Influence of tumor mutational burden, inflammatory gene expression profile, and PD-L1 expression on response to pembrolizumab in head and neck squamous cell carcinoma

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ABSTRACT

Background To characterize genomic determinants of response to pembrolizumab in recurrent/metastatic (R/M) head and neck squamous cell carcinoma (HNSCC) in the KEYNOTE-012 study.

Methods Associations between biomarkers (tumor mutational burden (TMB), neoantigen load (NL), 18-gene T-cell-inflamed gene expression profile (TcellGEP), and PD-L1 combined positive score (CPS)) and clinical outcomes with pembrolizumab were assessed in patients with R/M HNSCC (n=192). Tumor human papillomavirus (HPV) status was also evaluated with the use of p16 immunohistochemistry and whole exome sequencing (WES; HPV∗, mapping ≥20HPV reads) in pretreatment tumor samples (n=106).

Results TMB, clonality-weighted TMB, and TcellGEP were significantly associated with objective response (p=0.0276, p=0.0201, and p=0.006, respectively), and a positive trend was observed between NL and PD-L1 CPS and clinical response (p=0.0505 and p=0.0682, respectively). No correlation was observed between TMB and TcellGEP (Spearman ρ=−0.026) or TMB and PD-L1 (Spearman ρ=0.009); a correlation was observed between TcellGEP and PD-L1 (Spearman ρ=0.511). HPV status by WES and p16 immunohistochemistry showed concordance (84% x=0.573) among patients whose HPV results were available using both methods.

Conclusions TMB and inflammatory biomarkers (TcellGEP and PD-L1) may represent distinct and complementary biomarkers predicting response to anti-programmed death 1 therapies in HNSCC; further study of these relationships in randomized clinical trials is needed.

Trial registration number NCT01848834.

INTRODUCTION

Immune checkpoint inhibitors demonstrate antitumor activity in a variety of tumor types.1-4 Programmed death ligand 1 (PD-L1) expression can predict response to programmed death 1 (PD-L1) inhibition and is an approved diagnostic for some cancers.5,6 Despite the predictive value of PD-L1 expression, some patients with PD-L1-negative tumors experience clinical benefit with PD-L1-targeting/PD-L1-targeting regimens,7-9 and crossover of progression-free survival (PFS) or overall survival (OS) curves for patients with PD-L1-positive and PD-L1-negative tumors using various cutoffs has been observed,8,10 suggesting the existence of unidentified immunotherapy-responsive subpopulations. Greater understanding of the tumor microenvironment, beyond PD-L1 expression, is needed to predict clinical benefit more reliably.

Certain molecular signatures have been linked with clinical outcomes in several solid tumors.11 Tumor mutational burden (TMB), as determined by next-generation sequencing (NGS) or whole exome sequencing (WES), can predict response to immunotherapies (anti-cytotoxic T lymphocyte-associated protein 4 (CTLA-4) or anti-PD-1/PD-L1) as demonstrated in retrospective analyses across multiple tumor types.12-15 Additionally, the anti-PD-1 monoclonal antibody pembrolizumab is now approved as treatment for patients with TMB-high (TMB-H; ≥10 mutations/megabase) solid tumors.6 Neoantigen load (NL), a less validated biomarker, can also be determined by NGS and WES, but identifying criteria to further define neoantigens is needed.13 Some tumors exhibit a T-cell-inflamed phenotype, described by an 18-gene T-cell-inflamed gene expression profile (TcellGEP) composed of infiltrating T cells, chemokines, and an interferon gamma (IFN-γ) signature, and additional gene expression
T-cell inflammation signatures have been developed. A recent study suggested that tumor antigenicity, whether originating from somatic mutations or viral epitopes, and T-cell infiltration provide complementary information that predicts pembrolizumab activity. Markers of tumor T-cell inflammation are related to response to anti-PD-1/PD-L1 therapies, suggesting that tumors exhibit adaptive and cytotoxic T-cell responses of variable intensity. The primary objective of the evaluations presented here is to characterize response to pembrolizumab in head and neck squamous cell carcinoma (HNSCC) according to the landscape of these key mutational and inflammation biomarkers.

