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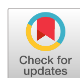

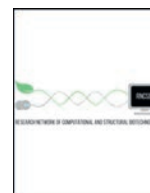

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Correlate the *TP53* Mutation and the *HRAS* Mutation with Immune Signatures in Head and Neck Squamous Cell Cancer

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ABSTRACT

Although immunotherapy has emerged as an effective therapeutic strategy for various cancers including head and neck squamous cell carcinomas (HNSCCs), only a subset of patients can benefit from such therapy. Hence, it is pressing to discover predictive biomarkers for cancer immunotherapy response. *TP53* and *HRAS* mutations frequently occur in HNSCC and correlate with a worse prognosis in HNSCC. We extensively characterized the associations of *TP53* mutations and *HRAS* mutations with HNSCC immunity based on multiple cancer genomics datasets. We compared the enrichment levels of 20 immune signatures between *TP53*-mutated and *TP53*-wildtype HNSCCs, and between *HRAS*-mutated and *HRAS*-wildtype HNSCCs, and found that *TP53* mutations were associated with depressed immune signatures while *HRAS* mutations were associated with enhanced immune signatures in HNSCC. Moreover, we found multiple p53- and RAS-mediated pathways showing significant correlations with HNSCC immunity. Furthermore, we demonstrated that the association between *TP53* mutation and tumor immunity was independent of the human papillomavirus (HPV) infection and smoking status in HNSCC. These data suggest that p53 and RAS may play important roles in regulating HNSCC immunity and that the *TP53* and *HRAS* mutation status could be useful biomarkers for stratifying HNSCC patients responsive to immunotherapy.

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1. Introduction

Head and neck cancer is the fifth most common cancer worldwide, most of which are squamous cell carcinomas (HNSCCs) [1]. HNSCC has a poor prognosis once the disease is not amenable to surgery, relapses or becomes metastatic [2]. Recently, cancer immunotherapy has achieved rapid clinical successes in treating multiple cancers including HNSCC [3]. Thus, the immunotherapy could be a promising treatment option for the HNSCC patients who have failed to surgery, radiation or chemotherapy. Unfortunately, thus far only approximately 20% cancer patients can benefit from immunotherapy such as immune checkpoint blockade [4]. A series of studies have focused on identifying molecular features that are associated with cancer immunotherapy response, such as tumor mutation burden (TMB), deficient mismatch-repair (dMMR), neoantigens, and PD-L1 expression in tumor cells [5–10]. A few studies have explored the associations of gene mutations with cancer immunotherapy response, e.g., the associations of *TP53* and *KRAS* mutations with immunotherapy response in lung cancer [11].

TP53 mutations frequently occur in cancer and are associated with poor prognosis in a wide variety of cancers [12]. In particular, *TP53* is the most frequently mutated gene in HNSCC and *TP53*-mutated HNSCCs have a worse overall survival (OS) prognosis than *TP53*-wildtype HNSCCs [13]. Myriad studies have shown that p53 plays an important role in tumor suppression via promoting cell cycle arrest and apoptosis [14]. A few studies have associated p53 with tumor immune regulation [15–18]. For example, p53 played a role in antitumor immunosurveillance by regulating *VISTA* [15]. p53 activation could enhance antitumor immunity [18]. The oncogenes of RAS family (*KRAS*, *HRAS*, and *NRAS*) are frequently mutated in various cancers and are associated unfavorable clinical outcomes in cancer [19]. Several studies have shown that the RAS signaling could promote tumor immunosuppression [20–22].

Abbreviations: APC, Antigen-Presenting Cell; BH, Benjami and Hochberg; DFS, Disease Free Survival; dMMR, Deficient Mismatch-Repair; EMT, Epithelial-Mesenchymal Transition; FDR, False Discovery Rate; GSEA, Gene-Set Enrichment Analysis; HLA, Human Leukocyte Antigen; HNSCC, Head and Neck Squamous Cell Carcinomas; HPV, Human Papilloma Virus; MHC, Major Histocompatibility Complex; NK, Natural Killer; OR, Odds Ratio; OS, Overall Survival; pDCs, Plasmacytoid Dendritic Cells; ssGSEA, single-sample Gene-Set Enrichment Analysis; TILs, Tumor-Infiltrating Lymphocytes; TIM, Tumor Immune Microenvironment; TMB, Tumor Mutation Burden.

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Since deregulation of the p53 and RAS pathways are significantly associated with tumor development and immune evasion, a comprehensive investigation of their associations with tumor immunity in HNSCC is worthwhile considering that such exploration remains lacking. To this end, we explored the associations of *TP53* mutations and *RAS* mutations with HNSCC immunity. We compared the enrichment levels of 20 immune signatures between *TP53*-mutated and *TP53*-wildtype HNSCCs, and between *HRAS*-mutated and *HRAS*-wildtype HNSCCs (we did not analyze *KRAS* and *NRAS* since both genes are rarely mutated in HNSCC) using several HNSCC multi-omics datasets [13,23]. Furthermore, we explored the phenotype and molecular features that were associated with the differential immune signatures between *TP53* (or *HRAS*) mutated and wildtype HNSCCs. This study aimed to identify biomarkers potentially effective for predicting responses to HNSCC immunotherapy.

2. Results

2.1. TP53 and HRAS Mutations are Associated with Depressed and Elevated Immune Signatures in HNSCC, Respectively

We found that 18 out of the 20 immune signatures analyzed showed significantly lower enrichment levels in *TP53*-mutated HNSCCs than in *TP53*-wildtype HNSCCs (Mann-Whitney *U* test, $P < 0.05$) (Fig. 1A; Supplementary Table S1). Moreover, *TP53*-mutated HNSCCs had significantly lower immune scores than *TP53*-wildtype HNSCCs (Mann-Whitney *U* test, $P = 4.25 \times 10^{-7}$) (Fig. 1B). In contrast, 18 of the 20 immune signatures showed significantly higher enrichment levels in *HRAS*-mutated HNSCCs than in *HRAS*-wildtype HNSCCs (Mann-Whitney *U* test, $P < 0.05$) (Supplementary Table S2), and *HRAS*-mutated HNSCCs had significantly higher immune scores (Mann-Whitney *U* test, $P = 0.005$) (Fig. 1C). Altogether, these data indicated that *TP53* mutations correlated with depressed tumor immunity, while *HRAS* mutations correlated with elevated tumor immunity in HNSCC.

Moreover, numerous marker genes of immune signatures showed decreased expression levels in *TP53*-mutated HNSCCs versus *TP53*-wildtype HNSCCs (Supplementary Tables S3–S7). For example, 80% of the B cell markers, the CD8+ T cell marker (*CD8A*), all NK cell markers, both cytolytic activity markers (*GZMA* and *PRF1*), and 104 (87%) of the 120 tumor-infiltrating lymphocytes (TILs) markers showed reduced expression in *TP53*-mutated HNSCCs relative to *TP53*-wildtype HNSCCs. In contrast, a considerable number of immune signature marker genes showed markedly increased expression in *HRAS*-mutated HNSCCs compared to *HRAS*-wildtype HNSCCs (Supplementary Tables S3–S7). For example, the CD8+ T cell marker and both cytolytic activity markers were more highly expressed in *HRAS*-mutated HNSCCs than in *HRAS*-wildtype HNSCCs. Of the 15 pro-inflammatory genes (*CD8B*, *TBX21*, *CD19*, *IFNG*, *IRF1*, *GZMB*, *IL12B*, *PRF1*, *IL12A*, *CXCL10*, *CXCL9*, *CXCL13*, *CCL5*, *GNLY* and *STAT1*) [24], 14 (except *STAT1*) showed significantly lower expression levels in *TP53*-mutated HNSCCs than in *TP53*-wildtype HNSCCs (Student's *t*-Test, false discovery rate: FDR < 0.05) (Supplementary Table S7). In contrast, 10 of the 15 pro-inflammatory genes showed significantly higher expression levels in *HRAS*-mutated HNSCCs than in *HRAS*-wildtype HNSCCs (Supplementary Table S7). Notably, *GZMB* (granzyme B) and *PRF1* (perforin 1) whose products are mainly secreted by NK cells and cytotoxic T lymphocytes [25], were downregulated in *TP53*-mutated HNSCCs versus *TP53*-wildtype HNSCCs, while were upregulated in *HRAS*-mutated HNSCCs versus *HRAS*-wildtype

