European Journal of Cancer 116 (2019) 67–76

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejcancer.com

Original Research

Interference of tumour mutational burden with outcome of patients with head and neck cancer treated with definitive chemoradiation: a multicentre retrospective study of the German Cancer Consortium Radiation Oncology Group

T. Eder [a], A.K. Hess [i], R. Konschak [a,i], C. Stromberger [i], K. Jöhrens [j], V. Fleischer [i], M. Hummel [a,j], P. Balermpas [d,q], J. von der Grün [d,q], A. Linge [b,k,l,m], F. Lohaus [b,k,l,m], M. Krause [b,k,l,m,n], M. Baumann [b,k,l,m,n,o], M. Stuschke [c,p], D. Zips [h,v], A.L. Grosu [e,r], A. Abdollahi [f,s], J. Debus [f,s], C. Belka [g,t], S. Pigorsch [g,u], S.E. Combs [g,u], V. Budach [a,i], I. Tinhofer [a,i,*] for the DKTK-ROG

[a] *German Cancer Research Center (DKFZ), Heidelberg and German Cancer Consortium (DKTK) Partner Sites, Berlin, Germany*
[b] *German Cancer Research Center (DKFZ), Heidelberg and German Cancer Consortium (DKTK) Partner Sites, Dresden, Germany*
[c] *German Cancer Research Center (DKFZ), Heidelberg and German Cancer Consortium (DKTK) Partner Sites, Essen, Germany*
[d] *German Cancer Research Center (DKFZ), Heidelberg and German Cancer Consortium (DKTK) Partner Sites, Frankfurt, Germany*
[e] *German Cancer Research Center (DKFZ), Heidelberg and German Cancer Consortium (DKTK) Partner Sites, Freiburg, Germany*
[f] *German Cancer Research Center (DKFZ), Heidelberg and German Cancer Consortium (DKTK) Partner Sites, Heidelberg, Germany*
[g] *German Cancer Research Center (DKFZ), Heidelberg and German Cancer Consortium (DKTK) Partner Sites, Munich, Germany*
[h] *German Cancer Research Center (DKFZ), Heidelberg and German Cancer Consortium (DKTK) Partner Sites, Tübingen Germany*
[i] *Department of Radiooncology and Radiotherapy, Charité University Hospital, Berlin, Germany*
[j] *Institute of Pathology, Charité University Hospital Berlin, Berlin, Germany*
[k] *OncoRay – National Center for Radiation Research in Oncology, Faculty of Medicine and University Hospital Carl Gustav Carus, Technische Universität Dresden, Helmholtz-Zentrum Dresden – Rossendorf, Dresden, Germany*
[l] *Department of Radiotherapy and Radiation Oncology, Faculty of Medicine and University Hospital Carl Gustav Carus, Technische Universität Dresden, Germany*
[m] *National Center for Tumor Diseases (NCT), Partner Site Dresden, Germany*

* *Corresponding author*: Charité University Hospital Berlin, Department of Radiooncology and Radiotherapy Translational Radiation Oncology Research Laboratory, Charitéplatz 1, 10117 Berlin, Germany. Fax: +49 30 450 7 527 074.
E-mail address: ingeborg.tinhofer@charite.de (I. Tinhofer).

https://doi.org/10.1016/j.ejca.2019.04.015

[n] Helmholtz-Zentrum Dresden – Rossendorf, Institute of Radiooncology – OncoRay, Germany
[o] German Cancer Research Center (DKFZ), Heidelberg, Germany
[p] Department of Radiotherapy, Medical Faculty, University of Duisburg-Essen, Essen, Germany
[q] Department of Radiotherapy and Oncology, Goethe-University Frankfurt, Germany
[r] Department of Radiation Oncology, University of Freiburg, Germany
[s] Heidelberg Institute of Radiation Oncology (HIRO), National Center for Radiation Research in Oncology (NCRO), University of Heidelberg Medical School and German Cancer Research Center (DKFZ), Germany
[t] Department of Radiotherapy and Radiation Oncology, Ludwig-Maximilians-Universität, Munich, Germany
[u] Department of Radiation Oncology, Technische Universität München, Germany
[v] Department of Radiation Oncology, Faculty of Medicine and University Hospital Tübingen, Eberhard Karls Universität Tübingen, Germany

Received 20 November 2018; received in revised form 9 April 2019; accepted 12 April 2019
Available online 4 June 2019

KEYWORDS
Tumour mutational burden;
Targeted next-generation sequencing;
Concurrent chemoradiation;
Immune expression profile;
Prognosis;
Biomarker

Abstract ***Background:*** Tumour mutational burden (TMB) estimated from whole exome sequencing or comprehensive gene panels has previously been established as predictive factor of response to immune checkpoint inhibitors (ICIs). Its predictive value for the efficacy of concurrent chemoradiation (cCRTX), a potential combination partner of ICI, remains unknown.
Methods: The accuracy of TMB estimation by an in-house 327-gene panel was established in the Cancer Genome Atlas (TCGA) head and neck squamous cell carcinoma (HNSCC) data set. Interference of TMB with outcome after cCRTX was determined in a multicentre cohort of patients with locally advanced HNSCC uniformly treated with cCRTX. Targeted next-generation sequencing was successfully applied in 101 formalin-fixed, paraffin-embedded pre-treatment tumour samples. In a subset of cases (n = 40), tumour RNA was used for immune-related gene expression profiling by the nanoString platform. TMB was correlated with *TP53* genotype, human papilloma virus (HPV) status, immune expression signatures and survival parameters. Results were validated in the TCGA HNSCC cohort.
Results: A high accuracy of TMB estimation by the 327-gene panel was established. High TMB was significantly associated with an increased prevalence of *TP53* mutations and immune gene expression patterns unrelated to T cell–inflamed gene expression profiles. Kaplan-Meier analysis revealed significantly reduced overall survival in the patient group with high TMB (hazard ratio for death: 1.79, 95% confidence interval: 1.02–3.14; $P = 0.042$) which remained significant after correcting for confounding factors in the multivariate model. The prognostic value of TMB was confirmed in the TCGA HNSCC cohort.
Conclusion: High TMB identifies HNSCC patients with poor outcome after cCRTX who might preferentially benefit from CRTX-ICI combinations.