Oncogenic viruses (eg, human papillomavirus (HPV), hepatitis B virus, Merkel cell polyomavirus, and Epstein-Barr virus) generate viral antigens distinct from somatic mutations. Response to immunotherapy is reportedly higher in some virus-associated cancers than in their virally unrelated counterparts. PD-L1 expression is increased in some virus-associated cancers, reflecting an inflamed tumor phenotype. However, genetic determinants of response to immunotherapy in virus-associated cancers are not well understood.

HNSCC includes HPV-associated cancers that might be highly immune cell infiltrated; the level of immune infiltration and activation varies according to HPV status, molecular subtype, and genomic instability. HNSCC tumors, irrespective of HPV status, benefit from immunotherapy. Pembrolizumab was well tolerated and conferred durable antitumor activity in patients with HNSCC in the nonrandomized phase 1b multicohort KEYNOTE-012 and phase 2 KEYNOTE-055 (NCT02255097) studies. In this exploratory analysis, the predictive value of TMB and inflammatory biomarkers (TcellinfGEP and PD-L1) were assessed in both KEYNOTE-012 cohorts (B1 and B2). The methods used to analyze TMB, NL, and TcellinfGEP have been reported. The cut-off of −0.318 used to define GEP low and GEP nonlow is synonymous with GEP low and GEP high used in Cristescu et al. Tumor PD-L1 expression was assessed by IHC combined positive score (CPS); CPS ≥1 was considered positive.

HPV status was confirmed by p16 IHC using the CINtec p16 Ventana assay on the BenchMark Ultra using formalin-fixed paraffin-embedded pretreatment clinical specimens and by WES of germline and tumor DNA. For HPV status determined by p16 IHC, HPV-positive status (defined as ≥70% tumor cells with positive nuclear and/or cytoplasmic diffuse staining and H score of 210) included patients with primary tumor locations in the oropharynx, and HPV-negative status included patients with non-HPV-associated oropharyngeal cancers and primary tumor locations outside the oropharynx. Additional details are described in online supplemental file 1.

**Statistical analysis**

Logistic regression modeling was used to test the association between TMB (and/or TcellinfGEP/PD-L1) and best overall response. A Cox model was used to assess the association between TMB (and/or TcellinfGEP/PD-L1) and PFS and OS; log or square root transformation was used when needed for TMB/PD-L1. Regression models were adjusted for Eastern Cooperative Oncology Group (ECOG) performance status and cohort. One-sided p values were calculated according to the hypothesized positive association between these exploratory biomarkers.
and improved clinical outcomes. The area under the receiver operating characteristic (AUROC) curve was used to measure discriminatory ability. A TMB cut-off of 175 mutations/exome (mut/exome) was used for illustrative purposes and was identified in previous exploratory analyses as the threshold with strong support for predicting response to pembrolizumab across multiple tumor types. Additional, this WES-based TMB cut-off of 175 mut/exome proved to be the most concordant with the FoundationOne®CDx (Foundation Medicine) 10 mutations/megabase cut-off, the current assay and cut-off for the tumor-agnostic indication for which pembrolizumab is approved. The cut-off for Tcell_infGEP was −0.318 (Tcell_infGEPlow ≤−0.318; Tcell_infGEPnonlow ≥−0.318); this was associated with the Youden Index in an ROC analysis of a pan-cancer data set using Tcell_infGEP to predict whether a tumor was inflamed, as defined by observations on the dendrogram from unsupervised clustering of the pan-cancer data and supported as an enriching cut-off across multiple tumor types. Correlations between TMB and inflammatory biomarkers were assessed using Spearman correlation. Nominal p values were reported for signature testing. No adjustment was made for multiplicity. The concordance of HPV status, defined by WES versus p16 IHC, was evaluated using a contingency table. The distribution of each biomarker (TMB/Tcell_infGEP/PD-L1) by HPV status was illustrated using boxplots, and the mean difference of each biomarker in HPV-positive versus HPV-negative subgroups was tested using a two-sample t-test, and the adjusted p values are reported for multiple testing across the three biomarkers (TMB/Tcell_infGEP/PD-L1). The Hochberg Step-up procedure was used for multiplicity to control the family-wise error rate. Testing for differential biomarker relationships according to HPV status was performed with an interaction term between the biomarker (TMB/Tcell_infGEP/...
using data from cohort B2.\textsuperscript{16} Median PFS was longer in the Tcell\textsubscript{inf}GEP\textsuperscript{nonlow} subgroup than the Tcell\textsubscript{inf}GEP\textsuperscript{low} subgroup (106 vs 57 days) (figure 3C). Similarly, median OS was longer in the Tcell\textsubscript{inf}GEP\textsuperscript{nonlow} subgroup than the Tcell\textsubscript{inf}GEP\textsuperscript{low} subgroup (385 vs 199 days) (figure 3D).

