Aus der Klinik für Radioonkologie und Strahlentherapie
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

# Characterization of the molecular mechanisms of resistance to radiochemotherapy of head and neck squamous cell carcinoma

zur Erlangung des akademischen Grades

Doctor rerum medicinalium (Dr. rer. medic.)

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# 1. Zusammenfassung

Das Plattenepithelkarzinom im Kopf-Hals Bereich zählt zu den sechs häufigsten bösartigen Tumoren weltweit mit einer Inzidenz von mehr als 650.000 Fällen pro Jahr und 330.000 Sterbefälle weltweit. Tumore, die sich in einem frühen Stadium befinden, werden primär operiert oder bestrahlt. Lokal fortgeschrittene Tumore werden mit einer radikalen Resektion, gefolgt von einer adjuvanten Radiotherapie (RT)/ Radiochemotherapie (RCT) oder einer primären RCT behandelt, wobei die lokoregionäre Rezidivrate bei 40% liegt. Die Gabe von Immuncheckpoint-Inhibitoren (ICI) stellt derzeit einen neuen Therapieansatz für Patienten mit rezidivierender, metastasierter Erkrankung dar. Ein wichtiger prädiktiver Faktor für das Ansprechen auf diese Therapie ist die Tumormutationslast (TML), welche entweder mittels Whole-Exome Sequenzierung (WES) oder Gen-Panels bestimmt werden kann. In der vorliegenden Studie wurde der prädiktive Wert der TML für die Wirksamkeit einer definitiven Radiochemotherapie (cCRTX-englisch: concurrent chemoradiation), welche als potentieller Kombinationspartner von ICI gesehen werden kann, untersucht.

Ein 327 Gene umfassendes und spezifisch für die Analyse von Tumoren im Kopf-Hals Bereich (HNSCC – englisch: head and neck squamous cell carcinoma) designtes Gen Panel wurde im Rahmen dieser Arbeit etabliert. Um die Präzision der TML Berechnung mittels eines Gen-Panels zu bestimmen, wurde zuerst der WES HNSCC Datensatz aus dem Cancer Genome Atlas (TCGA) verwendet. Anhand des 327-Gen Panel konnte die TML mit einer hohen Präzision berechnet werden. In einer multizentrischen Kohorte von Patienten mit lokal fortgeschrittenem HNSCC, welche einheitlich mittels cCRTX behandelt wurde, wurde die Interferenz der TML mit der Wirksamkeit einer cCRTX bestimmt. Von den insgesamt 158 Patienten konnten 101 Formalin-fixierte Paraffin-eingebettete (FFPE) Tumorproben, welche vor der Behandlung entnommen wurden, mittels gezielter Next Generation Sequenzierung (tNGS) analysiert werden. In 40 Fällen wurde zudem aus der Tumor RNA ein Genexpressionsprofil von Immun-assoziierten Genen mittels der nanoString Plattform erstellt.

Die TML wurde mit dem *TP53* Genotyp, dem HPV-Status, der Immun-Expressions-Signatur und verschiedenen Überlebensparametern korreliert. Auch für die Validierung der Ergebnisse wurde die TCGA HNSCC Kohorte verwendet.

Eine hohe TML zeigte eine signifikante Assoziation mit einer erhöhten Prävalenz von Mutationen im *TP53* Gen und Immungen-Expressionsmustern, welche unabhängig vom

inflammatorischen T-Zell Genexpressionsprofil waren. Außerdem zeigte sich eine signifikante Reduktion im Gesamtüberleben in der Patientengruppe mit hoher TML, was auch im multivariaten Modell bestätigt werden konnte. Der prognostische Wert der TML konnte in der TCGA HNSCC Kohorte bestätigt werden.

Zusammenfassend konnte in dieser Doktorarbeit gezeigt werden, dass HNSCC Patienten mit hoher TML eine schlechte Wirksamkeit der cCRTX aufweisen und womöglich von einer CRTX-ICI Kombinationsbehandlung profitieren würden.

## 2. Abstract

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignancy worldwide with an incidence of more than 650 000 cases per year and 330 000 cancer deaths worldwide. These tumors mainly arise in the squamous epithelium within the oral cavity, pharynx and salivary glands. Major risk factors are smoking, alcohol consumption and infection with human papilloma virus (HPV). Surgery or radiotherapy (RT) serve as a standard treatment for early stage tumors, while locoregionally advanced tumors are treated with radical surgery followed by subsequent adjuvant RT/chemoradiotherapy (CRT) or primary CRT. Despite advances in CRT, survival rates still remain poor with locoregional recurrences in up to 40% of patients. Novel therapeutic strategies like the administration of immune checkpoint inhibitors (ICI) in the recurrent and metastatic setting have been recently approved by the FDA. Tumor mutational burden (TMB) estimated from whole exome sequencing (WES) or comprehensive gene panels has previously been established as predictive factor of response to ICI. In the present study, TMB and its predictive value for the efficacy of concurrent chemoradiation (cCRTX), a potential combination partner of ICI was investigated.

The accuracy of TMB estimation by an in-house head and neck cancer-specific 327-gene panel was established in the Cancer Genome Atlas (TCGA) HNSCC WES dataset. A high accuracy of TMB estimation by the 327-gene panel could be observed. Subsequently, the interference of TMB with outcome after cCRTX was determined in a multicenter cohort including 158 patients with locally advanced HNSCC uniformly treated with cCRTX. Targeted next-generation sequencing (tNGS) was successfully applied in 101 formalin-fixed, paraffin-embedded pretreatment tumor samples. In a subset of cases (n=40), tumor RNA was used for immune related gene expression profiling by the nanoString platform. TMB was correlated with *TP53* genotype, HPV status, immune expression signatures and survival parameters. Results were validated in the TCGA HNSCC cohort.

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 which remained significant after correcting for confounding factors in the multivariate model. The prognostic value of TMB was confirmed in the TCGA HNSCC cohort.

In summary, it could be demonstrated that high TMB identifies HNSCC patients with poor outcome after cCRTX who might potentially benefit from CRTX-ICI combination.

# 3. Manteltext

Head and neck squamous cell carcinoma (HNSCC) represents the 6th most common cancer worldwide with an annual incidence of 0.65 million new cases, 0.33 million deaths worldwide and 140 000 newly diagnosed cases in Europe (1, 2). Main risk factors associated with HNSCC are tobacco and alcohol use (3). HNSCC can also be divided in two major subgroups, the human papilloma virus (HPV)-positive driven cancers, which are associated with a better prognosis, and HPV-negative cancers (4).