1. Introduction

The advent of chemotherapy to radiation has significantly improved outcome of locally advanced head and neck squamous cell carcinoma (HNSCC) [1]. As a result, concurrent chemoradiation (cCRTX) is the current standard of care for locally advanced unresectable disease. However, progression-free survival rates at 3 years of less than 40% in the intermediate-risk and high-risk patient subgroups [2] strongly underline the necessity of further treatment optimisation.

Recent promising results of programmed cell death protein-1 (PD-1) blockade in recurrent/metastatic (R/M) HNSCC [3–5] have stimulated the clinical development of antibodies to PD-1 or PD-1 ligand 1 as combination partner of CRTX in the curative setting. However, in unselected R/M HNSCC patient cohorts less than 25% of patients respond to immune checkpoint inhibitor (ICI) therapy, strongly underlining the urgent need for patient selection. Among the emerging biomarkers for ICI response are those related to tumour neo-epitope burden, such as microsatellite instability (MSI) or high tumour mutational burden (TMB). While the role of TMB for the efficacy of PD-1 blockade is well established in MSI+colon cancer and melanoma, and evidence is emerging in HNSCC [6], the interference of TMB with the efficacy of CRTX remains largely unknown.

In two recent targeted next-generation sequencing (tNGS) studies in locally advanced HNSCC, our group has evaluated the role of distinct somatic mutations for the efficacy of definitive [7] and adjuvant CRTX [8]. We could confirm previous reports of poor efficacy of radiotherapy in HNSCC tumours harbouring disruptive *TP53* mutations mainly enriched in HPV-negative carcinomas [9,10]. Here, using archival tumour samples from HNSCC patients who were included in a multicentre retrospective biomarker study of the German Cancer Consortium Radiation Oncology Group (DKTK-ROG) [11], we evaluated the role of TMB for outcome after cCRTX and its interference with the tumour immune micromilieu.

2. Material and methods

2.1. Patient samples

Patients with histologically proven locally advanced squamous cell carcinoma of the oral cavity (N = 27), oropharynx (N = 80) or hypopharynx (N = 51) and known HPV status (see supplementary information) from a retrospective multicentre DKTK-ROG biomarker study [11] were included in this study. The cohort comprised mainly HPV-negative cases since patients were diagnosed and treated between 2005 and 2011 when the incidence of HPV-positive tumours among oropharyngeal carcinomas was still low in Germany [12]. Treatment consisted of cCRTX according to standard protocols either based on cisplatin/5-fluorouracil (N = 129) or mitomycin-C/5-fluorouracil (N = 29). From the total patient cohort [11], assessment of TMB was possible in 101 of 158 (64%) cases. The flowchart depicting the sample selection for the biomarker analyses in this study is presented in Supplementary Fig. 1. Detailed patient characteristics are described in Supplementary Table 1.

2.2. Sequencing analysis

A detailed description of the isolation of genomic tumour DNA is given in the supplementary information. Mutational profiling was performed using an in-house gene panel targeting 327 genes (supplementary file 1) with the Haloplex^{HS} target enrichment system (Agilent, Santa Clara, CA). Sequencing analysis was carried out on the Illumina NextSeq500 platform (Illumina, San Diego, CA). Raw fastq files were further processed with Agilent SureCall software (version 3.5.1.46). A more detailed description of sequencing, data processing and analysis is provided in the supplementary information.

2.3. nanoString analysis

Immune-related mRNA expression profiles of tumour tissue samples were established using the nCounter PanCancer Immune Profiling Panel and the nCounter Digital Analyzer (nanoString Technologies, Seattle, WA) according to the manufacturer's protocol. RNA was isolated as described in the supplementary information. Data analysis using the nSolver Analysis Software 4.0 is described in more detail in the supplementary information. Raw and normalised expression data were deposited in Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) with accession number GSE122272.

2.4. Statistical analysis

The statistical analyses were performed using the SPSS software (v.25, SPSS Inc., IBM, Chicago, IL.). *P* values of <0.05 were considered statistically significant. TMB estimated by tNGS using our in-house 327-gene panel or the FoundationOne 315-gene panel were correlated with the mutational load determined by whole exome sequencing (WES) in the Cancer Genome Atlas (TCGA) project by linear regression analysis. Receiver operating characteristics (ROC) curve analysis was used to determine the accuracy of tNGS for patient stratification into low/high TMB groups, using TMB determined by WES as gold standard.

For assessment of the impact of TMB on prognosis, the following outcome parameters were used: overall survival (OS) where failure was defined as death because of any cause; locoregional failure-free survival (LRFFS), where failure was defined as local or regional progression and distant metastasis-free survival (DMFS) where failure was because of distant progression. Event times were measured from the date of diagnosis (OS) or the date of start of radiotherapy (LRFFS, DMFS) to the date of event occurrence or the last follow-up. Survival (OS, LRFFS, DMFS) was estimated using the Kaplan-Meier method. Survival of low and high TMB groups was compared using the log-rank test. Hazard ratios and interactions between risk parameters were estimated using Cox regression models.

