEAP1* expression were associated with modestly better overall survival compared to HPV^{+ve} HNSCC patients with low *KEAP1* (Fig. 6C). As reported in published literature (34), we also observed that low *TP53* expression among HPV^{+ve} HNSCC was associated with better overall survival as compared to high *TP53* expression (Fig. 6D). These lines of

evidence suggest that the co-expression pattern of $p16^{high}$, $NQO1^{low}$, and $p53^{low}$ can potentially have clinical implications in stratifying HNSCC patients into poor and better responders to therapy (Fig. 6E).

Discussion

Despite overwhelming evidence supporting better therapeutic responses in patients with HPV^{+ve} HNSCC, the underlying mechanisms remain elusive. Our study addressed this knowledge gap. In our prospective analysis of tumor samples, constitutive NRF2 activity as indicated by the mRNA expression of *NRF2* and its transcriptional targets in HPV^{+ve} HNSCC tumors was significantly reduced compared to HPV^{-ve} HNSCC tumors. Analysis of the TCGA database further confirmed our observation. The data indicated that in over 95% of HPV^{+ve} HNSCC tumors, the expression levels of *NQO1*, *GCLC*, and *GCLM* genes were below the mean of the respective gene in HPV^{-ve} HNSCC tumors. In our retrospective analysis, the NQO1 protein expression was markedly reduced in $p16^{high}$ tumors compared to $p16^{low}$ tumors, which agreed with our prospective cohort data. Subset analysis across different cancer types have revealed that patients with high constitutive NRF2 activity were associated with poor treatment responses and prognosis compared to patients with low constitutive NRF2 activity (8) of the same cancer type. Our findings suggest that low constitutive NRF2 activity may be the crucial mechanism mediating better treatment responses and prognosis in HPV^{+ve} HNSCC patients compared to patients with HPV^{-ve} HNSCC. This notion was further supported by our data from *in vitro* studies. Ectopic expression of HPV E6/E7 in A549 and HT-29 cells dramatically repressed constitutive NRF2 activation as indicated by diminished expression of *NRF2* and its target genes, low NQO1 enzyme activity, and low total GSH levels compared to cells transfected with plasmid control. Reduced NRF2 activity in HPV E6/E7 transfected A549 and HT-29 cells enhanced the sensitivity to cisplatin-induced cytotoxic effects. We also observed in the TCGA database that HPV^{+ve} HNSCC patients with 'low *NRF2*' or 'low *NQO1*' expression showed better overall survival as compared to HPV^{+ve} patients with 'high *NRF2*' and 'high *NQO1*' expression.

Inferior clinical outcomes of large phase III de-escalation trials (De-ESCALaTE trial (35) and RTOG 0129 trial (36)) have underscored the need of molecular markers, besides HPV status and smoking history for patient selection, who would benefit most from standard or de-escalated treatment modality. Clinical and pre-clinical evidence have demonstrated that basal NRF2 activity in cancers inversely correlates with sensitivity to chemotherapy and radiotherapy (37). Three lines of evidence from our study suggest that constitutive NRF2 activity status could aid in the segregation of HPV^{+ve} HNSCC patients into good responder vs. poor responder for treatment de-escalation trials. First, the TCGA database revealed that HPV^{+ve} HNSCC patients with low *NQO1* and low *NRF2* were associated with a better 3 year overall survival rate compared to HPV^{+ve} HNSCC patients with high *NQO1(*HR = 0.11, 95% C.I. = 0.01, 0.7) and high *NRF2* HR = 0.08, 95% C.I. = 0.01, 0.6). As expected, p53 levels inversely correlated with favourable outcomes among HPV^{+ve} HNSCC patients (HR = 0.21, 95% C.I. = 0.05, 0.9). Second, IHC analysis of FFPE specimens from the retrospective cohort revealed that 74% (32/43) of HPV^{+ve} HNSCC patients were $p16^{high}/NQO1^{low}/p53^{low}$. We observed a strong correlation between NQO1 and p53 expression in $p16^{high}$

HNSCC. Third, 100% of HPV^{+ve} HNSCC patients in our prospective cohort and 88.8% of HPV^{+ve} HNSCC patients in the TCGA cohort showed *NQO1* levels below the median *NQO1* in HPV^{-ve} HNSCC patients. Based on this evidence, we propose IHC panel of three molecular biomarkers: $p16^{high}/NQO1^{low}/p53^{low}$ for predicting better prognosis among HPV^{+ve} HNSCC and to aid in the selection of HPV^{+ve} HNSCC patients for de-escalation clinical trials.