Prognosis is still poor with 5-year survival rates ranging from 25% (hypo-and pharyngeal carcinoma) to 60% (laryngeal carcinoma) depending on the stage of disease and HPV status (2, 5). Initially, half of patients with HNSCC are diagnosed already at a locally advanced stage (2). Standard treatment involves different combinations of surgery, radiotherapy and chemotherapy. The addition of chemotherapy to radiation has significantly improved outcome of locally advanced HNSCC (6). Still, progression-free survival rates at 3 years in the intermediate-risk and high-risk patient subgroups are low, making optimization of treatment crucial (7, 8).

Focusing on targeted therapy, agents approved for HNSCC are the epidermal growth factor receptor (EGFR) targeted antibody cetuximab and programmed death receptor-1 (PD-1) antibodies nivolumab and pembrolizumab (9, 10). Recent studies could show promising results for administration of PD-1 antibodies in recurrent/ metastatic (R/M) HNSCC (9-11). The combination of PD-1 or PD-1 ligand 1 antibodies with CRTX in the curative setting is under clinical investigation (12). As in other tumor entities, there is also an urgent need for patient selection in R/M HNSCC, since response to immune checkpoint inhibitor (ICI) therapy is low in unselected patient cohorts (9-11, 13). In the KEYNOTE-012 study the overall response rate (ORR) in the initial cohort was 18% (13). A similar rate was also reported in the expansion cohort after a median follow-up of 9 months (13). Responses to ICI are influenced by the tumor neo-epitope burden, associated with microsatellite instability (MSI) and/or high tumor mutational burden (TMB). In MSI-positive colon cancer and melanoma the role of TMB for the efficacy of PD-1 blockade is well established with growing evidence in HNSCC too (14). However, the interference of TMB with the efficacy of CRTX remains largely unknown (8). It has been shown in previous studies, that a higher extent of CD8+ T-cell infiltration in pretreatment tumor samples has been identified as a biomarker for a more favorable clinical outcome after CRTX (8, 15, 16). Immune cell activation and upregulation of

immune checkpoint pathways have been linked to high mutational load (17). Therefore, it was assessed whether TMB interferes with immune-cell related expression patterns. An absence of any correlation between TMB and immune-cell related gene expression profiles in HNSCC was reported for HPV-negative oral squamous cell carcinoma from never-smokers and never-drinkers (18) as well as a biomarker study in patient cohorts of the Keynote-012 trial (8, 14). Only a modest correlation of TMB and immune-cell related gene expression patterns could be observed as well as the independent predictive value of these two factors of response to pembrolizumab. Both immune cell-related gene expression and TMB or the occurrence of distinct mutant variants can influence the sensitivity of tumors to radiation. 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 (8, 19). 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.

Publication of data from “The Cancer Genome Atlas” (TCGA) project included a large amount of data from the transcriptome, proteome and genome level in HNSCC (20). Still, we are lacking reliable biomarkers to be used in clinical routine, not only in terms of ICI therapy. The mutational load has been established as predictive biomarker of response to immune checkpoint therapy in a variety of cancers including HNSCC by whole-exome sequencing (WES) data (8, 14, 21-25). We developed a gene panel, including 327 genes associated with tumors in the head and neck region. In order to define the set of genes which are frequently altered in HNSCC and which should thus be included in the gene panel for TMB measurement, we analyzed 412 WES datasets from HNSCC from three independent patient cohorts in cBioPortal (Fig. 1).

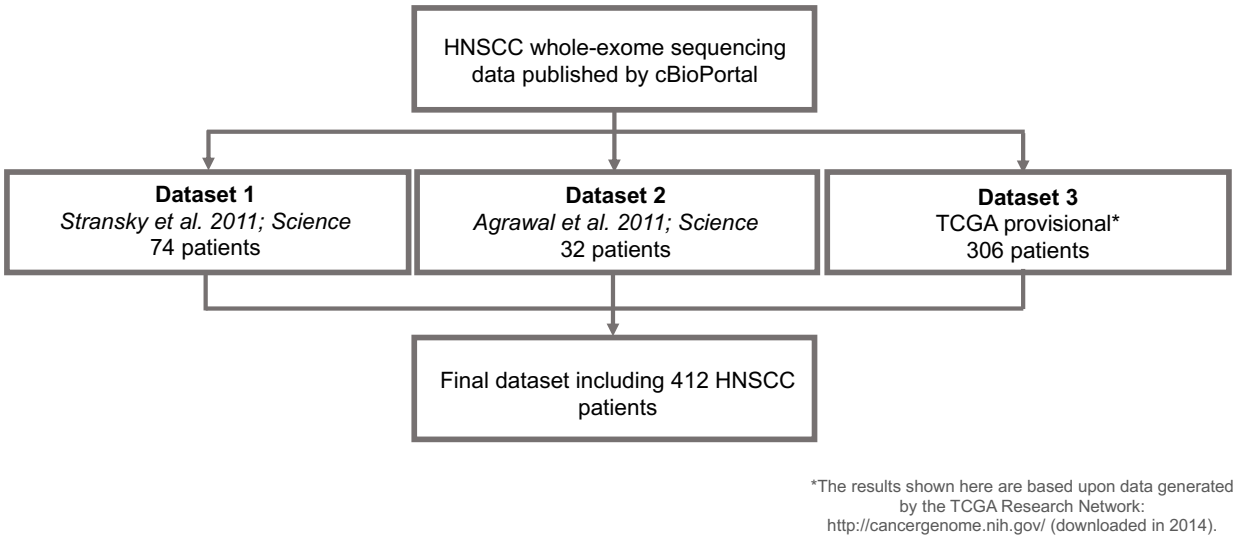



**Figure 1.** WES data from the cBioPortal including a total of 412 HNSCC cases were analyzed which were derived from three independent patient cohorts (26-30). In all datasets sequenced tumor DNA was matched against DNA from blood or healthy tissue for correction for germline variants. WES, whole-exome sequencing; TCGA, The Cancer Genome Atlas; HNSCC, head and neck squamous cell carcinoma.

As seen in Fig. 2, initial filtering included genes either mutated in more than 3% of the patients or genes fulfilling two out of three of the following criteria: (1) the gene is mutated in >1% of the patients; (2) the gene is affected by recurrent mutations that are seen in more than one patient; (3) the gene represents known hotspots mutations. After this first filtering step, 833 of 15 293 genes were left. Following, genes which were not mapped to biological pathway or had a GO annotation were excluded. After filtering out those genes with less than ten Pubmed listed publications, only 183 genes were left. Finally, significantly mutated genes from MutSig (31) and Cosmic database (32, 33) as well as handpicking of genes by a head and neck cancer expert resulted in a panel of 327 genes which represented the final HNSCC panel.