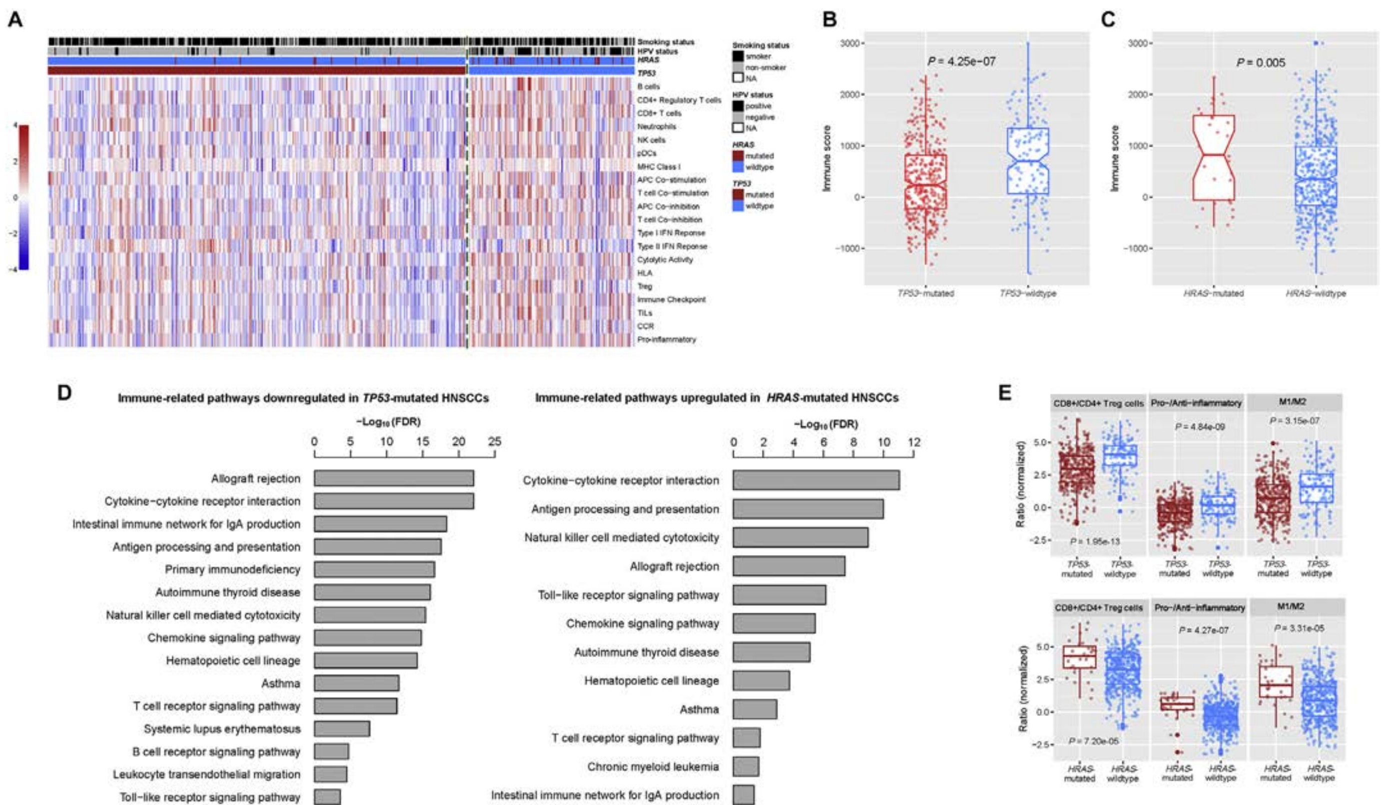

Fig. 1. *TP53*-mutated HNSCCs have reduced immune activity compared to *TP53*-wildtype HNSCCs while *HRAS*-mutated HNSCCs have increased immune activity compared to *HRAS*-wildtype HNSCCs. A. Heatmap showing the enrichment levels (ssGSEA scores) of 20 immune signatures in *TP53*-mutated HNSCCs versus *TP53*-wildtype HNSCCs. ssGSEA: single-sample gene-set enrichment analysis [49,50]. B. The immune infiltration levels (immune scores evaluated by ESTIMATE [53]) are significantly lower in *TP53*-mutated HNSCCs than in *TP53*-wildtype HNSCCs (Mann-Whitney *U* test, $P < 0.01$). C. The immune infiltration levels are significantly higher in *HRAS*-mutated HNSCCs than in *HRAS*-wildtype HNSCCs. D. GSEA [26] identifies numerous immune-related KEGG [52] pathways downregulated in *TP53*-mutated HNSCCs versus *TP53*-wildtype HNSCCs and immune-related pathways upregulated in *HRAS*-mutated HNSCCs versus *HRAS*-wildtype HNSCCs. E. The rations between immune-stimulatory signatures and immune-inhibitory signatures are significantly lower in *TP53*-mutated HNSCCs than in *TP53*-wildtype HNSCCs while significantly higher in *HRAS*-mutated HNSCCs than in *HRAS*-wildtype HNSCCs (Mann-Whitney *U* test *P*-values are shown). M1: M1 macrophages. M2: M2 macrophages.

HNSCCs. Overall, these findings suggest that *TP53* mutations may inhibit inflammatory and immune activity while *HRAS* mutations may promote them in HNSCC.

Furthermore, GSEA [26] analysis revealed that numerous immune-related pathways were significantly downregulated in *TP53*-mutated HNSCCs compared to *TP53*-wildtype HNSCCs. These pathways included antigen processing and presentation, allograft rejection, asthma, autoimmune thyroid disease, B cell receptor signaling, chemokine signaling, cytokine-cytokine receptor interaction, hematopoietic cell lineage, intestinal immune network for IgA production, leukocyte transendothelial migration, natural killer cell mediated cytotoxicity, primary immunodeficiency, systemic lupus erythematosus, T cell receptor signaling, and toll-like receptor signaling (Fig. 1D). In contrast, numerous immune-related pathways were significantly upregulated in *HRAS*-mutated HNSCCs compared to *HRAS*-wildtype HNSCCs, including cytokine-cytokine receptor interaction, antigen processing and presentation, natural killer cell mediated cytotoxicity, allograft rejection, toll-like receptor signaling, chemokine signaling, autoimmune thyroid disease, hematopoietic cell lineage, asthma, T cell receptor signaling, chronic myeloid leukemia, and intestinal immune network for IgA production (Fig. 1D). These results again demonstrate that *TP53* mutations are associated with depressed immune signatures while *HRAS* mutations are associated with enhanced immune signatures in HNSCC.