3. Results

3.1. Estimation of tumour mutational burden using targeted next-generation sequencing

Previously, accurate estimation of TMB by tNGS using comprehensive gene panels covering at least 1 Megabase (Mb) per exome [13] has been reported. We first checked the suitability of our in-house HNSCC 327-gene panel covering ~1.5 Mb per exome for estimation of TMB and compared its performance to the FDA-approved FoundationOne 315-gene panel targeting ~1.1 Mb. For this purpose, we performed an *in-silico* analysis based on the TCGA HNSCC data set [14] and correlated the number of non-synonymous variants

detected in the genes covered by either of the two panels with the mutational load determined by WES. As seen in Fig. 1, a high correlation between the number of mutations detected by either tNGS or WES was observed (327-gene panel: $R^2 = 0.81$, 315-gene panel: $R^2 = 0.87$). The accuracy of a tNGS approach for

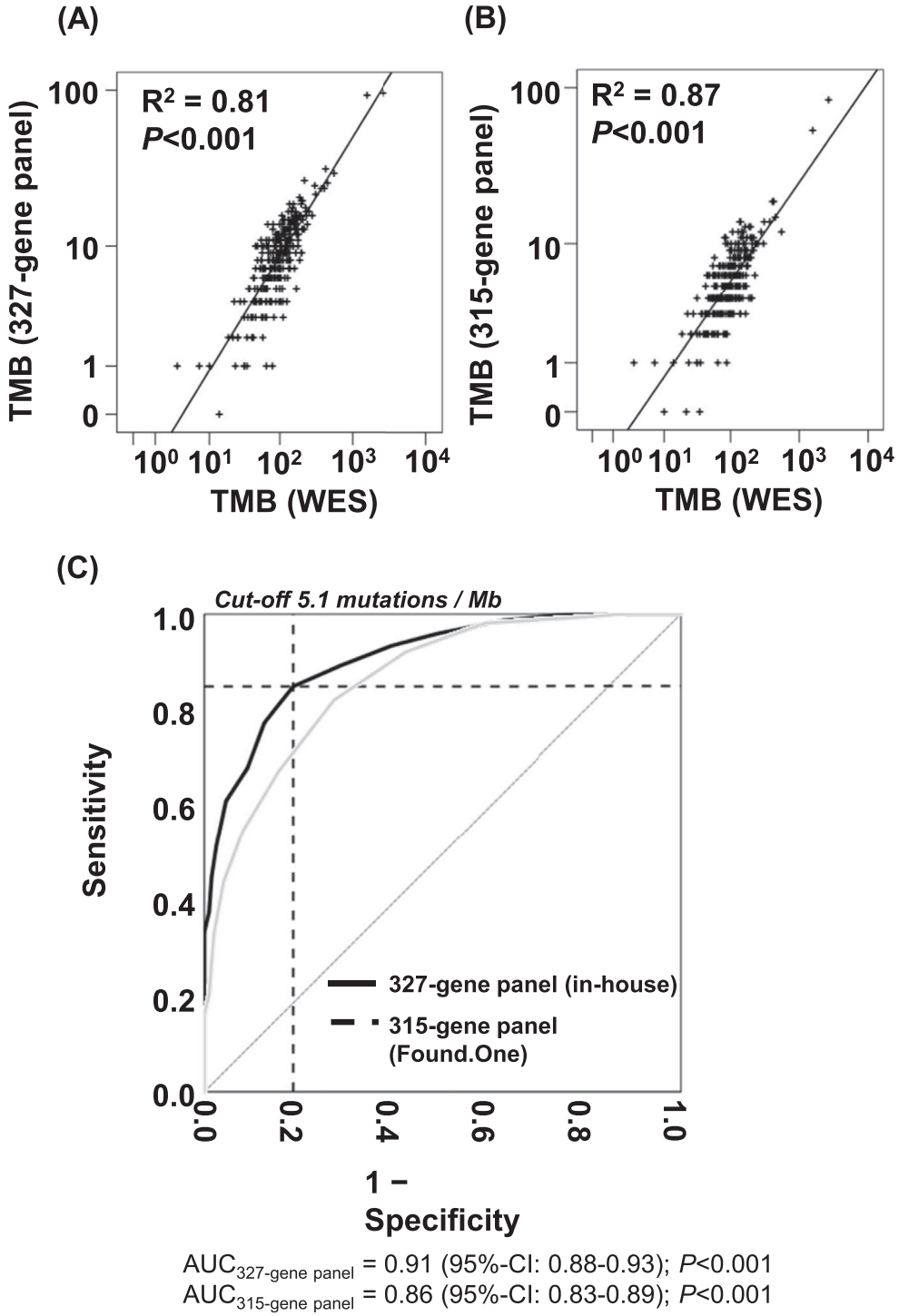

Fig. 1. TMB can be estimated from tNGS using comprehensive gene panels. The number of mutations found in genes covered by the in-house 327-gene panel (A) or the FoundationOne panel (B) was correlated with the overall number of non-synonymous mutations detected by WES in the TCGA HNSCC cohort (N = 510). (C) ROC curve analysis was used to determine sensitivity and specificity of the gene panels to stratify patients according to their TMB. As a gold standard, TMB determined by WES was used. AUC values, 95% confidence intervals and the cut-off for best discrimination between low and high TMB are given. TMB, tumour mutational burden; tNGS, targeted next-generation sequencing; WES, whole exome sequencing; TCGA, the Cancer Genome Atlas; HNSCC, head and neck squamous cell carcinoma; ROC, receiver operating characteristics; CI, confidence interval.