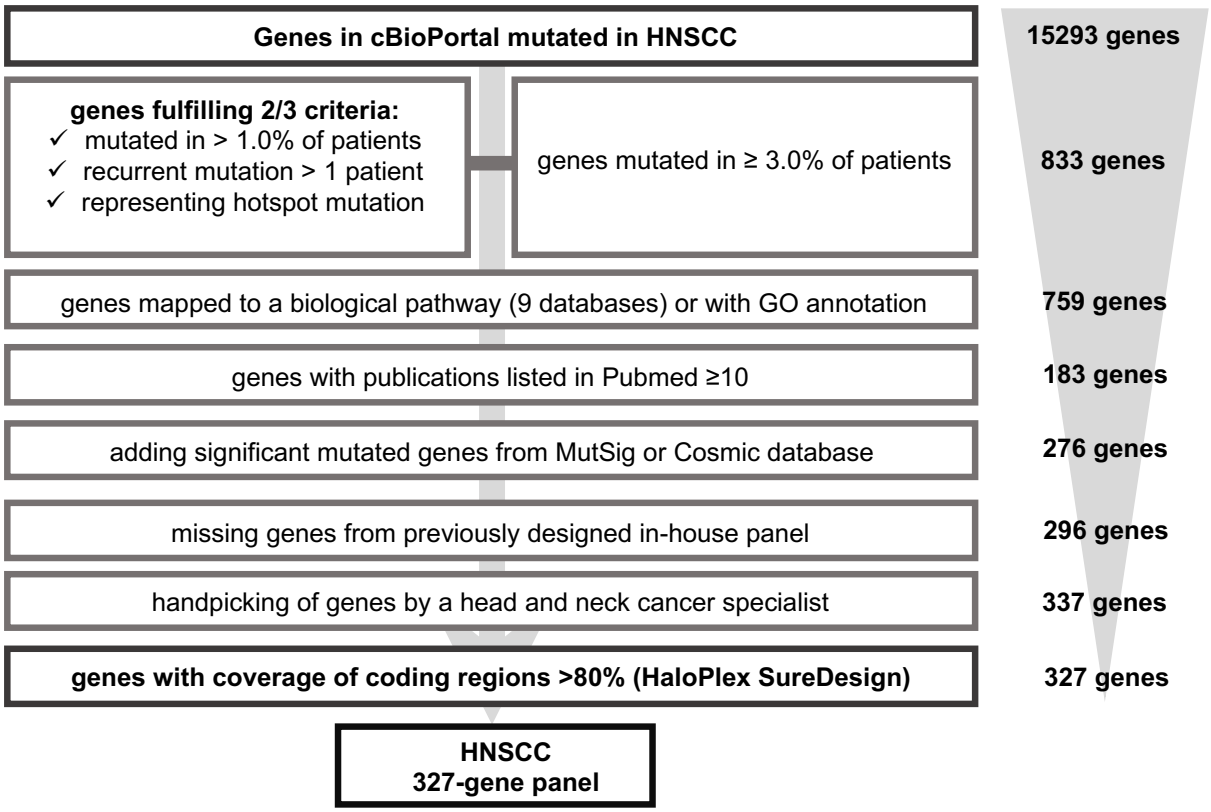



**Figure 2.** Flowchart depicting gene selection from cBioPortal, frequently mutated in HNSCC resulting in the 327-gene panel. HNSCC, head and neck squamous cell carcinoma.

HaloPlex Sure Design Software from Agilent was used for the panel design. The panel target region size was 1.47 mega base pairs (Mbp), consisting of 125 057 amplicons and a mean target coverage of 99.48%. Only genes of which the whole exonic sequence was covered more than 80% by the HaloPlexHS probes, were included. With the usage of the HaloPlexHS amplicon captured-based target enrichment, every molecule was tagged with an unique molecular barcode and captured DNA from both strands, which allowed to remove PCR errors during sequence data analysis (34).

Within the German Cancer Consortium Radiation Oncology Group (DKTK-ROG), archival tumor samples from HNSCC patients included in a multicenter retrospective biomarker study (35) could be used to evaluate the role of TMB for outcome after cCRTX and its interference with the tumor immune micromilieu (8).
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 were

included (36). Patients were treated between 2005 and 2011 at eight different DKTK partner sites (Berlin, Dresden, Essen, Frankfurt, Freiburg, Heidelberg, Munich and Tübingen) in Germany. Contrary to today, incidence of HPV+ cases was lower at this time, resulting in the low number of HPV positive (N=15) and high number of HPV negative (N= 143) cases in this study cohort (37).

Treatment with concurrent chemoradiation (cCRTX) according to standard protocols either based on cisplatin/5-fluorouracil (N=129) or mitomycin-C/5-fluorouracil (N=29) was carried out covering the tumor region and the regional lymph nodes (36). Sixty-nine patients received hyperfractionated accelerated radiotherapy (RT) up to 72 Gy, normofractionated RT up to 70 Gy was applied in 86 patients, whereas three patients received a simultaneously integrated boost technique. A minimum follow-up time of 24 months was required for all patients without progressive disease to be included in the retrospective study. Further inclusion criteria were the availability of RT treatment plans as well as computed tomography (CT) scans, magnetic resonance imaging (MRI) or positron emission tomography–CT (PET/CT) images of the location of the recurrent tumors. Being a biomarker study, the availability of formalin-fixed paraffin- embedded (FFPE) tumor material was crucial. Initially, it was planned to include 40 patients per DKTK partner site, but as FFPE material from tumor tissue was only available in six of eight DKTK partner sites, a total number of 158 cases could be included. In Table 1, clinicopathological characteristics of the DKTK patient cohort are presented. Most of the patients were male (N=133). Tumor stage was predominantly T4 (N=99). The majority of patients had a history of smoking (N=137) and drinking (N=88). The subcohort of patients in whom targeted next-generation sequencing (tNGS) analysis could be successfully performed (N=101) was representative with the exception of a lower number of female cases and never smokers.