Interestingly, we observed that the ratio between immune-stimulatory cells (CD8+ T cells with marker gene *CD8A*) and immune-inhibitory cells (CD4+ regulatory T cells with marker genes *C15orf53, CTLA4, FOXP3, GPR15, IL32, IL4,* and *IL5*) was significant reduced in *TP53*-mutated HNSCCs versus *TP53*-wildtype HNSCCs (Mann-Whitney *U* test, $P = 1.95 \times 10^{-13}$) (Fig. 1E). A significant decrease in the ratio between pro-inflammatory cytokines (marker genes *IFNG*, *IL-1A*, *IL-1B*, and *IL-2*) and anti-inflammatory cytokines (*IL-4*, *IL-10*, *IL-11*, and *TGFB1*) was also observed in *TP53*-mutated HNSCCs versus *TP53*-wildtype HNSCCs (Mann-Whitney *U* test, $P = 4.84 \times 10^{-9}$) (Fig. 1E). The ratio between immune-inciting M1 macrophages (*CD64*, *IDO*, *SOCS1*, and *CXCL10*) and immune-inhibiting M2 macrophages (*MRC1*, *TGM2*, *CD23*, and *CCL22*) was significantly reduced in *TP53*-mutated HNSCCs versus *TP53*-wildtype HNSCCs (Mann-Whitney *U* test, $P = 3.15 \times 10^{-7}$) (Fig. 1E). However, these ratios were significantly increased in *HRAS*-mutated HNSCCs versus *HRAS*-wildtype HNSCCs (Fig. 1E). These results again suggest that *TP53* mutations and *HRAS* mutations are associated with a reduced and an increased immune/inflammation activity in HNSCCs.

In another HNSCC multi-omics dataset GSE65858 [23], we observed that multiple immune signatures were significantly downregulated in *TP53*-mutated HNSCCs versus *TP53*-wildtype HNSCCs, e.g., CD8+ T cells, immune cytolytic activity, and immune score (Supplementary Fig. S1). It verified that *TP53* mutations were associated the reduced immune activity in HNSCC.

2.2. TP53 and HRAS Mutations are Associated with Reduced and Increased Expression of Human Leukocyte Antigen (HLA) Genes in HNSCC, Respectively

HLA genes encode MHC proteins which are involved in the regulation of the immune system in humans [27]. Of 24 HLA genes analyzed, 15 (62.5%) and zero were downregulated and upregulated in *TP53*-mutated HNSCCs compared to *TP53*-wildtype HNSCCs, respectively (Fig. 2A; Supplementary Table S6). In contrast, 13 (54%) and zero HLA genes were more highly and lowly expressed in *HRAS*-mutated HNSCCs than in *HRAS*-wildtype HNSCCs, respectively (Fig. 2B; Supplementary Table S6). These results suggest that *TP53* mutations may inhibit HLA expression while *HRAS* mutations may promote HLA expression in HNSCC. The finding of *TP53* mutations repressing HLA expression in HNSCC is in line with a previous report that p53 could increase expression of MHC proteins in cancer [28].

2.3. Correlations of TP53 Mutations and HRAS Mutations with TMB and Tumor Aneuploidy

TMB and tumor aneuploidy have been shown to significantly correlate with tumor immunity [29]. We found that *TP53*-mutated HNSCCs had significantly higher TMB than *TP53*-wildtype HNSCCs (Mann-Whitney *U* test, $P = 2.85 \times 10^{-5}$) while *HRAS* mutations had no significant correlation with TMB in HNSCC (Mann-Whitney *U* test, $P = 0.522$) (Fig. 3A). Moreover, gene mutations may yield neoantigens that are associated with tumor immunity [30]. We found that the mutations yielding predicted HLA-binding peptides [25] were much more in *TP53*-mutated HNSCCs than in *TP53*-wildtype HNSCCs (Mann-Whitney *U* test, $P = 0.014$) while showed no significant difference between *HRAS*-mutated and *HRAS*-wildtype HNSCCs (Mann-Whitney U test, $P = 0.621$). These results suggest that the reduced immunogenic activity in *TP53*-mutated HNSCCs may be ascribed to the depressed HLA function, but not to TMB or mutation-associated neoantigens. Interestingly, The aneuploidy levels were significantly higher in *TP53*-mutated HNSCCs than in *TP53*-wildtype HNSCCs (Mann-Whitney *U* test, $P = 5.50 \times 10^{-5}$) while had no significant differences between *HRAS*-mutated and *HRAS*-wildtype HNSCCs (Mann-Whitney *U* test, $P = 0.873$) (Fig. 3B). These findings are in line with previous studies showing that tumor aneuploidy correlated with reduced tumor immunity in cancer [29].

2.4. Prediction of Immune Signatures by TP53 Mutation, HRAS Mutation, TMB, and Tumor Aneuploidy in HNSCC

Based on the logistic regression model with four predictors (*TP53* mutation, *HRAS* mutation, TMB, and aneuploidy), we evaluated the contribution of *TP53* mutation and *HRAS* mutation in predicting immune cytolytic activity and immune infiltration levels (immune score) in HNSCC. We found that three of the four predictors significantly predicted immune cytolytic activity, including *TP53* mutation (β coefficient: $\beta = -1.214$, $P = 1.96 \times 10^{-4}$), *HRAS* mutation ($\beta = 1.619$, $P = 0.018$), and aneuploidy ($\beta = -0.664$, $P = 0.001$) (Fig. 4). As expected, both *TP53* mutation and aneuploidy were negative predictors and *HRAS* mutation was a positive predictor for immune cytolytic activity. In predicting immune infiltration levels, both *TP53* mutation ($\beta = -1.085$, $P = 6.95 \times 10^{-4}$) and aneuploidy ($\beta = -0.620$, $P = 0.002$) were significant negative predictors, and *HRAS* mutation was a positive predictor ($\beta = 0.650$, $P = 0.257$) (Fig. 4). These results confirmed the negative correlation between *TP53* mutations and immune signatures and the positive correlation between *HRAS* mutations and immune signatures in HNSCC. Meanwhile, these results confirmed the significant negative correlation between aneuploidy and immune signatures in cancer [29]. Interestingly, TMB showed minor contribution in predicting immune cytolytic activity ($\beta = 0.001$, $P = 0.134$) and immune infiltration levels ($\beta = 1.10 \times 10^{-4}$, $P = 0.794$) in HNSCC. It indicates that TMB is not significant in determining tumor immunity in HNSCC.

2.5. Identification of the Pathways that are Altered by TP53 or HRAS Mutations and are Significantly Associated with Immune Signatures in HNSCC

GSEA [26] showed that not only the immune-related pathways but also a number of cancer-associated pathways were disturbed upon *TP53* or *HRAS* mutations in HNSCC. These pathways included the p53, cell cycle, apoptosis, mismatch repair, Jak-STAT, focal adhesion, ECM-receptor interaction, calcium, MAPK, PI3K-Akt, mTOR, RAS, Wnt, Hedgehog, TGF-beta, ErbB, and glycolysis pathways. Interestingly, we found that most of these pathways were significantly associated with immune signatures in HNSCC (Fig. 5A, B). Notably, as a tumor suppressor, p53 prominently functions on promoting cell cycle arrest and apoptosis [14]. Accordingly, *TP53* mutations could lead to downregulation of the p53 and apoptosis pathways and upregulation of the cell cycle pathway in cancer. Our results showed that almost all 20 immune signatures

Fig. 2. Comparisons of the expression levels of human leukocyte antigen (HLA) genes between *TP53*-mutated and *TP53*-wildtype HNSCCs, and between *HRAS*-mutated and *HRAS*-wildtype HNSCCs. A. A number of HLA genes have significantly lower expression levels in *TP53*-mutated HNSCCs than in *TP53*-wildtype HNSCCs (Student's *t*-Test *P*-values are shown). B. A number of HLA genes have significantly higher expression levels in *HRAS*-mutated HNSCCs than in *HRAS*-wildtype HNSCCs.