**Association between PD-L1 and clinical outcomes**

PD-L1 tended to associate with higher response rates (p=0.0682) (figure 1E). The AUROC curve for PD-L1 response was 0.62 (figure 2). Median PFS was longer in the PD-L1 CPS\textgeq1 subgroup than the CPS<1 subgroup (97 vs 60 days) (figure 3E). Similarly, median OS was longer in the CPS\textgeq1 subgroup than the CPS<1 subgroup (353 vs 173 days) (figure 3F).

**Joint assessment of biomarkers**

There was no correlation between TMB and Tcell\textsubscript{inf}GEP (Spearman \(\rho=−.026\)) or between TMB and PD-L1 (Spearman \(\rho=0.009\)) (figure 4). Tcell\textsubscript{inf}GEP and PD-L1 were correlated with Spearman \(p=0.511\). When TMB, Tcell\textsubscript{inf}GEP, and PD-L1 were evaluated as continuous variables for their independent predictive values in a multivariate model with any two included into a model simultaneously, TMB and Tcell\textsubscript{inf}GEP showed independent predictive value with \(p<0.05\) after adjusting for another biomarker (TMB/ Tcell\textsubscript{inf}GEP/PD-L1); PD-L1 was not statistically significant after adjusting for Tcell\textsubscript{inf}GEP or TMB (\(p>0.05\)). Responses were enriched in the TMB \textgeq175 mut/exome subgroup (8/26 patients; 30.8%; 95\% CI 16.5\% to 50.0\%), the CPS\textgeq1 subgroup (19/88 patients; 21.6\%; 95\% CI 14.3\% to 31.3\%), and the Tcell\textsubscript{inf}GEP\textsuperscript{nonlow} subgroup (20/74 patients; 27.0\%; 95\% CI 18.2\% to 38.1\%). The TMB \textgeq175 mut/exome and PD-L1 CPS \textgeq1 (8/22 patients; 36.4\%; 95\% CI 19.7\% to 57.0\%) (figure 4B) and TMB \textgeq175 mut/exome and Tcell\textsubscript{inf}GEP\textsuperscript{nonlow} (7/18 patients; 38.9\%; 95\% CI 20.3\% to 61.4\%) (figure 4C) had the highest response rate.

**Distribution of biomarkers by HPV status**

Of 192 patients, 106 (55\%) had evaluable WES data. There was agreement between p16 IHC and WES for HPV status (89/106 (84\%); \(κ=0.573\)) (online supplemental table S2). Both WES-defined and p16 IHC-defined HPV status demonstrated mutual exclusivity with 7P53 mutation (online supplemental table S3).

The distribution of biomarkers (TMB/Tcell\textsubscript{inf}GEP/ PD-L1) was comparable in HPV-positive and HPV-negative subgroups defined by WES or p16 IHC (figure 5). Two-sample t-testing showed no significant difference between HPV-positive and HPV-negative subgroups defined by WES or p16 IHC for any biomarkers (adjusted \(p>0.6\)).

**Biomarker relationships with clinical outcomes by HPV status**

Evaluating trends for TMB, Tcell\textsubscript{inf}GEP, and PD-L1 within HPV status subtypes suggested an association between each biomarker and response in HPV-positive and HPV-negative subgroups (figure 6A–C). Trends for association between PD-L1 and response and Tcell\textsubscript{inf}GEP and response in HPV subgroups were observed regardless of methodology used to evaluate HPV status. In contrast,
Figure 3  Association between biomarkers and PFS and OS in the overall patient population at prespecified cut-offs. (A) TMB and PFS, (B) TMB and OS, (C) T-cell-inflamed GEP and PFS, (D) T-cell-inflamed GEP and OS, (E) PD-L1 CPS and PFS, and (F) PD-L1 CPS and OS. CPS, combined positive score; GEP, gene expression profile; OS, overall survival; PD-L1, programmed death ligand 1; PFS, progression-free survival; TMB, tumor mutational burden.
though a trend for association between TMB and response was seen in HPV subgroups analyzed by p16 IHC, a trend was observed only for the HPV-negative subgroup defined by WES.