**Table 1: Patient characteristics (modified after *Eder et al.* (8)).**

| Variable – N (%) | All cases | Patients with tNGS analysis | Patients without tNGS analysis |
|---|---|---|---|
| **All cases** | 158 (100) | 101 (100) | 57 (100) |
| Male | 133 (84) | 79 (78) | 54 (95) |
| Female | 25 (16) | 22 (22) | 3 (5) |
| Age in years (median, range) | 59 (39-82) | 58 (39-80) | 60 (44-82) |
| **Tumor site** | | | |
| Oropharynx | 80 (51) | 51 (50) | 29 (51) |
| Hypopharynx | 51 (32) | 31 (31) | 20 (35) |
| Oral cavity | 27 (17) | 19 (19) | 8 (14) |
| **T stage** | | | |
| T2-3 | 59 (37) | 41 (41) | 18 (32) |
| T4 | 99 (63) | 60 (59) | 39 (68) |
| **N stage** | | | |
| N0-N1 | 35 (22) | 23 (23) | 12 (21) |
| N2-N3 | 123 (78) | 78 (77) | 45 (79) |
| **Concurrent Chemotherapy** | | | |
| Cisplatin | 129 (82) | 84 (83) | 45 (79) |
| Mitomycin C | 29 (18) | 17 (17) | 12 (21) |
| **Never smoker** | | | |
| Yes | 21 (13) | 13 (13) | 8 (14) |
| No | 137 (87) | 88 (87) | 49 (86) |
| **Never drinker** | | | |
| Yes | 63 (40) | 49 (49) | 14 (25) |
| No | 88 (56) | 49 (49) | 39 (68) |
| Missing | 7 (4) | 3 (3) | 4 (7) |
| **HPV status** | | | |
| Positive | 15 (9) | 9 (9) | 6 (11) |
| Negative | 143 (91) | 92 (91) | 51 (89) |

As shown in Fig. 3 the assessment of TMB was feasible in 101 (64%) cases, while in 36% of the patients not sufficient tumor material for DNA isolation was available on the FFPE block. For the analysis of immune-related mRNA expression profiles with the nanoString PanCancer Immune Profiling Panel we could use in total 40 samples of which the three HPV-positive cases were excluded, due to the low number of cases.

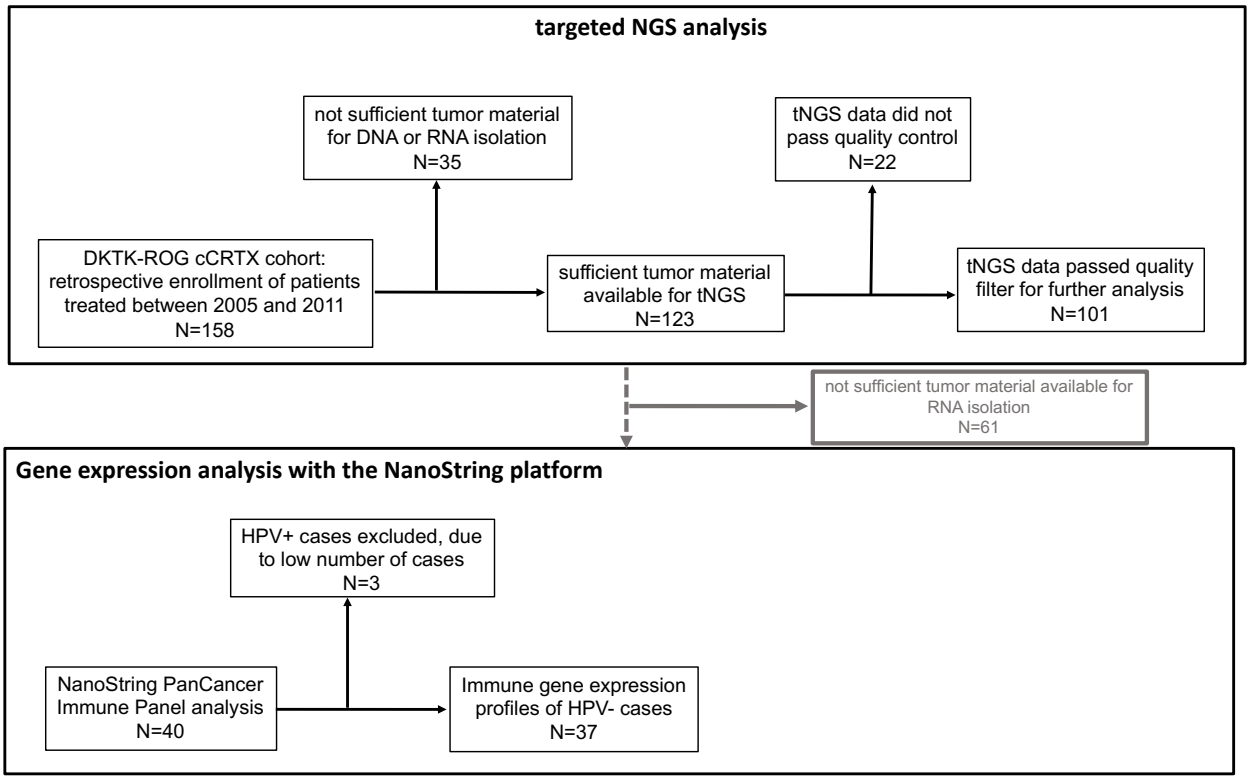



**Figure 3.** Sample selection workflow from the multicenter DKTK-ROG biomarker trial cohort (36) for NGS analysis with the in-house 327-gene panel and gene expression analysis with the nanoString platform using the PanCancer Immune Profiling Panel (8). DKTK ROG, German Cancer Consortium Radiation Oncology Group; cCRTX, concurrent chemoradiation; tNGS, targeted next-generation sequencing; HPV, human papilloma virus (modified after *Eder et al.* (8)).

Although sequencing costs are rapidly decreasing, requirements for WES regarding data storage capacity and experienced personnel for advanced bioinformatic analysis are still a major issue when using WES for assessment of TMB in clinical routine diagnostics. Given the retrospective nature of this study, fresh frozen tissue was not available, which would be the optimal input material for WES analysis. The only material available was archival FFPE tissue samples, therefore a tNGS approach was chosen.

First, we checked the suitability of our in-house 327-gene panel covering around 1.5 Mb per exome for estimation of TMB and compared its performance to the FDA-approved FoundationOne 325-gene panel targeting around 1.1 Mb (8). Therefore, we performed an *in-silico* analysis based on the TCGA HNSCC data set and correlated the number of non-synonymous variants detected in the genes covered by our in-house panel or the FoundationOne panel with the mutational load determined by WES (8). Download of the TCGA provisional HNSCC data set was done from the Firehose GDAC homepage of the Broad Institute (https://gdac.broadinstitute.org/; date of download: 12.02.2016) including survival data, HPV status, and mutation data (28-30). The observed correlation between the number of mutations detected by either tNGS or WES was high (327-gene panel: $R^2 = 0.81$, 315-gene panel: $R^2 = 0.87$), as seen in Fig. 4 (8).