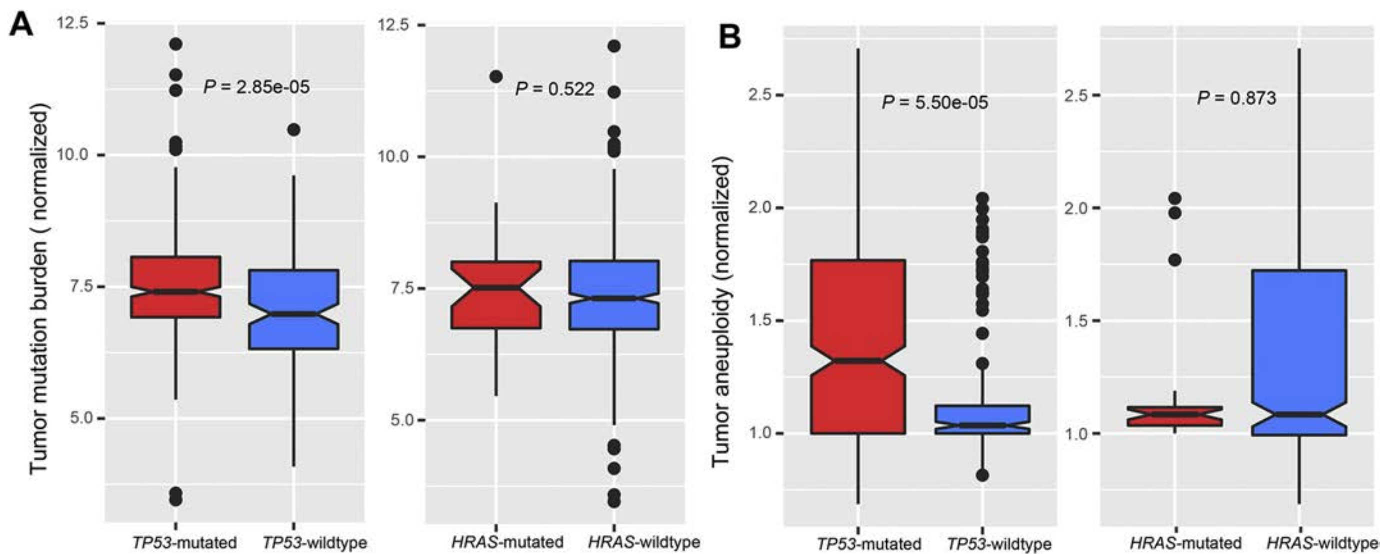

Fig. 3. Correlations of *TP53* mutations and *HRAS* mutations with tumor mutation burden (TMB) and tumor aneuploidy. A. *TP53*-mutated HNSCCs have significantly higher TMB than *TP53*-wildtype HNSCCs while *HRAS* mutations have no significant correlation with TMB in HNSCC (Mann-Whitney *U* test *P*-values are shown). B. *TP53*-mutated HNSCCs have significantly higher aneuploidy levels than *TP53*-wildtype HNSCCs while *HRAS* mutations have no significant correlation with tumor aneuploidy in HNSCC (Mann-Whitney *U* test *P*-values are shown). TMB is the total somatic mutation count in tumor and tumor aneuploidy is the tumor ploidy score evaluated by ABSOLUTE [54].

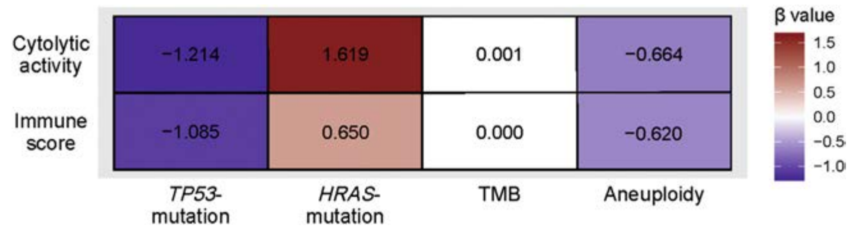

Fig. 4. Logistic regression analysis shows that *TP53* mutation and aneuploidy were negative predictors and *HRAS* mutation was a positive predictor for immune signatures in HNSCCs. β value: β coefficient.

A

Pro−inflammatory
CCR
TILs
Immune checkpoint
Treg
HLA
Cytolytic Activity
Type II IFN Reponse
Type I IFN Reponse
T cell Co−inhibition
APC Co−inhibition
T cell Co−stimulation
APC Co−stimulation
MHC Class I
pDCs
NK cells
Neutrophils
CD8+ T cells
CD4+ Regulatory T cells
B cells

p53.signaling.pathway
Cell.cycle
Apoptosis
Mismatch.repair
Jak.STAT.signaling.pathway
Focal.adhesion
ECM.receptor.interaction
Calcium.signaling.pathway
MAPK.signaling.pathway
PI3K.Akt.signaling.pathway
mTOR.signaling.pathway

B

Pro−inflammatory
CCR
TILs
Immune checkpoint
Treg
HLA
Cytolytic Activity
Type II IFN Reponse
Type I IFN Reponse
T cell Co−inhibition
APC Co−inhibition
T cell Co−stimulation
APC Co−stimulation
MHC Class I
pDCs
NK cells
Neutrophils
CD8+ T cells
CD4+ Regulatory T cells
B cells

Ras.signaling.pathway
Wnt.signaling.pathway
Hedgehog.signaling.pathway
TGF.beta.signaling.pathway
ErbB.signaling.pathway
Glycolysis_Gluconeogenesis

significant negative correlation
significant positive correlation
not significant
(Pearson's correlation test P>0.05)

C

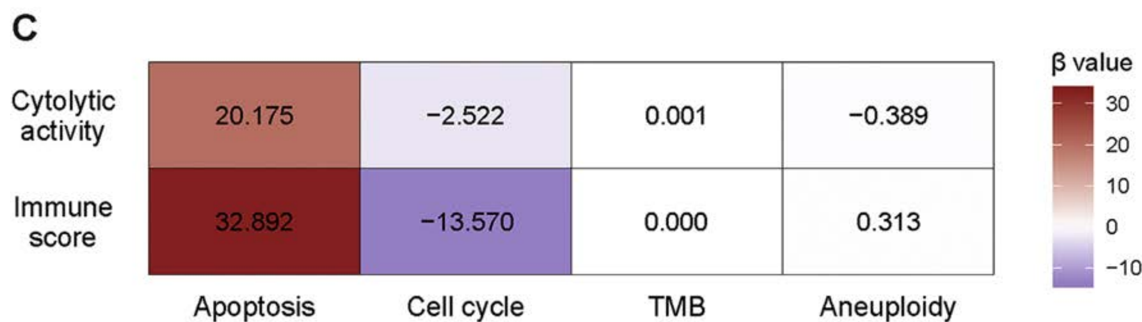

Fig. 5. Immune signatures are significantly associated with p53-mediated or RAS-mediated pathways in HNSCC. A. Immune signatures are significantly associated with p53-mediated pathways in HNSCC. B. Immune signatures are significantly associated with RAS-mediated pathways in HNSCC. The size of circles is proportional to the absolute values of correlation coefficients. C. Logistic regression analysis shows that the cell cycle score was a negative predictor and the apoptosis score was a positive predictor in predicting immune signatures in HNSCCs. The cell cycle score and apoptosis score were the ssGSEA scores [49,50] of the gene sets in the cell cycle and apoptosis pathways.

significantly positively correlated with the p53 and apoptosis pathways and inversely correlated with the cell cycle pathway (Fig. 5A). It conforms to our finding that *TP53* mutations were associated with depressed immune signatures in HNSCC. On the other hand, as an oncogene of RAS family, *HRAS* mutations may lead to hyperactivation of the RAS pathways. We found that almost all 20 immune signatures showed a significant positive correlation with the RAS pathways (Fig. 5B), consistent with our finding that *HRAS* mutations were associated with elevated immune signatures in HNSCC. Intriguingly, pro-oncogenic pathways were likely to exhibit a negative correlation with immune signatures in HNSCC, e.g., the cell cycle, mTOR, RAS, Wnt, Hedgehog, TGF-beta, ErbB, and glycolysis pathways (Fig. 5A, B). It indicates that the hyperactivation of pro-oncogenic pathways may promote tumor immunosuppression. Altogether, these results suggest that *TP53* mutations and *HRAS* mutations may alter the activity of their mediated pathways, thereby contributing to the depressed and elevated immune signatures in *TP53*-mutated HNSCCs and *HRAS*-mutated HNSCCs, respectively.