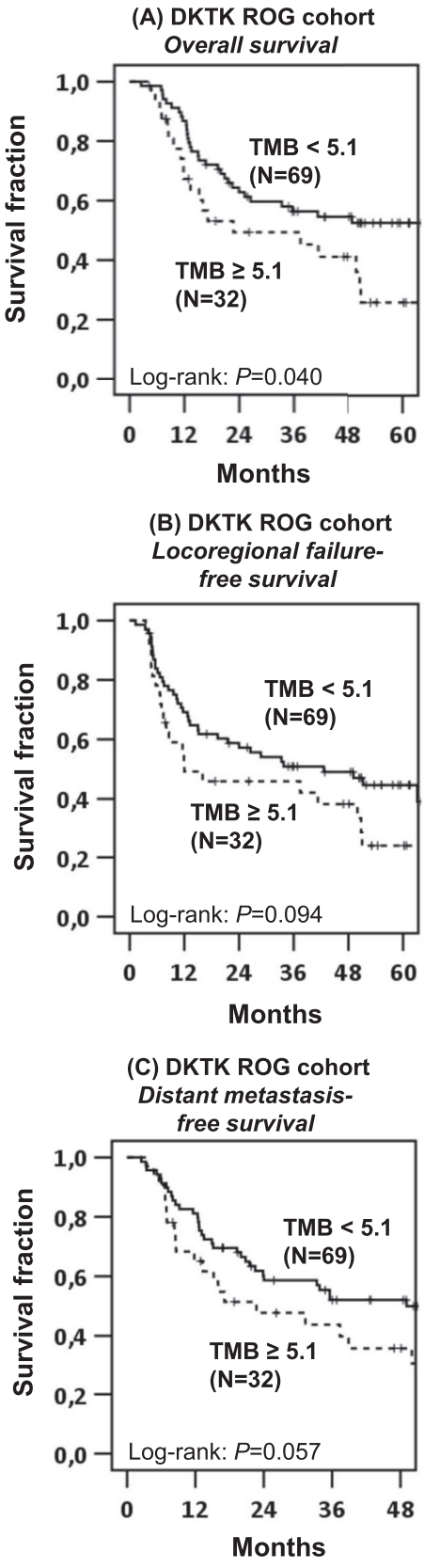

Fig. 2. TMB is a poor prognostic factor of survival after cCRTX. Patients from the DKTK-ROG cohort (including OPC, HPC and OCC cases) were stratified in two groups with high (≥5.1 mutations/Mb) and low (<5.1 mutations/Mb) TMB. Kaplan-Meier estimates of OS (A), LRFFS (B) and DMFS (C) for the TMB groups are presented. TMB, tumour mutational burden; cCRTX, efficacy of concurrent chemoradiation; DKTK-ROG, German Cancer Consortium Radiation Oncology Group; OS, overall survival; LRFFS, locoregional failure-free survival; DMFS, distant metastasis-free survival; OPC, oropharyngeal carcinoma; HPC, hypopharyngeal carcinoma; OCC, oral cavity carcinoma.

Table 1
Hazard ratios for overall survival, according to patient group.

Covariate	Hazard ratio	95% CI	*P* value
Stage (UICC IV versus III)	1.8	0.6 −5.1	0.257
TMB (high versus low)	1.9	1.1 −3.5	0.033
HPV status (HPV DNA-, p16-/discordant versus HPV DNA+, p16+)	2.8	0.9 −9.1	0.088
TP53 genotype (mutated versus wildtype)	0.6	0.3 −1.2	0.113

TMB, tumour mutational burden; CI, confidence interval.
Estimates for each covariate have been adjusted for all other covariates listed.

discrimination between patients with low versus high TMB was further determined by ROC analysis. Here, we used again TMB determined by WES as gold standard and the previously reported cut-off of 86 mutations per exome for classifying low and high TMB cases in HNSCC [6]. This analysis confirmed the high accuracy in estimating TMB based on mutational analysis by comprehensive cancer gene panels (327-gene panel: area under the curve (AUC) = 0.91; 315-gene panel: AUC = 0.86; Fig. 1C). ROC analysis based on the results from mutation analysis of the 327-gene panel revealed the highest sensitivity and specificity to stratify patients according to TMB at the value of 5.1 mutations/Mb. This value was used in all further analyses as cut-off for stratifying patients into low (<5.1 mutations/Mb) and high (≥5.1 mutations/Mb) TMB groups.

3.2. The value of TMB as a prognostic marker for outcome

Previously, chromosomal aberrations detected by comparative genomic hybridisation have been related to impaired treatment response [15] including reduced sensitivity to ionising radiation [16]. We here evaluated whether an increased number of somatic mutations also negatively interferes with the efficacy of cCRTX. Archival tumour samples from a multicentre DKTK-ROG biomarker study in which 158 patients with carcinomas of the oropharynx, hypopharynx or oral cavity uniformly treated with cCRTX had been included [11] were used for assessment of the prognostic role of TMB. tNGS using the 327-genel panel could be successfully applied to 101 samples. Detailed information