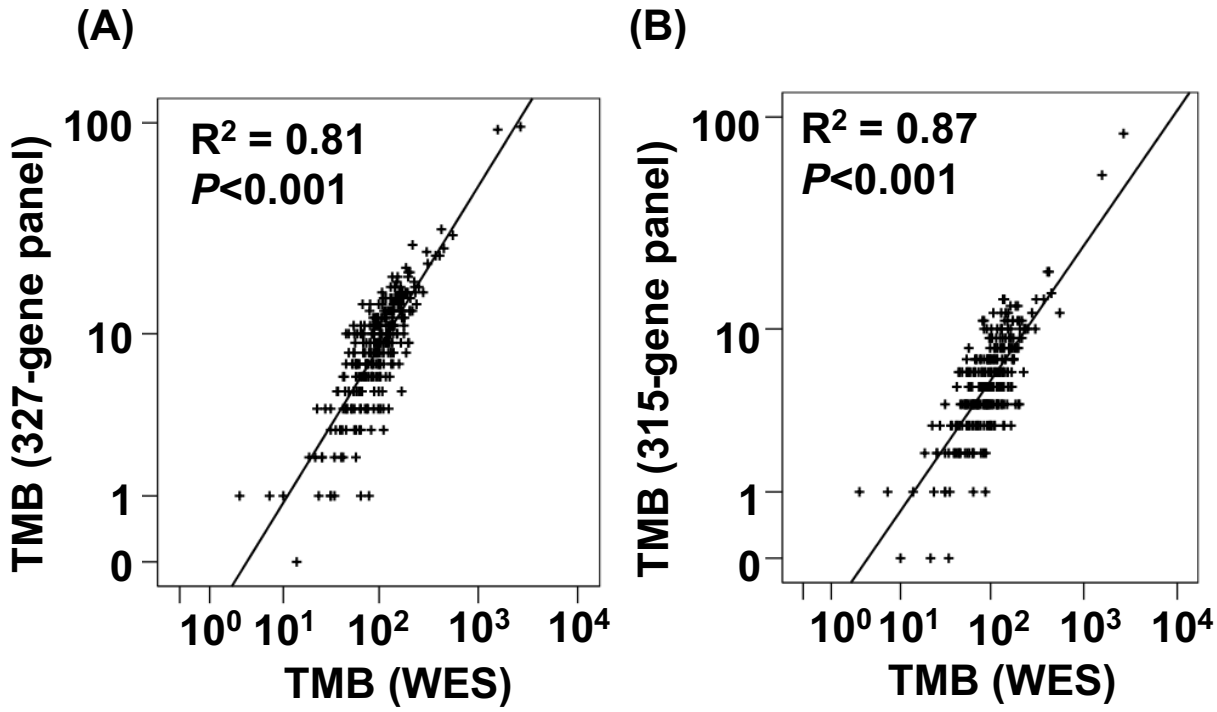
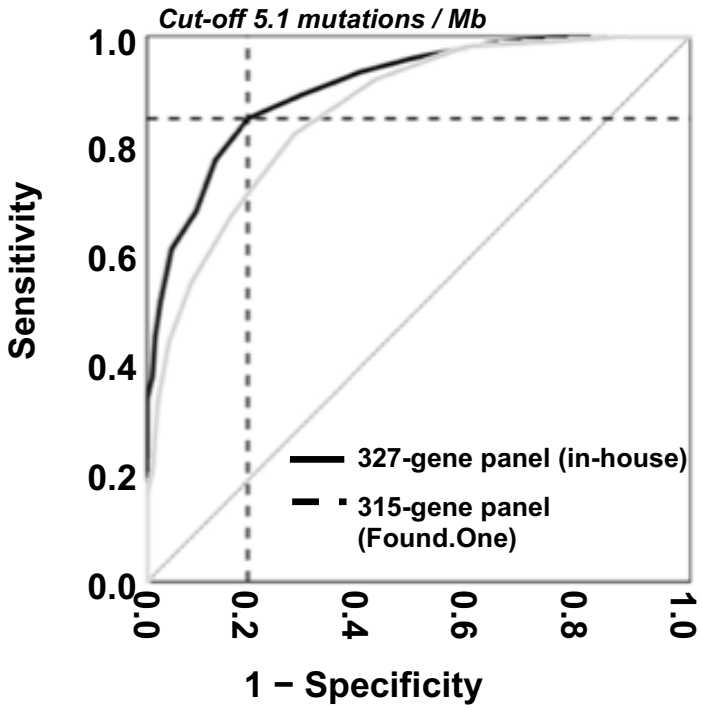



**Figure 4.** Estimation of TMB by tNGS by using two comprehensive gene panels. The overall number of non-synonymous mutations detected by WES in the TCGA HNSCC cohort (N = 510) was correlated with the number of mutations found in genes covered by the in-house 327-gene panel (A) and the 315-gene panel (B) (8). TMB, tumor mutational burden; tNGS, targeted next-generation sequencing; WES, whole-exome sequencing; TCGA, the Cancer Genome Atlas; HNSCC, head and neck squamous cell carcinoma (modified after *Eder et al.* (8)).

Our findings were in line with *Chalmers et al.*, who demonstrated a high correlation between TMB calculated by either tNGS or WES, when analyzing the same tumor specimens of one patient either with a 1.1 Mb comprehensive gene panel or WES (38). According to current literature, the precision of TMB estimation using tNGS is dependent on the panel size used. It was shown that using a 1.942 Mbp panel instead of a 0.533 Mbp panel, the range of confidence intervals was shortened. However, the usage of a 5 Mbp panel did not improve the test performance (39). Data from several studies suggested that TMB cut-offs should not only be panel specific but also entity specific, since TMB can vary over distinct cancer types (38, 39). When TMB for 167 distinct cancer types was assessed, the median TMB range was from 0.8 mutations/Mb in bone marrow myelodysplastic syndrome to 45.2 mutations/Mb in skin squamous cell carcinoma. Certain cancer types which are known to have significant mutagen exposure, like lung cancers and melanoma, were more highly mutated (median TMB 7.2 mutations/Mb and 13.5 mutations/Mb, respectively) (38).

To determine the accuracy of our tNGS approach for discriminating between patients with low versus high TMB, a ROC analysis was performed (Fig. 5). To classify patients in high and low TMB groups, TMB determined by WES and the cut-off described by *Cristescu et al.* of 86 mutations per exome served as gold standard (14). ROC analysis confirmed the high accuracy in assigning patients to the low/high TMB group based on mutational analysis by the in-house 327-gene panel (area under the curve (AUC) = 0.91) and the FoundationOne 315-gene panel (AUC = 0.86) (Fig. 5). For the 327-gene panel, ROC analysis 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 high (≥5.1 mutations/Mb) and low (<5.1 mutations/Mb) TMB groups (8).




$AUC_{327\text{-gene panel}}$ = 0.91 (95%-CI: 0.88-0.93); *P*<0.001
$AUC_{315\text{-gene panel}}$ = 0.86 (95%-CI: 0.83-0.89); *P*<0.001

**Figure 5.** To determine the sensitivity and specificity of the two gene panels for stratification of the patients according to their TMB, a ROC curve analysis was used. TMB determined by WES was used as a gold standard. Values of AUC, 95% confidence intervals and cut- off for the best discrimination between low and high TMB are shown (8). TMB, tumor mutational burden; WES, whole-exome sequencing; ROC, receiver operating characteristics; CI, confidence interval; AUC, area under the curve (modified after *Eder et al.* (8)).