To further prove that the negative association between *TP53* mutations and tumor immunity in HNSCC is associated with the cell cycle and apoptosis pathways, we used a logistic regression model with the predictors of cell cycle score and apoptosis score to predict immune signatures (immune cytolytic activity and immune infiltration levels). We found that the cell cycle score was a negative predictor and the apoptosis score was a positive predictor in predicting these immune signatures (Fig. 5C). These results indicate that *TP53* mutations lead to cell cycle activation and apoptosis inhibition, which in turn affect tumor immunity.

2.6. Identification of Proteins Whose Expression is Associated with TP53 Mutations as well as Immune Infiltration in HNSCC

We identified 8 proteins (Syk, Caspase-7, Cyclin B1, TIGAR, Bcl-2, Lck, VEGFR2, and PCNA) and 2 proteins (CK5 and Rb) having significantly lower and higher expression levels in *TP53*-mutated HNSCCs than in *TP53*-wildtype HNSCCs, respectively (Student's *t*-Test, FDR < 0.2). Of these proteins, Syk, Caspase-7, Bcl-2, and Lck had a significant positive expression correlation with immune infiltration levels (immune scores) in HNSCC (Spearman correlation, $P < 0.001$) (Fig. 6). Syk (spleen tyrosine kinase) is a non-receptor cytoplasmic enzyme that is primarily expressed in cells of hematopoietic lineage and regulates the biological processes that are associated with innate and adaptive immunity [31]. This protein functions as a tumor suppressor and is a p53 target [32,33]. The downregulation of Syk may be associated with the p53 dysfunction and contribute to the immunosuppression in the *TP53*-mutated HNSCC subtype. Lck is a member of the Src tyrosine kinase family and plays a key role in regulation of developing T-cells [34]. The downregulation of Syk may contribute to the depressed antitumor immunity

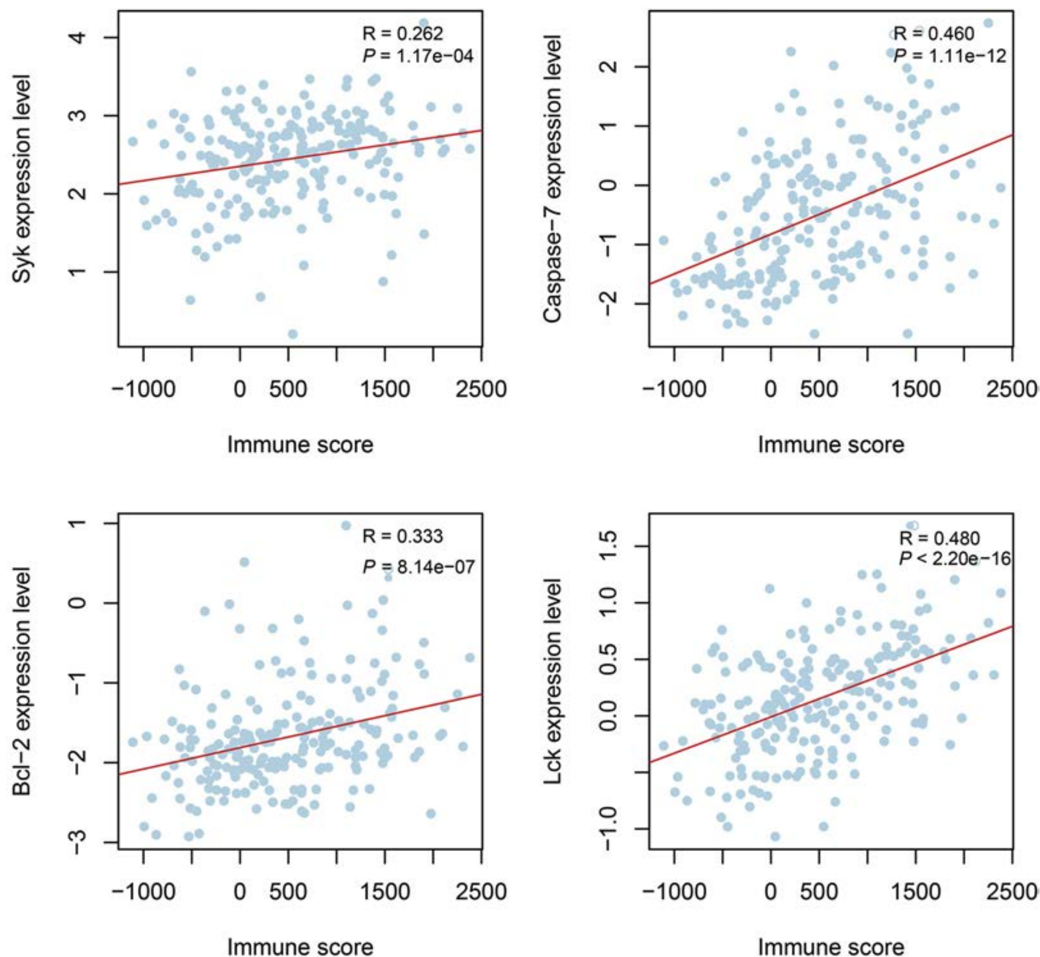

Fig. 6. Proteins which show significantly lower expression levels in *TP53*-mutated HNSCCs than in *TP53*-wildtype HNSCCs have significant positive expression correlations with immune infiltration levels in HNSCC.

in *TP53*-mutated HNSCCs. Caspase-7 and Bcl-2 are importantly involved in p53-regulated apoptosis [35,36]. The downregulation of both proteins indicated the reduced apoptosis activity that was associated the depressed immune activity in *TP53*-mutated HNSCCs. Taken together, these results demonstrate that the differential immune activity is significantly associated with the differential immune-associated protein expression between *TP53*-mutated and *TP53*-wildtype HNSCCs.

2.7. Immune Signatures are Positively Associated with Survival Prognosis in HNSCC

Survival analyses showed that the elevated enrichment of 12 immune signatures consistently correlated with better OS and/or DFS in HNSCC (log-rank test, $P < 0.05$) (Fig. 7A). These 12 immune signatures included B cells, CD4+ regulatory T cells, CD8+ T cells, neutrophils,