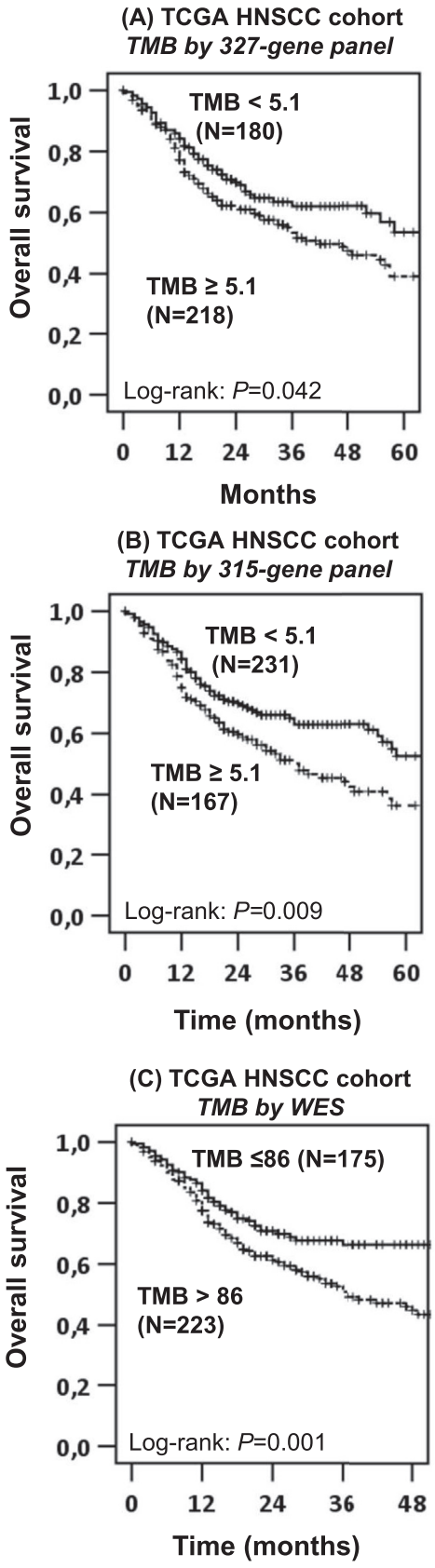

Fig. 3. Validation of the poor prognostic impact of high TMB in the TCGA HNSCC cohort. Cases with OPC, HPC and OCC of the TCGA HNSCC cohort (N = 398) were grouped according to their TMB estimated from mutational analysis of the genes covered by the in-house 327-gene panel (A), the FoundationOne 315-gene panel (B) or WES (C). The cut-off used for patient stratification and *P* values are given. TMB, tumour mutational burden; WES, whole exome sequencing; TCGA, the Cancer Genome Atlas; HNSCC, head and neck squamous cell carcinoma.

on the number of mutations per Mb, the detected single-nucleotide variants of the most frequently affected genes together with raw clinical and outcome parameter are provided in Supplementary Table 2. In 98% of the patient, at least one single nucleotide variant could be detected. The median number of mutations per patient was 7 translating into 4.7 mutations per Mb. Kaplan-Meier analysis revealed significantly reduced OS (log-rank: $P = 0.040$) in the patient group with high compared to low TMB (Fig. 2A) and a trend to reduced LRFFS ($P = 0.094$) and DMFS ($P = 0.057$) (Fig. 2B–C). The influence of TMB on OS remained significant in the multivariate Cox regression analysis after adjusting for potential confounding factors (Table 1).

The negative impact of high TMB on outcome after cCRTX is in contrast to the recent finding of an improved response of patients in this subgroup to PD-1 blockade [6,17]. For further validation of the negative association of high TMB with survival after standard non-immunomodulatory treatments, we used the TCGA HNSCC data set which comprises patients mainly treated with surgery alone or in combination with adjuvant radio(chemo)therapy. For better comparability with our results in the DKTK-ROG patient cohort, we only included patients with carcinomas of the oropharynx, hypopharynx or oral cavity. Consistently, the patient group with high TMB showed a significantly worse OS, irrespectively of whether results from mutation analysis of the gene panels (Fig. 3A and B) or WES (Fig. 3C) were used for TMB estimation. The independent prognostic value of TMB on OS in the TCGA HNSCC data set was confirmed by Cox regression analysis (Supplementary Table 3).

3.3. Differences in the mutation spectrum between patients with high and low TMB

After having established a negative association between high TMB and outcome after cCRTX, we next asked whether this could be explained by a more unfavourable mutation spectrum in this patient subgroup. Among the 327 analysed genes, 102 genes (31%) were commonly affected by mutations in both groups, whereas 105 genes (32%) were exclusively mutated in the high and 16 genes (5%) in the low TMB group (Supplementary Table 4). No selective enrichment of mutations in genes associated with cell cycle regulation and DNA damage repair was observed in the high compared with the low TMB group (Supplementary Table 4). As presented in Table 2 and Supplementary Table 2, the most frequently

Table 2
Frequency of mutations, according to TMB groups.

Affected Genes[a]	High TMB (n = 32)		Low TMB (n = 69)		*P* value[b]
	N	%	N	%	
TP53	22	69	29	42	0.033
SMARCA4	15	47	9	13	0.000
APC	12	38	8	12	0.008
ATRX	11	34	3	4	0.000
FBXW7	10	31	5	7	0.007
KDM6A	9	28	1	1	0.000
EGFR	8	25	1	1	0.000
MYH11	8	25	1	1	0.000
NOTCH1	8	25	4	6	0.017
PIK3R1	8	25	1	1	0.000
RB1	8	25	2	3	0.002
ATM	7	22	3	4	0.013
NF2	7	22	1	1	0.006
SMAD4	7	22	2	3	0.002

TMB, tumour mutational burden.
[a] Genes affected in ≥20% of cases in either the high-TMB or low-TMB group are listed.
[b] *P* values were adjusted for multiple testing using the Benjamini-Hochberg procedure.

mutated genes in either of the two groups were *TP53*, *SMARCA4* and *APC*. The significant enrichment of *TP53* mutations in cases with high TMB established in the DKTK cohort ($P = 0.033$, Table 2) was confirmed in the TCGA HNSCC data set ($P = 0.035$, Supplementary Fig. 2).

3.4. The mutational burden according to the immune signature of different tumour subsites

It has been postulated that a high number of mutations resulting in the expression of neoantigens promotes T-cell–mediated inflammation and consequently leads to the activation of immune checkpoints [18]. Hence, we determined if high TMB was associated with a distinct immune expression signature. For this analysis, only a subset of cases (n = 40) with sufficient tumour material for nanoString mRNA expression analysis was available. We excluded the HPV-positive cases (n = 3) because of their low number and the potential confounding factor of the viral infection on the immune expression pattern *per se*. Overall, only minor differences in the expression levels of the immune-related genes were observed between the low and high TMB groups. Specific features of immune checkpoint activation such as high CD8+ T-cell scores (Fig. 4A) and the interferon gamma (IFNG) signature (Fig. 4 B) were downregulated per trend in high compared with low TMB cases. In contrast, genes involved in neutrophil functions were found to be overexpressed in the high TMB group (Fig. 4 A).