First of all, we investigated the value of TMB as prognostic marker for outcome in our patient cohort. It has been described previously that chromosomal aberrations detected by comparative genomic hybridization are related to impaired treatment response (40) and reduced sensitivity to ionizing radiation (8, 41). Archival tumor samples from patients treated with cCRTX with carcinomas of the oropharynx, hypopharynx or oral cavity from the multicenter DKTK-ROG biomarker study (36) were used to evaluate whether an increased number of somatic mutations negatively interfered with the efficacy of cCRTX as well. We could observe in our patient cohort that a high TMB was a poor prognostic factor of survival after cCRTX (Fig. 6) (8).

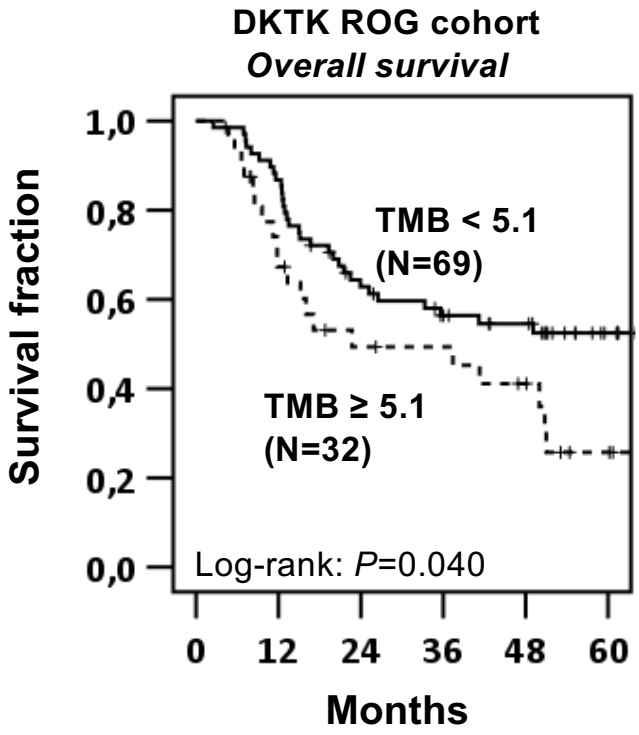



**Figure 6.** Patients were stratified into two groups according to their TMB. High TMB group was defined as more than 5.1 mutations/Mb and low TMB group was less than 5.1 mutations/Mb (8). TMB, tumor mutational burden; cCRTX, concurrent chemoradiation (modified after *Eder et al.* (8)).

The negative association between high TMB and outcome after cCRTX in our study could not be explained by the mutation spectrum in this patient subgroup. In cases with high TMB an enrichment of *TP53* mutations could be observed. However, *TP53*, *SMARCA4* and *APC* ranked among the most frequently mutated genes in the low TMB group as also seen in the high TMB group (8).

A high number of mutations has been described to result in the expression of neoantigens and thus should promote T-cell- mediated inflammation and the activation of immune checkpoints (42). Therefore, we investigated if high TMB was associated with a distinct immune expression signature in our patient cohort. For the nanoString mRNA expression analysis, sufficient tumor material was only available of a subset of cases (N=40). HPV-positive cases (N=3) were excluded from the analysis due to their low number and the potential confounding factor of the viral infection on the immune expression pattern. Slight changes in the expression levels of the immune-related genes were observed between low and high TMB groups. A trend toward downregulation could be observed for genes

associated with features of immune checkpoint activation such as high CD8+ T-cell scores (Fig. 7A) and the interferon gamma (IFNG) signature (Fig. 7B), when comparing high versus low TMB cases. Genes involved in neutrophil functions were overexpressed in high TMB cases (Fig. 7A) (8).

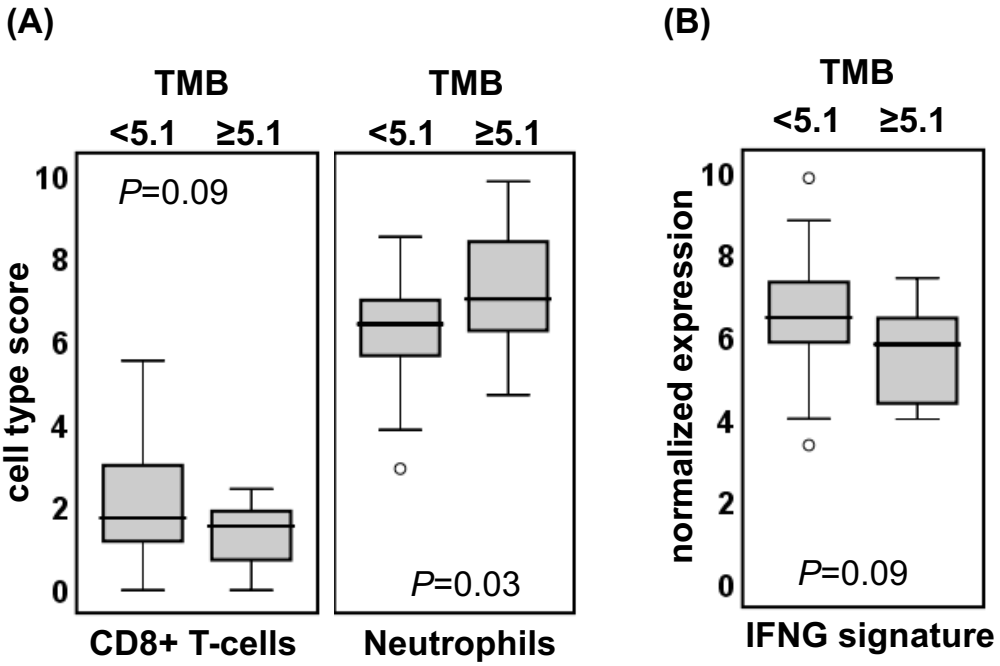



**Figure 7.** High TMB is associated with unfavorable immune expression signatures. The nanoString expression analysis of immune related genes was only performed in the HPV-negative patient cohort (N = 37). Cell type scores (i.e. the average log2 expression of cell type-specific marker genes) associated per trend with reduced CD8 positive 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 (8). TMB, tumor 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 harboring ≥5.1 mutations/Mb (modified after *Eder et al.* (8)).