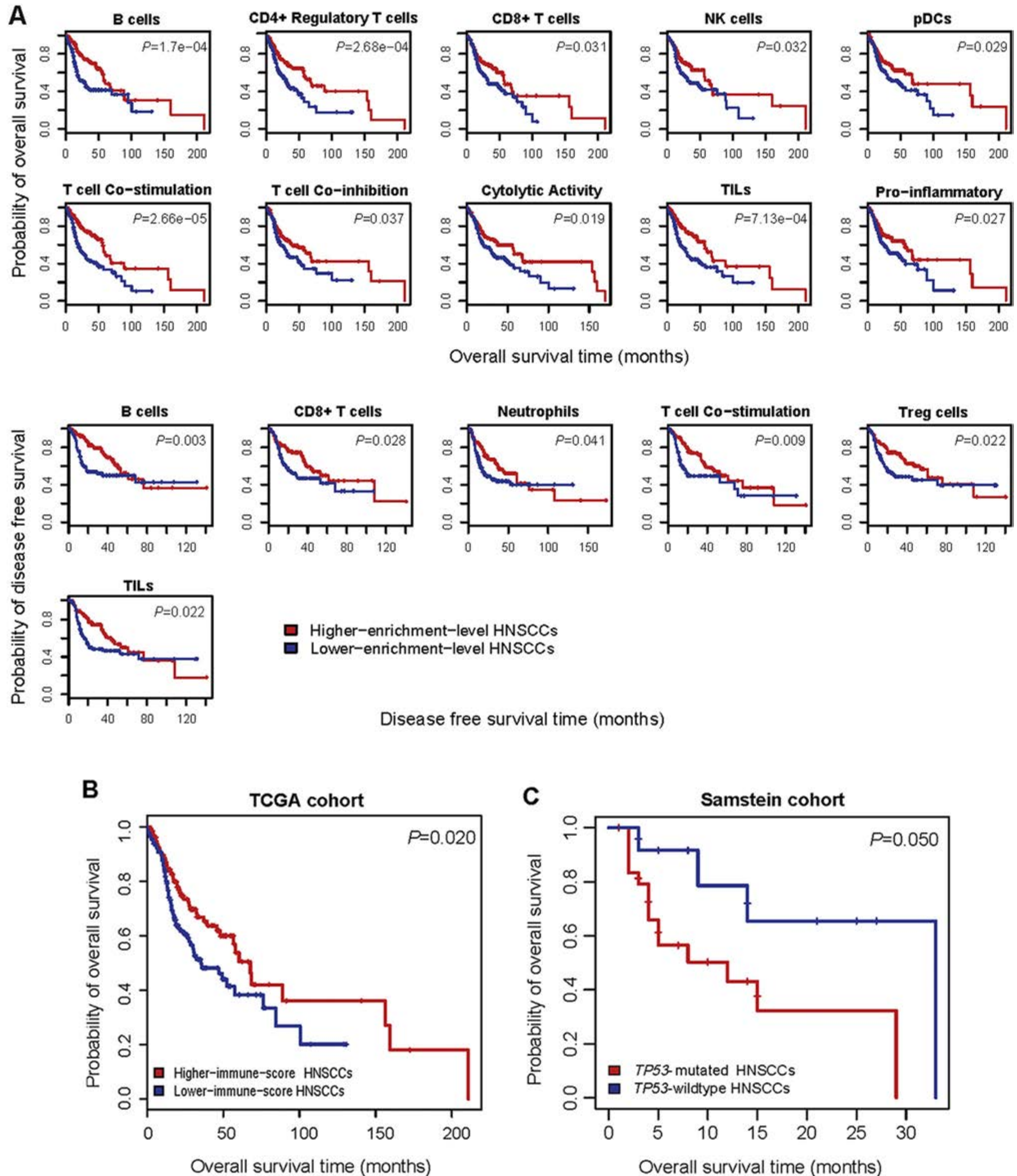

Fig. 7. Immune signatures are positively associated with survival prognosis in HNSCC. A. Kaplan-Meier survival curves show that the elevated enrichment of diverse immune signatures is associated with better survival prognosis in HNSCC (log-rank test, $P < 0.05$). B. Kaplan-Meier survival curves show that higher degree of immune cell infiltration is associated with better overall survival in HNSCC (log-rank test P value is shown). C. *TP53*-mutated HNSCC patients have significant worse overall survival than *TP53*-mutated HNSCC patients in Samstein cohort [40] receiving anti-PD-1/PD-L1/CTLA-4 immunotherapy (log-rank test, $P = 0.050$).

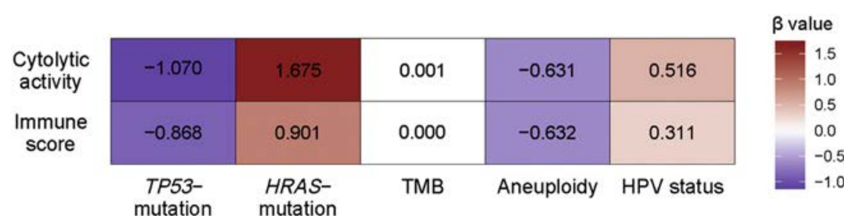

Fig. 8. Logistic regression analysis shows that *TP53* mutation is a significant negative predictor in predicting both immune signatures in HNSCC when the HPV infection status predictor is added into the predictive model.

NK cells, pDCs, T cell co-stimulation, T cell co-inhibition, cytolytic activity, Treg cells, TILs, and pro-inflammatory signatures. Notably, higher degree of tumor lymphocyte infiltration was associated with better OS and DFS in HNSCC. It is in agreement with previous studies showing that elevated levels of TILs were associated with improved survival in cancer patients [37,38]. Furthermore, higher density of CD8+ T cells or B cells was associated with better OS and DFS in HNSCC (Fig. 7A), bolstering the prognostic value of CD8+ T cell levels in cancer [39]. Moreover, we found that the HNSCCs with higher immune scores had better OS than the HNSCCs with lower immune scores (log-rank test, $P = 0.020$) (Fig. 7B), again suggesting that elevated antitumor immune activity is associated with better clinical outcomes in HNSCC.

Furthermore, using an HNSCC cohort (Samstein cohort [40]) receiving anti-PD-1/PD-L1/CTLA-4 immunotherapy, we examined the correlations of *TP53* mutations and *HRAS* mutations with OS prognosis. We found that *TP53*-mutated HNSCCs had worse OS than *TP53*-widtype HNSCCs (log-rank test, $P = 0.050$) (Fig. 7C). The negative correlation between *TP53* mutations and OS in the immunotherapy setting could be attributed to the unfavorable response to immunotherapy in *TP53*-mutated HNSCCs compared to *TP53*-wildtype HNSCCs. In the same cohort, because all HNSCCs were *HRAS*-wildtype, we did not analyze the correlation between *HRAS* mutations and survival prognosis.

2.8. The Negative Association between TP53 Mutations and Tumor Immunity is Independent of the Human Papillomavirus (HPV) Infection and Smoking Status in HNSCC

HPV and smoking are important factors in the rise of non-smoker HNSCCs and smoker HNSCCs, respectively [13,41]. We found that *TP53*-mutated HNSCCs had a significantly lower rate of HPV infection than *TP53*-wildtype HNSCCs (7% versus 46%, Fisher's exact test, $P = 1.60 \times 10^{-22}$, odds ratio: OR = 0.090). As expected, HPV+ HNSCCs likely had increased immune activity compared to HPV- HNSCCs (Supplementary Table S8). Thus, the lower immune activity in *TP53*-mutated HNSCCs could be due to the lower HPV infection rate relative to *TP53*-wildtype HNSCCs. Nevertheless, we found that a majority of the immune signatures showed significantly lower enrichment levels in *TP53*-mutated HPV- HNSCCs versus *TP53*-wildtype HPV- HNSCCs, as well as in *TP53*-mutated HPV+ HNSCCs versus *TP53*-wildtype HPV+ HNSCCs (Supplementary Table S9). To further investigate how the association between *TP53* mutations and tumor immunity is affected by the HPV infection factor, we constructed a logistic regression model with the predictors of *TP53* mutation and HPV infection to predict immune signatures (immune cytolytic activity and immune infiltration levels). We found that *TP53* mutation was a significant negative predictor in predicting both immune signatures ($\beta = -1.070$ and $P = 0.003$ in predicting immune cytolytic activity; $\beta = -0.868$ and $P = 0.019$ in predicting immune infiltration levels) (Fig. 8). All together, these analyses consistently demonstrate that *TP53* mutations and tumor immunity have a strong inverse correlation regardless of the HPV infection status in HNSCC.