4. Discussion

The mutational load determined by WES has previously been established as predictive biomarker of response to immune checkpoint therapy in some cancers including HNSCC [6,19–23]. Despite rapidly decreasing sequencing costs, the specific requirements for WES in terms of data storage capacity and experienced personnel for advanced bioinformatics analysis still represent a relevant hurdle in the introduction of WES for assessment of TMB in clinical routine diagnostics. Here, we corroborate previous evidence that estimation of TMB from comprehensive genomic profiling targeting the entire coding region of several hundred genes can accurately assess whole exome mutational burden [13,24,25]. Comparative analysis of our in-house HNSCC gene panel which we had specifically designed to capture mutations in HNSCC and the FoundationOne 315-gene panel which was developed as a pan-cancer diagnostic tool showed a similar performance of both panels. This suggests that the size of the captured genomic area rather than the selection of distinct gene loci determines the accuracy of TMB estimation. Our findings clearly indicate that TMB determined by a gene panel covering at least 1.1 Mb represents an accurate, cost-effective and clinically feasible tool for measuring TMB in HNSCC. This conclusion is further corroborated by a previous study in which comparison of TMB estimation by targeted gene panels versus WES demonstrated a relevant deviation in the measured compared to the expected mutation counts per MBase only if less than 0.5 Mbase was covered by the targeted NGS approach [13].

In one third of FFPE tumour samples in our cohort, target NGS analysis was not possible due to poor quality of DNA. This high drop-out rate can be explained by the long storage (>6 years) of FFPE samples which is known to negatively affect DNA yield and integrity [26]. No such limitations are to be expected for routine TMB assessment if fresh FFPE samples will be used for targeted NGS. Indeed, quality assessment of clinical mutation detection in a large series of fresh FFPE neoplastic tissues revealed that NGS assays were successfully conducted and reported in more than 95% of specimens [27].

Using TMB assessment by our in-house HNSCC gene panel, we showed for the first time that high TMB identifies patients with reduced OS and is associated with a lower efficacy of cCRTX in terms of locoregional and distant tumour control. Although high TMB was significantly associated with the occurrence of *TP53* mutations, its negative influence on outcome was independent of this important risk parameter. Importantly, for definition of high TMB, we used a cut-off of >86 mutations per exome which was used for identification of patients with prolonged PFS under pembrolizumab

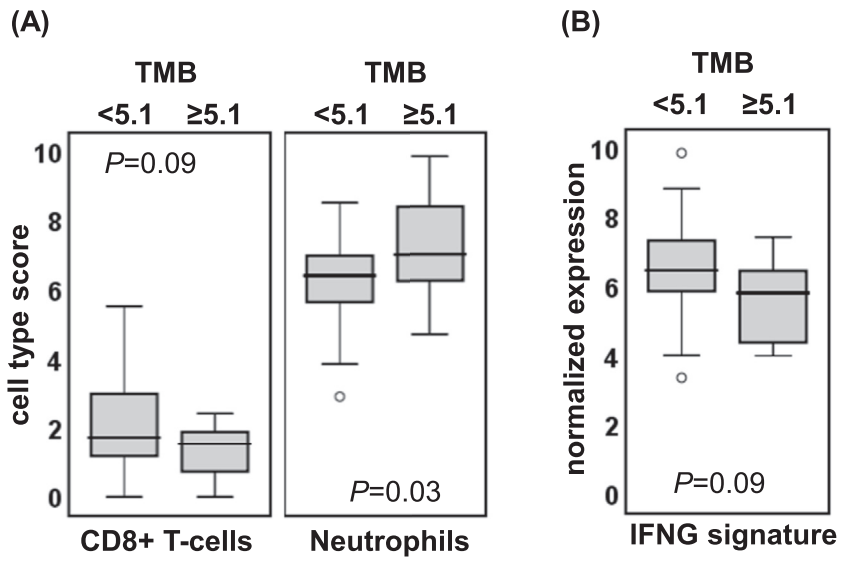

Fig. 4. High TMB is associated with unfavourable immune expression signatures. NanoString mRNA expression analysis of immune-related genes was performed in the HPV-negative DKTK-ROG cohort (N = 37). Cell type scores (i.e. the average log2 expression of cell type-specific marker genes) associated per trend with reduced CD8+ T-cells ($P = 0.09$) and lower expression of the IFNG signature as well as significantly elevated neutrophil infiltrates ($P = 0.03$) were observed in the high compared with the low TMB groups. TMB, tumour mutational burden; DKTK-ROG, German Cancer Consortium Radiation Oncology Group; IFNG, interferon gamma; <5.1, low TMB group harboring <5.1 mutations/Mb; ≥5.1, high TMB group harbouring ≥5.1 mutations/Mb.

treatment in the Keynote-012 trial [6,17]. Given the higher response rates to ICI in HNSCC patients with high TMB in the R/M setting, combination of CRTX with immune-modulating therapy might be an interesting option for optimisation of curative treatment for this patient subgroup.