The advantage of a targeted sequencing approach is not only relevant in terms of TMB estimation but also for routine molecular diagnostics for personalized medicine. With relatively small personnel and bioinformatic efforts, genes of interest can be sequenced in a fairly short time, speaking of days for tNGS compared to several weeks for WES analysis. For example, in routine diagnostics NGS analysis is implemented easily for targeted mutational testing of different tumor entities and sequencing results can be

provided to the treating physician for further therapy planning. Commercially available gene panels normally cover cancer genes, which are relevant for targeted therapy or give a hint on therapy resistance. Besides the time factor, the cost factor is also much lower when using a tNGS approach. However, gene panels are always restricted to certain genes and cannot be adjusted in a flexible manner. If a gene turns out to be an interesting cancer gene or a new druggable target, WES or WGS datasets can be reanalyzed. Commercially available gene panels are still being improved in terms of genes covered on the panel, but also the TMB and MSI-status can now also be derived from sequencing of only one tumor sample (43). Normally, a reference tissue from the patient is needed to asses MSI status but certain tNGS panels can assess this from one tumor tissue biopsy (43). The use of large gene panels (up to 500 genes) allow to have a closer look on many cancer related genes. FDA approvals have been given for three genes panels, Oncomine DX Target Test (23 genes), MSK-IMPACT (468 genes) and FoundationOne CDx (324 genes), which are also available in Europe (44).

A major issue in HNSCC is that many genes are mutated only at very low allele frequencies, and functional consequences of these mutations are often unclear. The lack of druggable targets makes targeted treatment of the tumor impossible. Furthermore, many of these genes are currently only candidates, since they are without functional studies and also not functionally linked to carcinogenesis (45).

Sequencing of a whole genome or exome from a tumor patient are still not standard in routine diagnostics and are currently mainly performed within clinical studies or molecular tumor boards for a small group of patients who suffer from advanced-stage disease. Several studies were conducted in the past years, addressing the feasibility and implementation of genomic data beyond standard molecular routine diagnostics. One example is the MASTER (Molecularly Aided Stratification for Tumor Eradication Research) program of the DKTK in which patients of young age, with rare tumors of all histologies at advanced stage are included. The purpose of MASTER is to assess the feasibility of prospective WES and RNA sequencing in a clinical setting, and to demonstrate that patients can benefit from comprehensive genomic analysis. The study is still ongoing but first data of 550 patients throughout several entities including head and neck, breast, gastrointestinal, urogenital, hematologic and other tumors have already been published (46). Turnaround time from biopsy to decision making in the molecular tumor board was less than six weeks, although WES or WGS and transcriptome sequencing were carried out. Based on molecular profiling, patients could be stratified

into seven different baskets of specific molecular pathways and cellular processes. The majority of patients were assigned to the tyrosine kinases basket (35%) followed by the immune evasion basket (17%) (47). The MASTER program includes nine comprehensive cancer centers within the DKTK. At the DKTK partner site in Berlin, data from the first 100 patients, which were discussed at the weekly molecular tumor board (MTB) had been published recently (48). This included not only patients from DKTK MASTER program, but also patients treated within the Treat20Plus program, where both WES and mRNA sequencing data were available. Data available from panel sequencing or immunohistochemistry (e.g. programmed cell death 1 ligand 1 (PD-L1) or MSI) using FFPE tissue were also taken into consideration. Median patient age was 51 and tumor types were heterogenous but the majority of patients were suffering from gastrointestinal tumors. In 63% of the cases treatment options could be recommended by the MTB. The median number of treatment options per patient was two (48). The aforementioned studies could demonstrate the feasibility of implementing tNGS approaches as well as complex genomic techniques like WES, WGS or transcriptome analysis into clinical routine with first evidence of benefit for the patients (47, 48). Since there is not always sufficient amount of tissue available for WES or WGS, tNGS continues to be an important technique for clinical routine diagnostic. Gene panels provide the treating oncologist with the information about the molecular characteristics of every tumor, so that they can plan the therapy individually to fit every patients needs.

Another case to mention is those of a young woman diagnosed with a squamous cell carcinoma of the tongue at the age of 26 in 2015. After initial sole surgery, followed by surgery combined with adjuvant chemoradiation at the first regional recurrence, the patient developed distant metastases and was presented to the MTB at Charité Cancer Comprehensive Center in October 2017 for molecular profiling. Panel sequencing was done with our 327-HNSCC gene panel, due to high treatment pressure to identify a molecular target, since the waiting time for the WES results was several weeks. Gene amplification in the *EGFR*, *CCND1* and *ANO1* gene could be observed. Furthermore, panel sequencing revealed somatic mutations in the *CDKN2A* and *TP53* genes and germline mutation in the *FANCM* gene. In addition, immunohistochemical staining with PD-L1 antibody revealed high expression levels in 70% of the tumor cells in the lung metastasis and 50% in the lymph node metastasis. Therapy with PD-1 antibody Nivolumab was started two years ago and is still ongoing, with no evidence for disease recurrence (status June 2020).

Despite initial responses, treatment with cisplatin-based chemotherapy often leads to chemoresistance in HNSCC. The role of treatment-induced clonal selection in the development of cisplatin resistance remains largely unknown. In order to investigate this matter, a FaDu cell line was used in our laboratory as a model for studying cisplatin-induced clonal evolution in HNSCC (49). Drug sensitivity testing and detailed molecular characterization including mutational profiling with our 327-gene panel, was carried out. It could be shown that this cell line is composed of multiple genetically different clones, which were distinct in their sensitivity to cisplatin. Panel sequencing results of FaDu subclones resistant to cisplatin revealed single nucleotide variants (SNVs) in the *TP53* gene: the intronic mutation c.673 G>A leading to an early stop codon was detected at decreased allele frequency compared with FaDu cells sensitive to cisplatin. The *TP53* p.R248L missense mutation was only found in FaDu subclones resistant to cisplatin but not in FaDu subclones sensitive to cisplatin even when allele frequency down to 0.01 were used as a filter for variant calling (49). This data supported the hypothesis of intratumoral genetic heterogeneity and treatment-induced selection of tumor subclones which carry genetic features to promote primary resistance to cisplatin (49). These results underline the importance of using a deep sequencing technique, to be able to detect minor tumor subclones with primary drug resistance within the tumor biopsy.

To conclude, the use of a gene panel seems to be a better approach for initial molecular characterization of the tumor in terms of time and costs to decide on further treatment strategies in contrast to WES or WGS. Not only druggable gene targets can be detected with panel sequencing, but also mutations predicting drug resistance. Tumors may also contain minor subclones with primary drug resistance and those mutations can only be detected by a deep sequencing approach.