Although HPV^{+ve} HNSCC tumors were associated with lower constitutive activation of NRF2 compared to HPV^{-ve} HNSCC, it does not inform whether HPV infection per se repressed NRF2 activity. Several pathogenic viruses are reported to modulate NRF2 signaling in the host cells for their advantage (38). Compared to wild-type cells, *NRF2*-deficient cells were associated with greater viral burden following infection with Herpes simplex virus 1, Zika virus, vaccinia virus, respiratory syncytial virus, influenza virus, rotavirus, HIV, Enterovirus 71, and severe acute respiratory syndrome coronavirus-2 suggesting that repression of NRF2 signaling is advantageous for supporting viral life cycle (27, 39). In general, host cells activate NRF2 as an adaptive response to acute oxidative stress elicited by viral infections (40). However, few viruses directly regulate NRF2 activation by modifying KEAP1 protein or altering *KEAP1* gene expression. For example, Marburg viral protein VP24 binds to KEAP1 protein and disrupts NRF2-KEAP1 interaction resulting in NRF2 activation (41). In contrast, Enterovirus 71 infection in Vero cells repressed NRF2 activation by increasing KEAP1 expression, which was essential for its replication and propagation (18). Our transfection experiments using HPV E6/E7 indicated that HPV infection represses constitutive NRF2 activity. The question whether repression of NRF2 activity favour progression of HPV infection remains to be elucidated. Indirect evidence suggests that repression of NRF2 activity may favour HPV replication, HPV integration, and HPV-induced cellular transformation by sustaining oxidative stress milieu in the host cells by depleting cellular antioxidants such as GSH. Depletion of GSH by L-buthionine-sulfoximine increased the rate of HPV integration in the host genome whereas exogenous antioxidants such as resveratrol and vitamin E suppressed HPV integration into the host genome (42).

Although the underlying mechanism of how HPV E6/E7 repress NRF2 activity needs further investigation, our initial observations indicate that HPV infection may repress NRF2 activation by increasing KEAP1 expression. Data from prospective cohort analysis and TCGA dataset revealed that HPV^{+ve} tumors were associated with significantly greater expression of *KEAP1* than HPV^{-ve} tumors. Our in vitro studies validated that ectopic expression of HPV-E6/E7 plasmid in A549 cells and HT29 cells significantly increased the mRNA and protein expression of KEAP1 as compared to mock or control plasmid. Increased KEAP1 expression corresponded with decreased NRF2-transcriptional activity in HPV-E6/E7 transfected A549 or HT29 cells as compared to mock or control plasmid. Previous studies have demonstrated that elevated expression of KEAP1 inversely correlates with NRF2 expression and NRF2-transcriptional activity in cancers (43, 44).

In conclusion, our study demonstrated that HPV^{+ve} HNSCC cancers are associated with lower constitutive NRF2 activity compared to HPV^{-ve} HNSCC cancers and amongst HPV^{+ve} HNSCC patients who were associated with lower NRF2 activity showed better prognosis and overall survival. Our in vitro studies

using HPV-E6/E7 substantiate that HPV infection represses constitutive NRF2 signaling in cancer cells and enhanced sensitivity to anticancer drugs. These findings led us to propose a panel of predictive IHC markers, $p16^{high}/NQO1^{low}/p53^{low}$, which can be of immediate clinical relevance for patients' stratification in ongoing and future de-escalation trials.

Abbreviations

FFPE: Formalin Fixed Paraffin-Embedded; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; GCLC: Glutamate cysteine ligase catalytic subunit; GCLM: Glutamate-cysteine ligase modifier subunit; GSH: Glutathione; HMOX1: Heme oxygenase 1; HNSCC: Head and Neck squamous cell carcinoma; HPV: Human Papillomavirus; IHC: Immunohistochemistry; KEAP1: Kelch-like ECH-associated protein 1; NQO1: NAD(P)H quinone dehydrogenase 1; NRF2: Nuclear erythroid 2 Related Factor 2 ; PCR: Polymerase Chain Reaction; ROS: Reactive Oxygen Species; TCGA: The Cancer Genome Atlas

Declarations

Ethics approval and consent to participate

The study was conducted in accordance with the declaration of Helsinki and the protocol was approved by the Institutional Ethics Committee of JSS Medical College, Mysore (JSSMC/IEC/25/1991/2017-2018). A signed informed consent was collected from all the participants.

Consent for publication

Not applicable

Availability of data and materials

The data generated in this study are available within the article and its supplementary data files. Expression profile and survival data analysed in this study were obtained from publicly available online resource The Cancer Genome Atlas (TCGA) Head & Neck cohort by using the cBioPortal for Cancer Genomics (http://www.cbioportal.org/).