Interaction testing did not confirm evidence of unique relationships for these biomarkers with clinical outcome, depending on HPV status. A test of the interaction effect, TMB by HPV, showed no difference of TMB association with response in HPV-positive versus HPV-negative subgroups defined by WES (TMB*HPV interaction, adjusted p=0.8184) or p16 IHC (adjusted p=0.4879). Last, a test of the interaction effect, PD-L1 by HPV, showed no difference of PD-L1 association with response in HPV-positive versus HPV-negative subgroups defined by WES (PD-L1*HPV interaction, adjusted p=0.3129) or p16 IHC (adjusted p=0.3069).

**DISCUSSION**

Pembrolizumab is approved in the USA for patients with R/M HNSCC\(^6\), however, not all patients respond

![Figure 4](image-url)
to treatment. Establishing patient-specific and tumor-specific factors that predict response can help identify patients likely to achieve clinical benefit and may accelerate the study of novel strategies for patients less likely to benefit from therapy. In the current study, we explored genomic determinants of response, including by HPV status, to pembrolizumab; this is a comprehensive analysis reporting the impact of TMB and inflammatory biomarkers (TcellinfGEP and PD-L1) on response to pembrolizumab in patients with HNSCC. Results indicate that higher mutation and inflammation levels are associated with improved propensity to respond.

In the overall population, our data show that TMB and TcellinfGEP were orthogonal, independently significant predictors of response. Responses were higher in patients with TMB≥175 mut/exome or TcellinfGEPnonlow tumors; a similar pattern was observed for PFS. OS was prolonged in the TcellinfGEPnonlow compared with the TcellinfGEPlow subgroups; no significant associations between TMB and OS were observed. TMB-H is associated with response and prolonged survival in patients receiving immune checkpoint inhibitors.

The distributions of TMB, TcellinfGEP, and PD-L1 were not dramatically different between HPV-positive and HPV-negative subgroups, and no significant statistical evidence supporting HPV-specific relationships between these biomarkers and clinical outcome was observed. In the small subset of patients with WES-defined HPV-positive tumors, TMB did not appear to be associated with response, possibly because of the dominance of viral neoepitopes not measured by WES compared with somatic neoepitopes captured by WES and potentially reflected by that fact that HPV-positive tumors exhibit a characteristic somatic mutation signature (apolipoprotein B editing catalytic polypeptide, potentially virally induced) more frequently than do HPV-negative tumors. Nevertheless, the general statistical shortcomings of evaluating biomarker distributions across small HPV subgroups must be acknowledged. The small sample size of the HPV-positive subgroup in this study precludes reliable conclusions, but emerging evidence on establishing HPV infection status to inform treatment decisions highlights its importance in HNSCC. HPV-positive HNSCC is associated with improved survival and response to cytotoxic therapy; it is now recommended that HPV status be reported before therapy is chosen. Similarly, in a recent pooled analysis, HPV-positive tumors were associated with greater clinical benefit from PD-1/PD-L1 inhibitors than were HPV-negative tumors. HPV infection promotes T-cell infiltration, immune effector cell activation, and T-cell receptor diversity in HNSCC, suggesting that HPV-positive tumors may be more amenable to immunotherapy. The current analysis, though limited by sample size, highlights the importance of evaluating genomic signatures in HNSCC and of trying to understand whether those genomic correlates of response operate similarly in their associations regardless of HPV status.

Genomic correlates of response to immune checkpoint inhibitors have been studied in several tumor types. An 18-gene TcellinfGEP predicted response to pembrolizumab across multiple solid tumor types, including HNSCC, and other T-cell inflammation/IFN-γ-related signatures are being developed. In metastatic melanoma, TMB, NL, and expression of cytolytic markers (genes encoding granzyme A and perforin) were predictors of clinical benefit with ipilimumab (anti-CTLA-4). In patients with advanced melanoma, TcellinfGEP predicted best overall response with anti-PD-1 but not with anti-CTLA-4 therapy.