In previous studies, a higher extent of CD8+ T-cell infiltration in pretreatment tumour samples has been identified as a biomarker for a more favourable clinical outcome after CRTX [28,29]. High mutational load has been causally linked to immune cell activation and upregulation of immune checkpoint pathways [18]. We therefore assessed whether TMB interferes with immune cell–related expression patterns but failed to observe a strong correlation between the two features. This is in line with results from a recent study in which the establishment of a robust threshold in the mutational load associated with evidence of immune checkpoint activation was not possible for HNSCC, while it was successful in eight other solid cancer types in TCGA including cutaneous melanoma, lung, colon and bladder cancer [18]. The reason for disqualifying as a robust model in this study was not the absence of an association between mutational load and immune checkpoint activation in HNSCC but the low relative number of cases (<5%) above the immune checkpoint-activating mutation threshold in this disease entity [18]. An absence of any correlation between TMB and immune cell–related gene expression profiles in HNSCC was also reported for HPV-negative oral squamous cell carcinoma from never-smokers and never-drinkers [30] as well as a biomarker study in patient cohorts of the Keynote-012 trial [6]. This lack of correlation, combined with the observed individual predictive values, suggested that TMB and immune cell–related gene expression profiles are independent predictive measures of response to pembrolizumab. Together with the results from our study, these data further imply that both TMB and immune expression signatures might have potential diagnostic utility for identifying patients with benefit from CRTX and CRTX-ICI combinations.

Sensitivity of tumours to radiation is not only influenced by the overall load of mutations or the occurrence of distinct mutant variants but is also affected by gene expression. Recently, a gene expression-based radiation-resistance score was established which was associated with poor disease-free survival in HPV-negative HNSCC patients treated by surgery and radiotherapy (with or without chemotherapy) but not in patients treated with surgery alone [31]. The use of a radiation resistance score in combination with TMB and immune signatures might allow further refinement of patient selection for CRTX-ICI combinations.

5. Conclusion and outlook

The major strength of our study is the analysis of the prognostic value of TMB in a uniformly treated HNSCC patient cohort and the independent validation of our findings in the TCGA data set. Limitations are the lack of information on potential confounding factors such as performance status and smoking because of the retrospective nature of the study and the small

number of HPV-positive cases in the DKTK-ROG cohort. The value of TMB assessment by our comprehensive gene panel and the established cut-off of 5.1 mutations/Mb for selection of patients with potential preferential benefit from CRTX-ICI combinations will need to be rigorously evaluated in prospective trials. These studies should include sequencing of germline DNA because our approach of filtering against public collections of somatic alterations in cancer for correction for germline variants—though being perfectly able to unravel driver mutations—might be less pertinent when looking also for passenger mutations for TMB calculation. We propose that future studies should also evaluate whether the inclusion of distinct genes associated with resistance to or higher efficacy of PD-1 blockade such as *JAK1* or *JAK2* [32] or *MLH1*, *MSH2*, *MSH6* and *PMS2* [21], respectively, in tNGS panels might additionally improve biomarker-based patient selection in this setting.

Conflicts of interest statement

The authors declare no potential conflicts of interest.

Funding

This study was financed by a Joint Funding Grant within the German Cancer Consortium (DKTK) which was given to the DKTK-ROG (principal investigator: M. Baumann). The DKTK is funded as one of the National German Health Centres by the Federal German Ministry of Education and Research.

See manuscript for details and attached ICMJE forms.

Acknowledgements

The assistance of the radiation oncologists and pathologists from the clinical centres in retrospective FFPE sample collection is gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejca.2019.04.015.

References

[1] Pignon JP, le Maitre A, Maillard E, Bourhis J. Meta-analysis of chemotherapy in head and neck cancer (MACH-NC): an update on 93 randomised trials and 17,346 patients. Radiother Oncol 2009;92(1):4–14.

[2] Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tan PF, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. N Engl J Med 2010;363(1):24–35.

[3] Ferris RL, Blumenschein Jr G, Fayette J, Guigay J, Colevas AD, Licitra L, et al. Nivolumab for recurrent squamous-cell carcinoma of the head and neck. N Engl J Med 2016;375(19):1856–67.

[4] Ferris RL, Blumenschein Jr G, Fayette J, Guigay J, Colevas AD, Licitra L, et al. Nivolumab vs investigator's choice in recurrent or metastatic squamous cell carcinoma of the head and neck: 2-year long-term survival update of CheckMate 141 with analyses by tumor PD-L1 expression. Oral Oncol 2018;81:45–51.

[5] Seiwert TY, Burtness B, Mehra R, Weiss J, Berger R, Eder JP, et al. Safety and clinical activity of pembrolizumab for treatment of recurrent or metastatic squamous cell carcinoma of the head and neck (KEYNOTE-012): an open-label, multicentre, phase 1b trial. Lancet Oncol 2016;17(7):956–65.

[6] Cristescu R, Mogg R, Ayers M, Albright A, Murphy E, Yearley J, et al. Pan-tumor genomic biomarkers for PD-1 checkpoint blockade-based immunotherapy. Science 2018;362(6411).

[7] Tinhofer I, Stenzinger A, Eder T, Konschak R, Niehr F, Endris V, et al. Targeted next-generation sequencing identifies molecular subgroups in squamous cell carcinoma of the head and neck with distinct outcome after concurrent chemoradiation. Ann Oncol 2016;27(12):2262–8.

[8] Tinhofer I, Budach V, Saki M, Konschak R, Niehr F, Johrens K, et al. Targeted next-generation sequencing of locally advanced squamous cell carcinomas of the head and neck reveals druggable targets for improving adjuvant chemoradiation. Eur J Cancer 2016;57:78–86.

[9] Lindenbergh-van der Plas M, Brakenhoff RH, Kuik DJ, Buijze M, Bloemena E, Snijders PJ, et al. Prognostic significance of truncating TP53 mutations in head and neck squamous cell carcinoma. Clin Cancer Res 2011;17(11):3733–41.

[10] Skinner HD, Sandulache VC, Ow TJ, Meyn RE, Yordy JS, Beadle BM, et al. TP53 disruptive mutations lead to head and neck cancer treatment failure through inhibition of radiation-induced senescence. Clin Cancer Res 2012;18(1):290–300.