Competing interests

The authors declare that they have no competing interests.

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None

Authors' contributions

P.S. Ramesh, D. Devegowda, and R.K. Thimmulappa **conceptualized and designed the study**. P.S. Ramesh, V.R. Bovilla, and V.H. Swamy **performed the experiments**. N.N. Manoli, S.M. Siddegowda, S. Chandrashekarappa, V.M. Somasundara, and K.B. Dasegowda **acquired the patient data and managed the patients**. P.S. Ramesh, N.N. Manoli, R.K. Thimmulappa **analysed and interpreted the data**. P.S. Ramesh, D. Devegowda, R.K. Thimmulappa **wrote the original draft and edited the manuscript**. **All the authors read and approved the final manuscript**.

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Figures

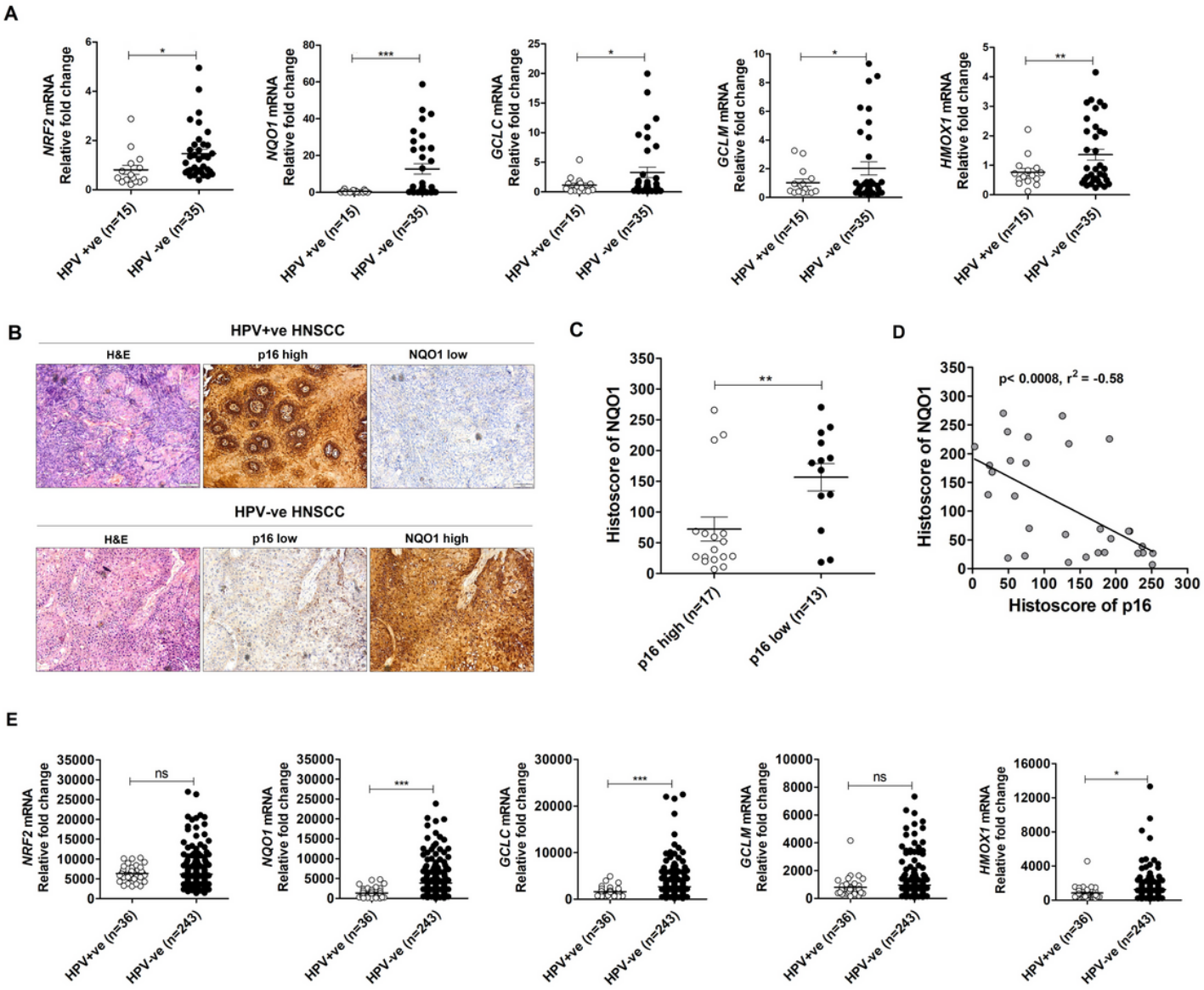

Figure 1

HPV^{+ve} HNSCC are associated with lower expression of NRF2 and its target antioxidant genes as compared to HPV^{-ve} HNSCC. A. Quantification of mRNA levels of *NRF2* and its target genes, *NQO1*, *GCLC*, *GCLM* and *HMOX1* in HPV^{+ve} (n=15) and HPV^{-ve} (n=35) HNSCC tumor biopsy samples. Observed values were normalized to internal control (mean of *β-ACTIN* and *GAPDH*). **B.** Representative microscopy images (20x) showing expression of p16 and NQO1 in HPV^{+ve} HNSCC and HPV^{-ve} HNSCC. **C.** Comparison of NQO1 histoscores between p16^{high} tumors and p16^{low} tumors. **D.** Pearson correlation coefficient for the expression of NQO1 and p16 in HNSCC tumors. **E.** Comparison of mRNA levels of *NRF2* and its target genes between HPV^{+ve} (n=36) HNSCC and HPV^{-ve} (n=243) HNSCC from TCGA database. All values were statistically significant at a minimum of P<0.05 as determined by unpaired t test with Welch's correction.