In addition, we found that *TP53*-mutated HNSCCs contained a higher proportion of heavy smokers compared to *TP53*-wildtype HNSCCs (Fisher's exact test, $P = 0.043$, OR = 1.582). Unexpectedly, we found that heavy-smoker HNSCCs were inclined to have depressed immune activity compared to light-smoker and non-smoker HNSCCs (Supplementary Table S10), although the former had significantly higher TMB than the latter (Mann-Whitney *U* test, $P = 5.11 \times 10^{-6}$). This suggests that the heavy-smoking may dampen the immune function of HNSCCs. Furthermore, we found that most of the immune signatures had significantly lower enrichment levels in *TP53*-mutated heavy-smoker HNSCCs versus *TP53*-wildtype heavy-smoker HNSCCs, as well as in *TP53*-mutated non-heavy-smoker HNSCCs versus *TP53*-wildtype non-heavy-smoker HNSCCs (Supplementary Table S11). It demonstrates that the smoking factor solely cannot explain the differential immune activity between *TP53*-mutated and *TP53*-wildtype HNSCCs.

3. Discussion

TP53 has a high mutation rate (>70%) in HNSCC and the *TP53* mutation is associated with a worse prognosis in HNSCC [12]. Strikingly, we found that almost all the immune signatures analyzed showed significantly lower activities in *TP53*-mutated HNSCCs versus *TP53*-wildtype HNSCCs. In contrast, the mutation of *HRAS*, which is the most frequently mutated RAS gene in HNSCC (>6%), was likely associated with higher immune activities in HNSCC. These results demonstrate the opposed effect of the tumor suppressor gene (*TP53*) mutation and the oncogene (*HRAS*) mutation on the tumor immune microenvironment (TIM) in HNSCC. It has been shown that high immune signatures in the TIM, e.g., dense infiltration of lymphocytes such as CD8+ T cells and B cells, often indicate favorable clinical outcomes and active response to immunotherapy in cancer patients [37,38]. Thus, our data have potential clinical implications that *TP53*-wildtype HNSCCs more likely respond to immunotherapy than *TP53*-mutated HNSCCs and that *HRAS*-mutated HNSCCs may be more responsive to immunotherapy as compared to *HRAS*-wildtype HNSCCs.

TP53 mutations often result in the deregulation of p53 function in regulating cell cycle, apoptosis, and genome stability [14,42]. Thus, *TP53*-mutated HNSCCs likely have increased cell cycle, reduced apoptosis, and high genome instability. Our results and reports from other studies [29,43,44] consistently showed that cell cycle inhibited tumor immunity, apoptosis promoted tumor immunity, and genome instability suppressed tumor immunity. Thus, the depressed tumor immunity in *TP53*-mutated HNSCCs could be attributed to the deregulation of

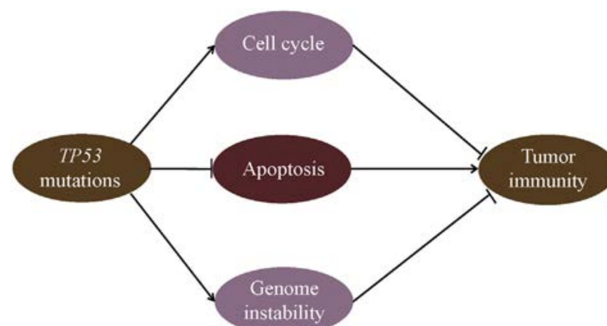

Fig. 9. *TP53*-mutations result in the deregulation of p53-mediated cell cycle, apoptosis, and genome stability thereby contributing to the depressed immune activity in HNSCC.

these p53 functions (Fig. 9). Moreover, we found plentiful p53- and RAS-mediated pathways whose activity was significantly associated with immune activity in HNSCC (Fig. 5). These pathways are involved in various cancer-associated activities, including DNA damage repair, proliferation, metabolism, inflammation, epithelial-mesenchymal transition (EMT), angiogenesis, and metastasis, suggesting that the disturbance of a wide range of cancer-associated pathways may alter the TIM in cancer and that the effective intervention of these pathways may enhance antitumor immunity and cancer immunotherapy response.

We found that *HRAS* mutations were associated with elevated antitumor immune signatures in HNSCC. However, the *HRAS* mutation was not associated with a better survival prognosis in the TCGA HNSCC cohort (log-rank test, $P = 0.516$, 0.632 for OS and DFS, respectively). A possible explanation is that *HRAS* mutations also promoted immunosuppressive signatures, such as PD-L1 expression. However, we believe that the *HRAS* mutation could be a predictive biomarker for favorable response to anti-PD-1/PD-L1 immunotherapy since both the elevated antitumor immune infiltration [45] and PD-L1 expression [46] indicate a more active response to anti-PD-1/PD-L1 inhibitors.

The relationship between gene mutations and tumor immunity could vary with tumor progression. We compared the enrichment levels of the 20 immune signatures between *TP53*-mutated and *TP53*-wildtype HNSCCs within early- and late-stage cancers, respectively. We found that 18 immune signatures displayed significantly lower enrichment levels in *TP53*-mutated early-stage HNSCCs than in *TP53*-wildtype early-stage HNSCCs, and in *TP53*-mutated late-stage HNSCCs than in *TP53*-wildtype late-stage HNSCCs (Mann-Whitney *U* test, $P < 0.05$). It suggests that the association between *TP53* mutations and tumor immunity does not vary with tumor progression.

Interesting, of the 47 immune checkpoint genes [47], 28 (60%) showed significantly lower expression levels in *TP53*-mutated HNSCCs than in *TP53*-wildtype HNSCCs (Student's *t*-Test, FDR < 0.05), and included many established or potential immunotherapeutic targets such as *CTLA4*, *PD1*, *PD-L1*, *LAG3*, *IDO1/2*, *BTLA*, *TIM3*, and *TIGIT* (Fig. 10A; Supplementary Table S5). In contrast, many notable immune checkpoint genes were more highly expressed in *HRAS*-mutated HNSCCs than in *HRAS*-wildtype HNSCCs, including *PD1*, *PD-L1*, *PD-L2*, *LAG3*, *IDO1*, and *TNFSF9* (Student's *t*-Test, FDR < 0.05) (Fig. 10B; Supplementary Table S5). Altogether, these results suggest that the *TP53* mutation and the *HRAS* mutation could be a negative and a positive indicator for active response to the immune checkpoint blockade therapy of HNSCC, respectively.

4. Material and Methods

4.1. Material

Three cancer multi-omics datasets were used in this study, including TCGA HNSCC dataset [13], an HNSCC gene expression profiling dataset GSE65858 [23], and an HNSCC cohort (Samstein cohort [40]) with anti-PD-1/PD-L1/CTLA-4 immunotherapy. The TCGA RNA-Seq gene expression profiles (Level 3), gene somatic mutations (Level 3), protein expression profiles (Level 3), and clinical data for HNSCC were downloaded from the genomic data commons data portal (https://portal.gdc.cancer.gov/), and GSE65858 was downloaded from the NCBI gene expression omnibus (https://www.ncbi.nlm.nih.gov/geo/). The gene mutation and clinical data for Samstein cohort [40] were from the associated publication. We obtained 20 immune signatures (represented by 20 different gene sets) from a previous publication [48].