The widely used method of detecting HPV infection—p16 IHC—is readily available and evaluable in pathology laboratories. p16 positivity is commonly defined as strong and diffuse nuclear and cytoplasmic staining in ≥70% of the tumor specimen, although there has been some debate with regard to the cut-off
and staining patterns. Potential problems exist with p16 IHC detection of HPV, which include p16 expression in 5% of HPV-negative head and neck cancers as well as p16 staining heterogeneity, false-positive staining, and lack of a standardized cut-off for positivity. Moreover, WES-defined and p16-defined HPV status demonstrated mutual exclusivity with TP53 mutation in this data set; such mutual exclusivity has been reported in major head and neck molecular studies, which include The Cancer Genome Atlas (TCGA). Increased sensitivity of NGS methods for HPV detection over p16 IHC was demonstrated for HNSCC, but no direct comparison was made. In the first study to describe large-scale WES in HNSCC, Stransky et al identified more HPV-positive cases by use of WES than by use of p16/CDKN2A detection. Further validation of the usefulness of WES could be achieved by determining whether the natural history of patients with WES-defined HPV-positive disease is the same as that for p16 IHC-defined patients. Because WES HPV genetic material detection is accurate, the absence of HPV genetic material is considered reliable for determining HPV-negative status; however, the presence of HPV DNA is considered less accurate than HPV RNA (the gold standard for HPV determination). Nevertheless, DNA-based and RNA-based methods showed similar accuracy in a TCGA analysis in HNSCC, and discrepancies between HPV DNA-based methods and p16 IHC similar to those found in our analysis have been observed.

Sample size is an important limitation of this study, particularly for subset analysis based on HPV status.
and attendant lack of power for exploring HPV-specific biomarker relationships. Further evaluation in larger studies will provide greater insight into potential interactions between mutation, inflammation, and HPV status in their association with response to pembrolizumab monotherapy.

Conclusion
TMB and inflammatory markers (Tcell_GEP and PD-L1) in HNSCC appear to represent distinct, fairly uncorrelated measures within the tumor microenvironment, providing complementary information for propensity to respond to pembrolizumab monotherapy. In this limited data set, TMB and inflammatory measures appeared to follow similar distributions in HPV-positive and HPV-negative tumors, and no evidence of HPV-specific relationships with clinical outcome for these biomarkers was observed. Evaluation in larger, randomized HNSCC studies will facilitate further understanding of the role TMB and inflammatory markers have as biomarkers of response to anti-PD-1 therapies in HNSCC and, when assessed separately or jointly, potentially aid in the identification of patients who will benefit from treatment.

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Contributors
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Patient consent for publication
Not required.

Ethics approval
The KEYNOTE-012 study was conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonization Good Clinical Practice guidelines and was approved by the institutional review boards or ethics committees at all sites. All patients provided written informed consent.

Provenance and peer review
Not commissioned; externally peer reviewed.

Data availability statement
Merck Sharp & Dohme Corp., a subsidiary of Merck & Co., Inc., Kenilworth, New Jersey, USA (MSD) is committed to providing qualified scientific researchers access to anonymized data and clinical study reports from the company’s clinical trials for the purpose of conducting legitimate scientific research. MSD is also obligated to protect the rights and privacy of trial participants, and as such, has a procedure in place for evaluating and fulfilling requests for sharing company clinical trial data with qualified external scientific researchers. The MSD data sharing website (available at: http://msd.com/clinical_trials/) outlines the process and requirements for submitting a data request. Applications will be promptly assessed for completeness and policy compliance. Feasible requests will be reviewed by a committee of MSD subject matter experts to assess the scientific value of the request and the qualifications of the requestors. In line with data privacy legislation, submissions of approved requests must enter into a standard data-sharing agreement with MSD before data access is granted. Data will be made available for request after

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Competing interests
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product approval in the US and EU or after product development is discontinued. There are circumstances that may prevent MSD from sharing requested data, including country or region-specific regulations. If the request is declined, it will be communicated to the investigator. Access to genetic or exploratory biomarker data requires a detailed, hypothesis-driven statistical analysis plan that is collaboratively developed by the requestor and MSD subject matter experts; after approval of the statistical analysis plan and execution of a data-sharing agreement, MSD will either perform the proposed analyses and share the results with the requestor or will construct biomarker covariates and add them to a file with clinical data that is uploaded to an analysis portal so that the requestor can perform the proposed analyses.