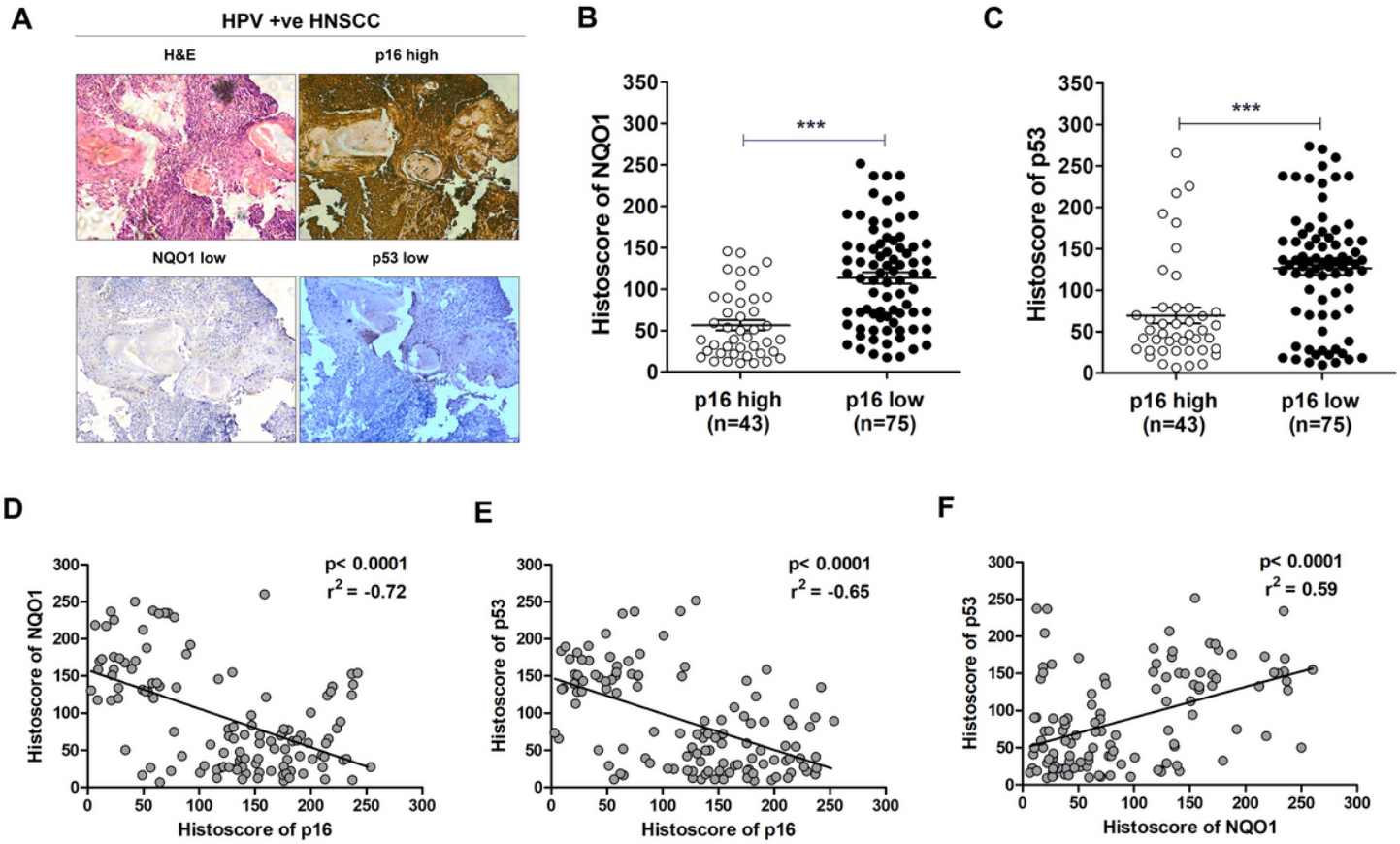

Figure 2

p16^{high} HNSCC harbours low co-expression of NQO1 and p53. A. representative microscopy images of p16, NQO1 and p53 IHC staining in HPV^{+ve} HNSCC tumors. Sections from the same tissue were probed with p16, NQO1 and p53 antibodies and stained with DAB chromogen. **B.** Comparison of NQO1 histoscores between p16^{high} and p16^{low} HNSCC. **C.** Comparison of p53 histoscores between p16^{high} and p16^{low} HNSCC. (P< 0.05 was considered statistically significant as determined by unpaired t test with Welch's correction). **D and E.** Correlation of NQO1 and p53 histoscores with p16 in HNSCC using the Pearson correlation coefficient test. **F.** Correlation of NQO1 and p53 in HNSCC tumors using the Pearson correlation coefficient test. Linear regression graphs with 95% confidence band set as best fit line and regression range set automatically using Graph Pad Prism 5.0 software.