[11] Linge A, Lohaus F, Lock S, Nowak A, Gudziol V, Valentini C, et al. HPV status, cancer stem cell marker expression, hypoxia gene signatures and tumour volume identify good prognosis subgroups in patients with HNSCC after primary radiochemotherapy: a multicentre retrospective study of the German Cancer Consortium Radiation Oncology Group (DKTK-ROG). Radiother Oncol 2016;121(3):364–73.

[12] Tinhofer I, Johrens K, Keilholz U, Kaufmann A, Lehmann A, Weichert W, et al. Contribution of human papilloma virus to the incidence of squamous cell carcinoma of the head and neck in a European population with high smoking prevalence. Eur J Cancer 2015;51(4):514–21.

[13] Chalmers ZR, Connelly CF, Fabrizio D, Gay L, Ali SM, Ennis R, et al. Analysis of 100,000 human cancer genomes reveals the landscape of tumor mutational burden. Genome Med 2017;9(1):34.

[14] Comprehensive genomic characterization of head and neck squamous cell carcinomas. Nature 2015;517(7536):576–82.

[15] Bauer VL, Braselmann H, Henke M, Mattern D, Walch A, Unger K, et al. Chromosomal changes characterize head and neck cancer with poor prognosis. J Mol Med (Berl) 2008;86(12):1353–65.

[16] Ashman JN, Patmore HS, Condon LT, Cawkwell L, Stafford ND, Greenman J. Prognostic value of genomic alterations in head and neck squamous cell carcinoma detected by comparative genomic hybridisation. Br J Canc 2003;89(5):864–9.

[17] Haddad RI, Seiwert TY, Chow LQM, Gupta S, Weiss J, Gluck I, et al. Genomic determinants of response to pembrolizumab in head and neck squamous cell carcinoma (HNSCC). J Clin Oncol 2017;35(15_suppl). 6009-6009.

[18] Panda A, Betigeri A, Subramanian K, Ross JS, Pavlick DC, Ali S, et al. Identifying a clinically applicable mutational burden threshold as a potential biomarker of response to immune

checkpoint therapy in solid tumors. JCO Precis Oncol 2017;(1):1–13.
[19] Snyder A, Makarov V, Merghoub T, Yuan J, Zaretsky JM, Desrichard A, et al. Genetic basis for clinical response to CTLA-4 blockade in melanoma. N Engl J Med 2014;371(23):2189–99.
[20] Van Allen EM, Miao D, Schilling B, Shukla SA, Blank C, Zimmer L, et al. Genomic correlates of response to CTLA-4 blockade in metastatic melanoma. Science 2015;350(6257):207–11.
[21] Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, et al. PD-1 blockade in tumors with mismatch-repair deficiency. N Engl J Med 2015;372(26):2509–20.
[22] Rizvi NA, Hellmann MD, Snyder A, Kvistborg P, Makarov V, Havel JJ, et al. Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. Science 2015;348(6230):124–8.
[23] Rosenberg JE, Hoffman-Censits J, Powles T, van der Heijden MS, Balar AV, Necchi A, et al. Atezolizumab in patients with locally advanced and metastatic urothelial carcinoma who have progressed following treatment with platinum-based chemotherapy: a single-arm, multicentre, phase 2 trial. Lancet 2016;387(10031):1909–20.
[24] Campesato LF, Barroso-Sousa R, Jimenez L, Correa BR, Sabbaga J, Hoff PM, et al. Comprehensive cancer-gene panels can be used to estimate mutational load and predict clinical benefit to PD-1 blockade in clinical practice. Oncotarget 2015;6(33):34221–7.
[25] Johnson DB, Frampton GM, Rioth MJ, Yusko E, Xu Y, Guo X, et al. Targeted next generation sequencing identifies markers of response to PD-1 blockade. Cancer Immunol Res 2016;4(11):959–67.
[26] Watanabe M, Hashida S, Yamamoto H, Matsubara T, Ohtsuka T, Suzawa K, et al. Estimation of age-related DNA degradation from formalin-fixed and paraffin-embedded tissue according to the extraction methods. Exp Ther Med 2017;14(3):2683–8.
[27] Zheng G, Tsai H, Tseng L-H, Illei P, Gocke CD, Eshleman JR, et al. Test feasibility of next-generation sequencing assays in clinical mutation detection of small biopsy and fine needle aspiration specimens. Am J Clin Pathol 2016;145(5):696–702.
[28] Balermpas P, Michel Y, Wagenblast J, Seitz O, Weiss C, Rodel F, et al. Tumour-infiltrating lymphocytes predict response to definitive chemoradiotherapy in head and neck cancer. Br J Canc 2014;110(2):501–9.
[29] Balermpas P, Rodel F, Rodel C, Krause M, Linge A, Lohaus F, et al. CD8+ tumour-infiltrating lymphocytes in relation to HPV status and clinical outcome in patients with head and neck cancer after postoperative chemoradiotherapy: a multicentre study of the German cancer consortium radiation oncology group (DKTK-ROG). Int J Cancer 2016;138(1):171–81.
[30] Puisieux A, Caux C, Michallet MC, Bendriss-Vermare N, Foy JP, Saintigny P, et al. The immune microenvironment of HPV-negative oral squamous cell carcinoma from never-smokers and never-drinkers patients suggests higher clinical benefit of Ido1 and PD1/PD-L1 blockade. Ann Oncol 2017;28(8):1934–41.
[31] Foy J-P, Bazire L, Ortiz-Cuaran S, Deneuve S, Kielbassa J, Thomas E, et al. A 13-gene expression-based radioresistance score highlights the heterogeneity in the response to radiation therapy across HPV-negative HNSCC molecular subtypes. BMC Med 2017;15(1):165.
[32] Shin DS, Zaretsky JM, Escuin-Ordinas H, Garcia-Diaz A, Hu-Lieskovan S, Kalbasi A, et al. Primary resistance to PD-1 blockade mediated by JAK1/2 mutations. Cancer Discov 2017;7(2):188–201.