Supplemental material
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SUPPLEMENTARY FILE

RNA isolation and gene expression analysis

Total RNA was isolated from 5-μm–thick formalin-fixed, paraffin-embedded tumor sections using the Ambion RecoverAll kit (Thermo Fisher Scientific) according to the manufacturer’s instructions and was quantified using the NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific). Total RNA was mixed with a 3’ biotinylated capture probe and a 5’ reporter probe tagged with a fluorescent barcode from the custom gene expression code set. Probes and target transcripts were hybridized overnight at 65°C. Resultant samples were run on the NanoString nCounter platform (NanoString Technologies) using the high-sensitivity protocol and were analyzed using a custom code set consisting of a 680-gene panel related to T-cell biology, immune regulation, and cellular markers of tumor-infiltrating lymphocytes and tumor-associated macrophages. The samples were scanned at maximum resolution on the nCounter Digital Analyzer (NanoString Technologies).

Analysis of tumor mutational burden

Single-nucleotide variants

Whole exome sequence (WES) reads were aligned to reference human genome GRCh37; the method is described elsewhere.\textsuperscript{1-3} MuTect (v1.1.7-3-gcb7069b) from The Cancer Genome Analysis suite released by Appistry (http://www.appistry.com/) was used to generate somatic single-nucleotide variant (SNV) calls.\textsuperscript{4} Those present in the Single Nucleotide Polymorphism Database (dbSNP,v141)\textsuperscript{5} but not in the Catalogue of Somatic Mutations in Cancer (COSMIC496v68)\textsuperscript{6} were filtered out, as were SNVs with fewer than four mutant reads in tumor samples. Tumor mutational burden (TMB) for a patient was defined as the sum of somatic nonsynonymous SNVs remaining after the filtering process.
Clonality and clonality-weighted TMB

Cellular prevalence for all somatic SNVs was estimated by inputting somatic SNVs with variant allele frequency information obtained using MuTect and Sequenza output allele-specific copy number and cellularity estimation into PyClone software. Mutational clonality was inferred through the clustering process of PyClone. Clonality for each sample was determined using the method of McGranahan et al. Clonality-weighted TMB was calculated by multiplying the clonality by TMB as an estimate of the TMB restricted to clonal mutations.

SNV annotation and neoantigen detection

TMB was defined as nonsynonymous mutations in protein coding regions. Of the possible 9-mer peptide sequences with mutated amino acid inside for each nonsynonymous mutation locus, that with the highest binding affinity with the human leukocyte antigen (HLA) alleles from a nonsynonymous mutation locus was selected as the representative antigen for the mutation. HLA-I major loci, A, B, and C, were typed at four-digit resolution by using OptiType (v1.0) as previously described. Further, for output-typed alleles not found in the NetMHC (v3.4) input list, the corresponding supertype was identified for each allele and the supertype-representative allele was used for NetMHC. Neoantigens were defined as representative antigens with an HLA-A or HLA-B binding affinity of <50 nM.

Mutational signature analysis

Mutational signature analysis was performed using the deconstructSigs package (v1.6.0), and exome regions were defined by Sureselect V5 target region (Agilent). Only somatic mutations in exome regions were considered, and trinucleotide counts were normalized by the number of times each trinucleotide context was observed in the exome region. Mutational signatures, as defined in Alexandrov et al, were the target signature set to be screened, and the relationships
between clinical outcomes and these various mutational signatures, including specific nucleotide changes, DNA repair, smoking, neoantigen, tp53, and apolipoprotein B mRNA editing enzyme, were evaluated in samples across the pan-tumor cohort.

Assessment of tumor PD-L1 expression

Tumor programmed death ligand 1 (PD-L1) expression was assessed using PD-L1 IHC 22C3 pharmDx (Agilent) combined positive score (CPS), where CPS is defined as the ratio of the number of all PD-L1–staining cells (tumor cells, lymphocytes, macrophages) to the number of all viable tumor cells, multiplied by 100.

Association between TMB, T-cell–inflamed gene expression profile, and PD-L1 with clinical response

Agreement between p16 immunohistochemistry (IHC)-determined and WES-determined HPV status was established. Clinical response associations were assessed based on objective response rate (ORR) and progression-free survival (PFS) per RECIST v1.1. Associations of ORR, PFS, and overall survival with TMB, neoantigen load (NL), clonality, PD-L1 CPS, and T-cell–inflamed gene expression profile (TcellinfGEP) were assessed in all patients for whom transcriptomic data were available.