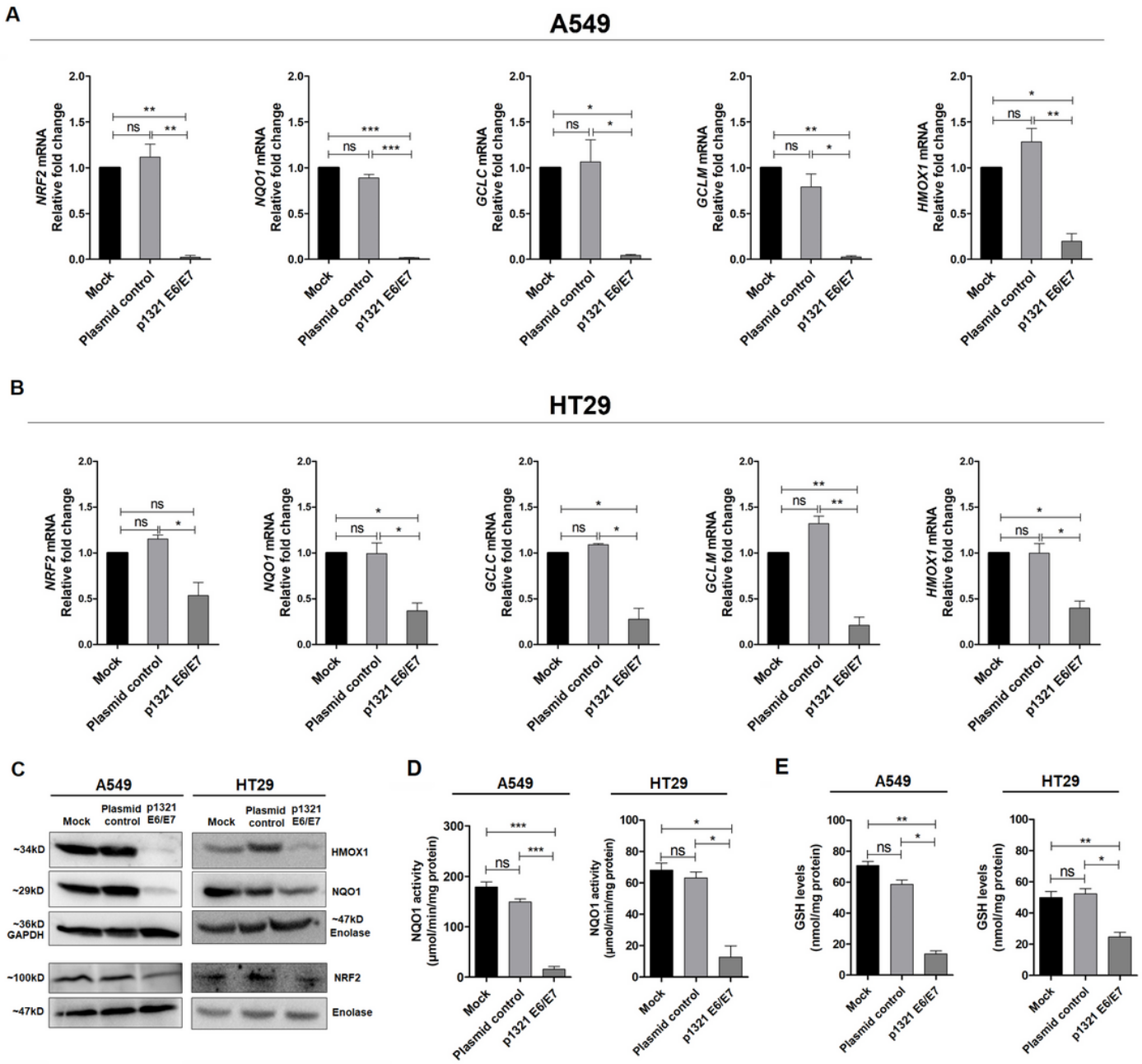

Figure 3

Ectopic expression of HPV E6/E7 markedly repressed the constitutive NRF2 activation in cancer cells and decreased total GSH levels. Analysis of mRNA levels of *NRF2 and* its target genes (*NQO1*, *GCLC*, *GCLM* and *HMOX1*) in A549 **(A)** cells and HT29 cells **(B)** after transfection with p1321 HPV-16 E6/E7 plasmid or control plasmid. The data is mean±SEM of three independent experiments (***P<0.0001, **P<0.001, *P<0.01, ns= not significant, as determined by One-way ANOVA with Tukey's multiple comparison test). **C.** Immunoblot analysis of NRF2, NQO1, and HMOX1 protein in A549 and HT29 cells transfected with HPV

E6/E7 plasmid in comparison to control plasmid or mock (control). GAPDH and or Enolase were used as loading control as indicated. **D.** NQO1 enzyme activity in A549 and HT29 cells transfected with HPV E6/E7 plasmid, plasmid control or mock. The data is mean±SEM of three independent experiments (***P<0.0001, *P<0.01, and ns= not significant, as determined by One-way ANOVA with Tukey's multiple comparison test) **E.** Total GSH levels in A549 and HT29 cells transfected with HPV E6/E7 plasmid in comparison to plasmid control or in mock. The data is mean±SEM of two independent experiments (**P<0.01, *P<0.01, and ns= not significant, as determined by One-way ANOVA with Tukey's multiple comparison test).

Figure 4

Ectopic expression of HPV E6/E7 oncogene sensitizes A549 cells to cisplatin toxicity. A. Dose-response curves of A549 cell line upon cisplatin treatment (0-200μM) and or in combination with plasmid control or p1321 HPV E6/E7 for 48hr time point as determined by the Sulforhodamine B (SRB) assay. **B.** IC_{50} values of cisplatin in A549 cells transfected with plasmid control or p1321 E6/E7 plasmid as indicated. **C.** Colony forming assay showing representative images of A549 cells treated with either plasmid control or p1321 E6/E7 or cisplatin alone and in combinations as indicated. **D.** Quantification of the colonies as performed by ImageJ software. The data are the mean ± SEM of three independent experiments performed in triplicate. P< 0.05 was considered significant as determined by one way ANOVA with Tukey's multiple comparison test.