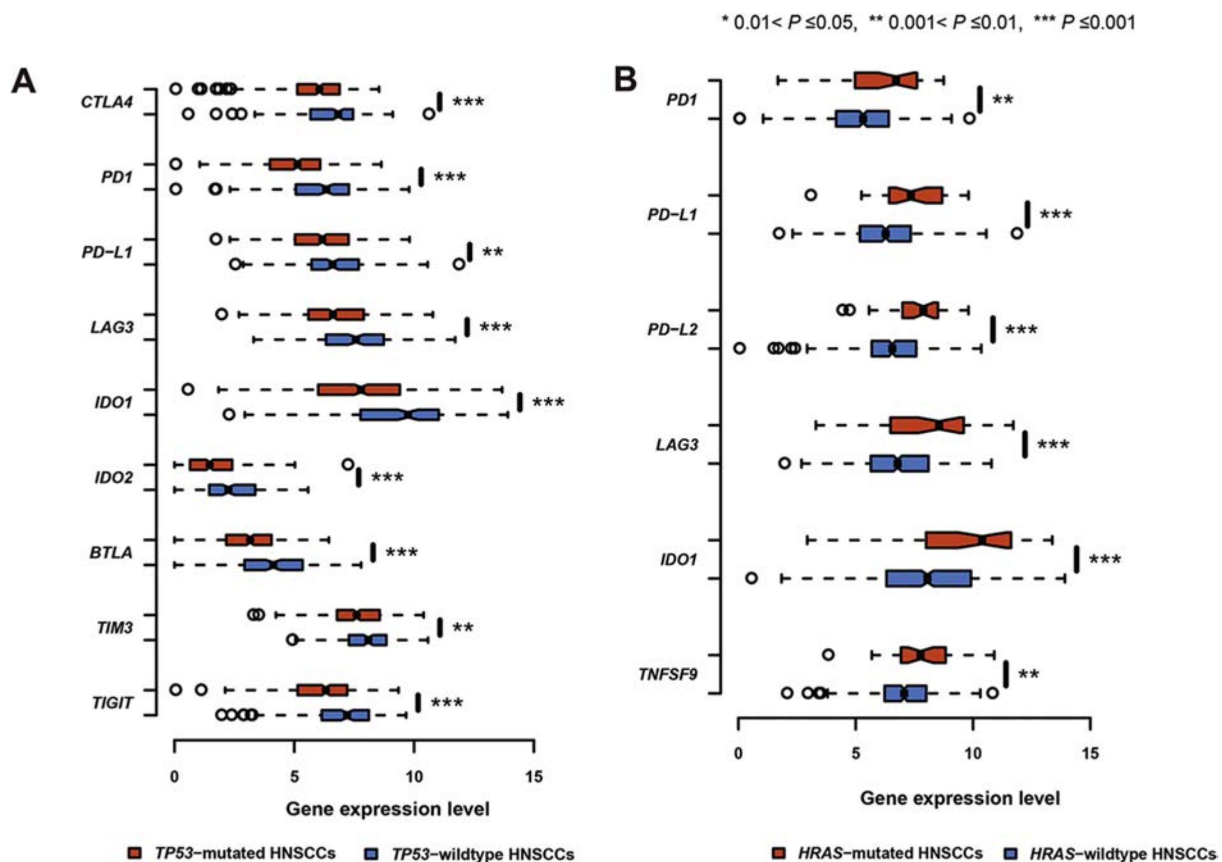

Fig. 10. A number of immune checkpoint genes are differentially expressed between *TP53*-mutated and *TP53*-wildtype HNSCCs, and between *HRAS*-mutated and *HRAS*-wildtype HNSCCs (Student's *t*-Test, $P < 0.01$). A. Numerous immune checkpoint genes have significantly lower expression levels in *TP53*-mutated HNSCCs than in *TP53*-wildtype HNSCCs. B. A number of immune checkpoint genes have significantly higher expression levels in *HRAS*-mutated HNSCCs than in *HRAS*-wildtype HNSCCs.

4.2. Comparisons of Gene Expression Levels, Immune Signature Enrichment Levels, and Protein Expression Levels Between Two Classes of Samples

For HNSCC gene expression profiling data, we normalized the gene expression values by base-2 log transformation and compared the expression levels of a single gene between two classes of samples using Student's *t*-Test. We quantified the enrichment level of an immune signature represented by a gene set in a sample by the single-sample gene-set enrichment analysis (ssGSEA) score [49,50] and compared the enrichment levels (ssGSEA scores) of an immune signature between two classes of samples using Mann-Whitney *U* test. We compared the ratios between immune-stimulatory signatures and immune-inhibitory signatures between two classes of samples based on the ratios between the average expression levels of marker genes of immune-stimulatory signatures and the average expression levels of marker genes of immune-inhibitory signatures (CD8+/CD4+ Treg cells, pro−/anti-inflammatory cytokines, and M1/M2 macrophages). We compared protein expression levels between two classes of samples based on the normalized HNSCC protein expression profiles dataset in TCGA using Student's *t*-Test. The false discovery rate (FDR) was calculated by the Benjamini and Hochberg (BH) method [51] to obtain the adjusted *P*-values in multiple tests. The threshold of FDR < 0.05 indicates the statistical significance.

4.3. Gene-Set Enrichment Analysis

We performed gene-set enrichment analysis of the HNSCC gene expression profiling data by GSEA [26] and identified the KEGG [52] pathways that were differentially expressed between *TP53*-mutated and *TP53*-wildtype HNSCCs or between *HRAS*-mutated and *HRAS*-wildtype HNSCCs (FDR < 0.05).

4.4. Comparison of Immune Cell Infiltration Levels between Two Classes of Samples

We quantified the immune cell infiltration level of each HNSCC sample using the immune score evaluated by ESTIMATE [53]. The Mann-Whitney *U* test was used to compare the immune scores between two groups of HNSCCs.

4.5. Logistic Regression Model for Predicting Immune Signature Levels

To evaluate the contributions of different molecular features in predicting immune signature levels, we used the logistic regression model with multiple predictors (*TP53* mutation, *HRAS* mutation, cell cycle score, apoptosis score, HPV infection status, TMB, and aneuploidy). *TP53* mutation and *HRAS* mutation were binary variables (mutated or wildtype), cell cycle score and apoptosis score were continuous variables (ssGSEA scores of the gene sets in the cell cycle and apoptosis pathways), HPV infection status was a binary variable (HPV+ or HPV-), TMB (defined as the total somatic mutation count in tumor) was a discrete variable, and tumor aneuploidy (defined as the tumor ploidy score generated by ABSOLUTE [54]) was a continuous variable. The tumors with high (upper quarter) versus low (bottom quarter) immune signature scores were predicted.

4.6. Investigation of the Correlation Between Pathway or Protein Activity and Immune Signatures in HNSCC

We investigated the correlation between p53- or RAS-mediated pathways and immune signatures in HNSCC. The ssGSEA score [49,50] was used to quantify the activity of a pathway in an HNSCC sample on the basis of the set of genes included in the pathway. The first-order partial correlation [55] was used to assess the correlations between pathways and immune signatures in order to correct for the strong correlation between the p53 pathway and the p53-mediated pathways and between the RAS pathway and the RAS-mediated pathways by control of the p53 and RAS pathways, respectively. The significance of the correlation between a pathway and an immune signature was determined with the threshold of FDR < 0.05. The Spearman correlation test was used to evaluate the correlation between protein expression levels and immune signature enrichment levels.

4.7. Survival Analyses

We classified HNSCC patients into two different classes based on immune signature enrichment levels (higher-enrichment-level (ssGSEA scores > the third quartile) versus lower-enrichment-level (ssGSEA scores < the first quartile), or immune scores (higher-immune-score (immune scores > the third quartile) versus lower-immune-score (immune scores < the first quartile). We used Kaplan-Meier survival curves to exhibit the survival (OS or disease free survival (DFS)) differences and the log-rank test to evaluate the survival-time differences between two classes of patients with a significance threshold of $P < 0.05$.

5. Conclusions

The *TP53* mutation inhibited tumor immunity while the *HRAS* mutation promoted tumor immunity in HNSCC. These findings have potential clinical implications that the *TP53* mutation and the *HRAS* mutation status could be useful biomarkers for identifying HNSCC patients responsive to immunotherapy.

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Ethics Approval and Consent to Participate

Ethical approval and consent to participate was waived since we used only publicly available data and materials in this study.

Consent for Publication

Not applicable.

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Authors' Contributions

HL performed data analyses and helped prepare for the manuscript. ML performed data analyses and helped prepare for the manuscript. ZJ performed data analyses. ZL performed data analyses. XW conceived the research, designed analysis strategies, and wrote the manuscript. All the authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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