Determination of human papillomavirus status by whole exome sequencing

Cancer-associated DNA virus signals were identified from Exome-Seq data using virus reference genomes for 189 human papillomavirus (HPV) types from the Human Papillomavirus Episteme combined with the human reference genome hg19/GRCh37 as a chimeric reference genome. All Exome-Seq reads were mapped to this chimeric reference genome using BWA (v0.7.5a-r405) aln with default parameters. Using cohort B as a training set, a cutoff of 20 reads was identified.
by maximizing concordance of WES-based HPV with clinical HPV assay; subsequently, patients were classified as positive for a particular virus if ≥20 reads were properly mapped to the reference genome for this virus.

**Statistical analysis**

For ORR, a responder was defined as a patient with a “complete response” or a “partial response” as determined by RECIST v1.1, assessed by central review. Models include covariate terms for Eastern Cooperative Oncology Group performance status and cohorts. TMB and clonality-weighted TMB were evaluated on a log scale because of the skewness of TMB score. However, NL was evaluated on a square root scale because two patients had a zero count. PD-L1 was also evaluated on a square root scale. Joint modeling that incorporated both the TMB and an inflammatory biomarker (Tcell\(_{\text{int}}\)GEP or PD-L1) were conducted to establish whether TMB confers additional value after adjustment for the inflammatory biomarker.
Table S1  Baseline characteristics of the patients from cohorts B1 and B2 of the KEYNOTE-012 trial

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Overall(^a) N=192</th>
<th>WES(^b) n=106</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median (range), years</td>
<td>60 (20-84)</td>
<td>61 (25-83)</td>
</tr>
<tr>
<td>Male</td>
<td>159 (83)</td>
<td>87 (82)</td>
</tr>
<tr>
<td>ECOG PS 1</td>
<td>135 (70)</td>
<td>71 (67)</td>
</tr>
<tr>
<td>Metastatic staging (M1)</td>
<td>165 (86)</td>
<td>91 (86)</td>
</tr>
<tr>
<td>Prior therapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>34 (18)</td>
<td>21 (20)</td>
</tr>
<tr>
<td>1</td>
<td>41 (21)</td>
<td>18 (17)</td>
</tr>
<tr>
<td>2</td>
<td>45 (23)</td>
<td>33 (31)</td>
</tr>
<tr>
<td>≥3</td>
<td>72 (38)</td>
<td>34 (32)</td>
</tr>
<tr>
<td>HPV status: positive p16 IHC</td>
<td>45 (23)</td>
<td>25 (24)</td>
</tr>
<tr>
<td>HPV status: positive WES</td>
<td>NA</td>
<td>28 (26)</td>
</tr>
</tbody>
</table>

All values are n (%) unless stated otherwise.

ECOG PS, Eastern Cooperative Oncology Group performance status; HPV, human papillomavirus; IHC, immunohistochemistry; NA, not applicable; WES, whole exome sequencing.

\(^a\)Overall KEYNOTE-012 study cohort (B1 + B2).

\(^b\)Patients with available WES data.
**Table S2** HPV status by p16 immunohistochemistry and WES methods

<table>
<thead>
<tr>
<th>Method</th>
<th>HPV-negative (p16)</th>
<th>HPV-positive (p16)</th>
<th>Total (WES)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV-negative (WES)</td>
<td>71</td>
<td>7</td>
<td>78/106 (74%)</td>
</tr>
<tr>
<td>HPV-positive (WES)</td>
<td>10</td>
<td>18</td>
<td>28/106 (26%)</td>
</tr>
<tr>
<td>Total HPV (p16)(^b)</td>
<td>81/106 (76%)</td>
<td>25/106 (24%)</td>
<td>—</td>
</tr>
</tbody>
</table>

HPV, human papillomavirus; WES, whole exome sequencing.

\(^a\)Defined as >20 reads mapping to the HPV genome, all tumor sites.

\(^b\)Oropharynx.
Table S3  *TP53* mutation by HPV status using p16 IHC or WES methods

<table>
<thead>
<tr>
<th>Mutation</th>
<th>WES</th>
<th>p16 IHC</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HPV-negative</td>
<td>HPV-positive</td>
<td>HPV-negative</td>
</tr>
<tr>
<td><em>TP53wt</em></td>
<td>36</td>
<td>26</td>
<td>40</td>
</tr>
<tr>
<td><em>TP53mut</em></td>
<td>42</td>
<td>2</td>
<td>41</td>
</tr>
</tbody>
</table>

HPV, human papillomavirus; IHC immunohistochemistry; WES, whole exome sequencing.
REFERENCES