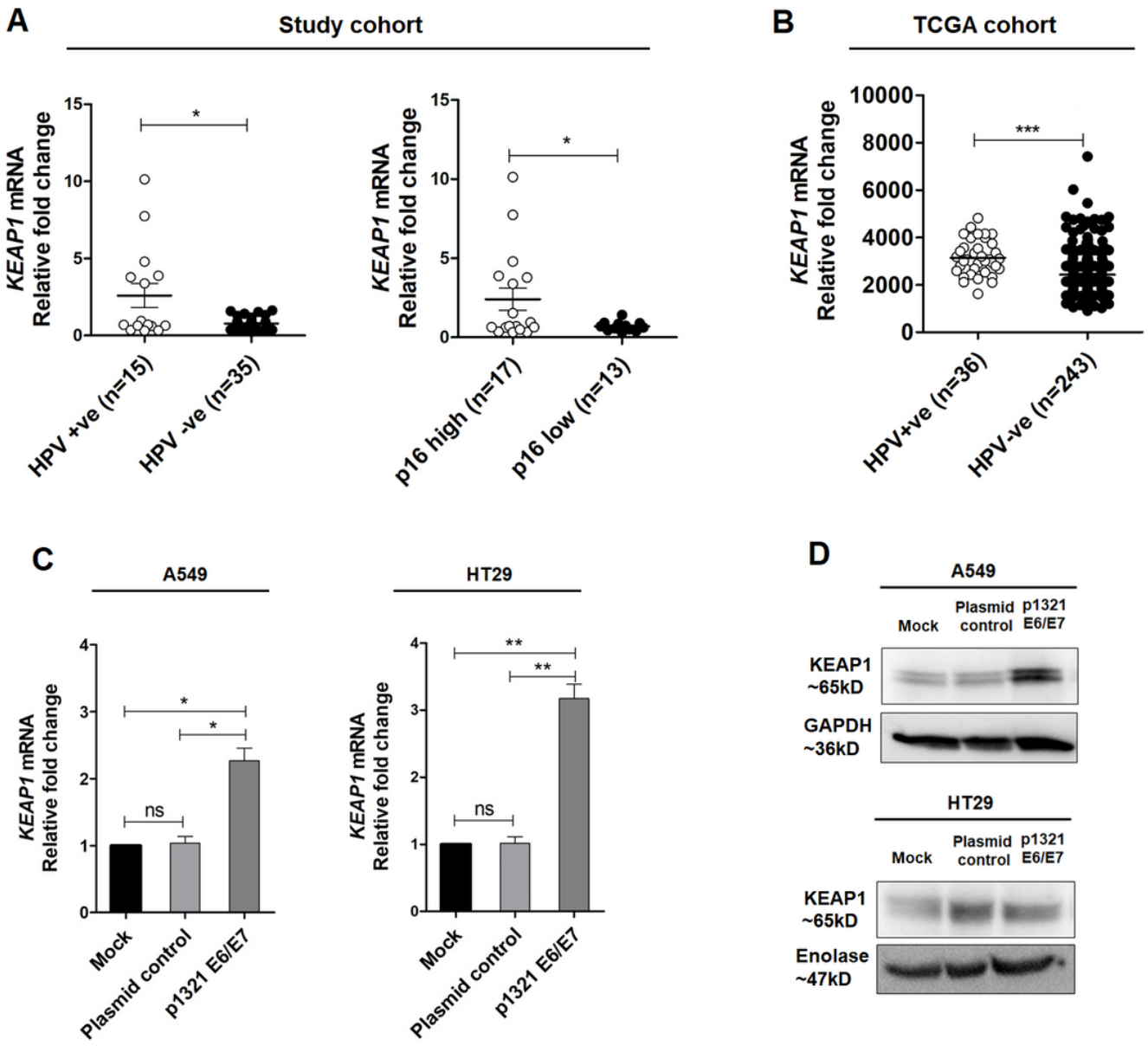

Figure 5

High expression of KEAP1 in HPV^{+ve} HNSCC patients and in HPV E6/E7 plasmid transfected cancer cells. **A.** Quantification of mRNA levels of *KEAP1* in HPV^{+ve} and p16^{+ve} HNSCC tumors from our study cohort. Observed values were normalized to internal control (mean of *β-ACTIN* and *GAPDH*). **B.** Comparison of mRNA levels of *KEAP1* gene between HPV^{+ve} and HPV^{-ve} HNSCC patients from TCGA database. P< 0.05 was considered statistically significant as determined by unpaired t test with Welch's correction. Ectopic expression of HPV E6/E7 significantly increased mRNA (**C**) and **(D)** protein expression of KEAP1 in A549 and HT29 cells as compared to respective mock or plasmid control transfected cells. The experiments were performed two times and error bars indicate the standard errors of the means (**P<0.01, ns= not significant as determined by one-way ANOVA with Tukey's multiple comparison test).

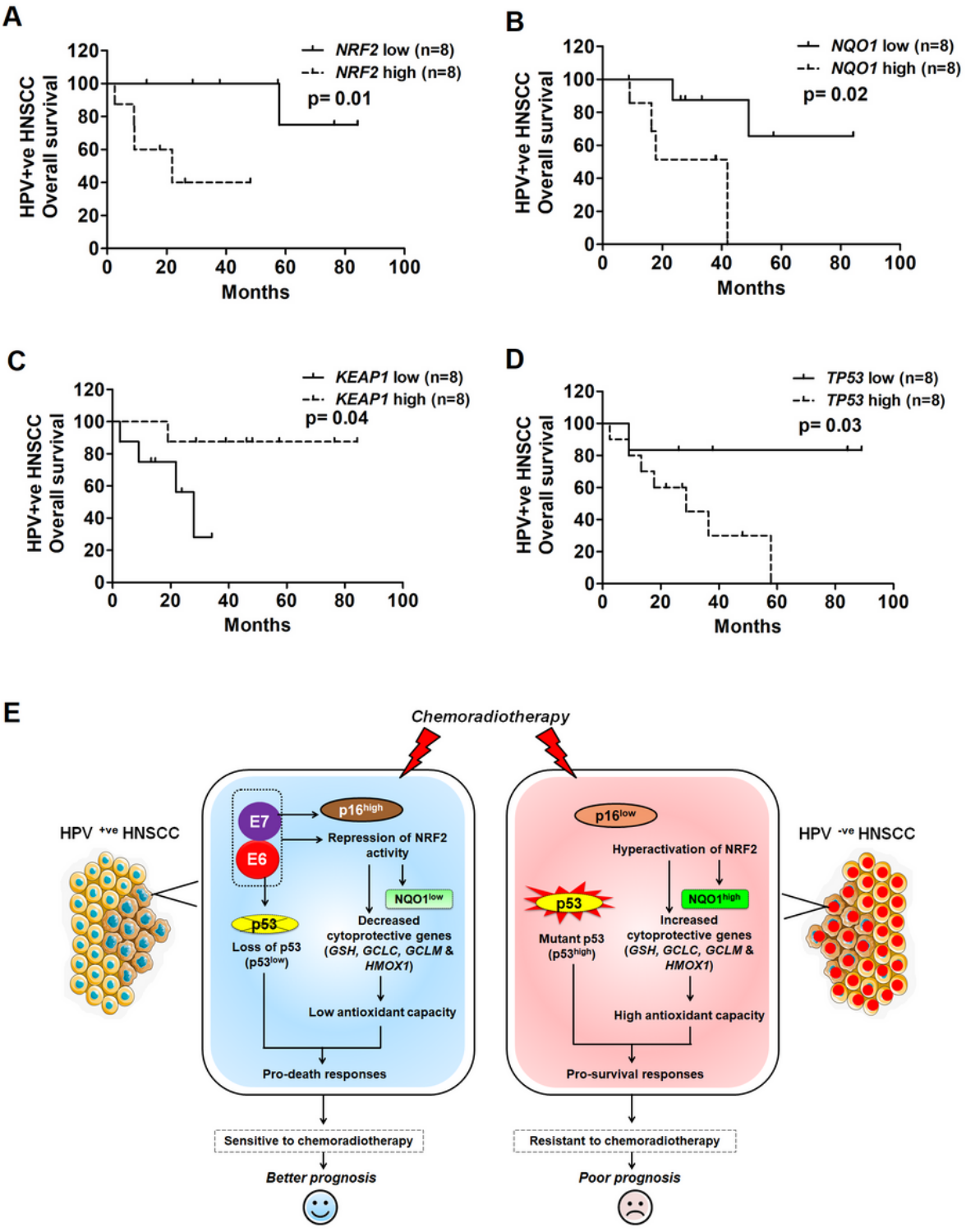

Figure 6

Low *NRF2*, *NQO1* and *TP53* expression correlates with increased overall survival in HPV^{+ve} HNSCC patients. Overall survival rate associated with *NRF2* **(A)**, *NQO1* **(B)**, *KEAP1* **(C)** and *TP53* **(D)** expression in HPV^{+ve} HNSCC patients from the TCGA network using the cBioPortal for cancer genomics database.

Patient cohorts were divided into “High” and “Low” gene expression groups based on median gene expression. The raw data from this analysis was plotted as a Kaplan-Meier overall survival plot in GraphPad Prism and statistical significance was calculated using the log-rank test with indicated p value. **E.** Schematics illustrating differences in HPV^{+ve} and HPV^{-ve} HNSCC cells. In HPV^{+ve} HNSCC, expression of HPV-E7 leads to increased p16 ($p16^{high}$) and HPV-E6 leads to proteosomal degradation of p53 ($p53^{low}$). Ectopic expression of HPV-E6/E7 also contributes to repression of NRF2 activity which further reduces NQO1 ($NQO1^{low}$), and other cytoprotective genes. The combined result is activation of pro-death responses making the cancer cells more sensitive to chemoradiotherapy leading to better prognosis of the patients. On the other hand, in HPV^{-ve} HNSCC, there is decreased p16 expression ($p16^{low}$). Also, mutated p53 ($p53^{high}$) and hyperactivation of NRF2 pathway ($NQO1^{high}$, and increase in other cytoprotective genes) increases pro-survival responses which confers cancer cells with resistance to chemoradiotherapy leading to poorer prognosis of the patients.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1.docx
- Additionalfile2.docx
- Additionalfile3.xlsx