Our retrospective biomarker study provides comprehensive data of the prognostic value of TMB in a uniformly treated HNSCC patient cohort, which could also be validated in the TCGA data set. The usage of a tNGS approach for TMB assessment was successfully applied to our patient cohort. Due to the retrospective nature of the study there is a lack of information on potential confounding factors such as performance status and smoking. The small number of HPV-positive cases in the DKTK-ROG cohort represents another limitation of the study. Further prospective studies should address whether the inclusion of distinct genes associated with resistance to or higher efficacy of PD-1 blockade such

as *JAK1* or *JAK2* (50) or *MLH1*, *MSH2*, *MSH6* and *PMS2* (23), might additionally improve biomarker-based patient selection with tNGS panels. The assessment of the TMB value by our 327-gene panel and the establishment of the cut-off with 5.1 mutations/Mb to select patients with potential benefit from CRTX-ICI combination therapy will need to be precisely evaluated in prospective trials.

# 4. Tabellenverzeichnis



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# 5. Abbildungsverzeichnis

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Figure 5. To determine the sensitivity and specificity of the two gene panels for stratification of the patients according to their TMB, a ROC curve analysis was used. TMB determined by WES was used as a gold standard. Values of AUC, 95% confidence intervals and cut- off for the best discrimination between low and high TMB are shown (8). TMB, tumor mutational burden; WES, whole-exome sequencing; ROC, receiver operating characteristics; CI, confidence interval; AUC, area under the curve (modified after Eder et al. (8)). ……15
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Figure 6. Patients were stratified into two groups according to their TMB. High TMB group was defined as more than 5.1 mutations/Mb and low TMB group was less than 5.1 mutations/Mb (8). TMB, tumor mutational burden; cCRTX, concurrent chemoradiation (modified after Eder et al. (8)). ……16
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Figure 7. High TMB is associated with unfavorable immune expression signatures. The nanoString expression analysis of immune related genes was only performed in the HPV-negative patient cohort (N = 37). Cell type scores (i.e. the average log2 expression of cell type-specific marker genes) associated per trend with reduced CD8 positive 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 (8). TMB, tumor 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 harboring ≥5.1 mutations/Mb (modified after Eder et al. (8)). ……..17
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# 7. Eidesstattliche Versicherung

„Ich, Theresa Eder-Pinggera, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „*Characterization of the molecular mechanisms of resistance to radiochemotherapy of head and neck squamous cell carcinoma*“ („Charakterisierung der molekularen Resistenzmechanismen gegen Radiochemotherapie beim Plattenepithelkarzinom im Kopf-Hals Bereich“) selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.
Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren/innen beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) werden von mir verantwortet.

[Für den Fall, dass Sie die Forschung für Ihre Promotion ganz oder teilweise in Gruppenarbeit durchgeführt haben:] Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe (siehe Anteilserklärung). Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich gemacht.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Erstbetreuer/in, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.

Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

Datum Unterschrift

# 8. Anteilserklärung an der erfolgten Publikation

Publikation: Eder T, Hess AK, Konschak R, Stromberger C, Jöhrens K, Fleischer V, Hummel M, Balermpas P, von der Grün J, Linge A, Lohaus F, Krause M, Baumann M, Stuschke M, Zips D, Grosu AL, Abdollahi A, Debus J, Belka C, Pigorsch S, Combs SE, Budach V, Tinhofer I; DKTK-ROG.

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. European Journal of Cancer, 2019 July

Beitrag im Einzelnen:
Literaturrecherche, Planung und Durchführung der Experimente (Next-Generation Sequencing Analyse, teilweise nanoString RNA Expressions-Profiling). Durchführung der Datenanalyse und statistischen Auswertung sowie Erstellung der Tabellen (Supplementary Table 1, Table 1, Table 2 (unterstützt durch Prof. Dr. Ingeborg Tinhofer-Keilholz)) und Abbildungen (Supplementary Figure 1, Supplementary Figure 2, Supplementary File 1, Figure 1a/b, Figure 1c (unterstützt durch Prof. Dr. Ingeborg Tinhofer-Keilholz), Figure 2, Figure 3), Verfassung und Revision des Manuskripts unterstützt durch Prof. Dr. Ingeborg Tinhofer-Keilholz.

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Unterschrift, Datum und Stempel des/der erstbetreuenden Hochschullehrers/in

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Unterschrift des Doktoranden/der Doktorandin

# 9. Journal Summary List (ISI Web of Knowledge SM)

Journal Data Filtered By: **Selected JCR Year: 2017** Selected Editions: SCIE,SSCI
Selected Categories: **"ONCOLOGY"** Selected Category Scheme: WoS
**Gesamtanzahl: 222 Journale**

| Rank | Full Journal Title | Total Cites | Journal Impact Factor | Eigenfactor Score |
|---|---|---|---|---|
| 1 | CA-A CANCER JOURNAL FOR CLINICIANS | 28,839 | 244.585 | 0.066030 |
| 2 | NATURE REVIEWS CANCER | 50,407 | 42.784 | 0.079730 |
| 3 | LANCET ONCOLOGY | 44,961 | 36.418 | 0.136440 |
| 4 | JOURNAL OF CLINICAL ONCOLOGY | 156,474 | 26.303 | 0.285130 |
| 5 | Nature Reviews Clinical Oncology | 8,354 | 24.653 | 0.026110 |
| 6 | Cancer Discovery | 11,896 | 24.373 | 0.065350 |
| 7 | CANCER CELL | 35,217 | 22.844 | 0.096910 |
| 8 | JAMA Oncology | 5,707 | 20.871 | 0.027770 |
| 9 | ANNALS OF ONCOLOGY | 38,738 | 13.926 | 0.095780 |
| 10 | JNCI-Journal of the National Cancer Institute | 37,933 | 11.238 | 0.052550 |
| 11 | Journal of Thoracic Oncology | 15,010 | 10.336 | 0.033280 |
| 12 | CLINICAL CANCER RESEARCH | 81,859 | 10.199 | 0.132210 |
| 13 | SEMINARS IN CANCER BIOLOGY | 6,330 | 10.198 | 0.010740 |
| 14 | LEUKEMIA | 25,265 | 10.023 | 0.059580 |
| 15 | NEURO-ONCOLOGY | 10,930 | 9.384 | 0.030350 |
| 16 | Cancer Immunology Research | 4,361 | 9.188 | 0.021180 |
| 17 | CANCER RESEARCH | 139,291 | 9.130 | 0.130190 |
| 18 | Journal for ImmunoTherapy of Cancer | 1,675 | 8.374 | 0.007130 |
| 19 | BIOCHIMICA ET BIOPHYSICA ACTA-REVIEWS ON CANCER | 5,276 | 8.220 | 0.009300 |
| 20 | Blood Cancer Journal | 1,804 | 8.125 | 0.007660 |
| 21 | CANCER TREATMENT REVIEWS | 7,870 | 8.122 | 0.015820 |
| 22 | Molecular Cancer | 10,301 | 7.776 | 0.017280 |
| 23 | INTERNATIONAL JOURNAL OF CANCER | 51,800 | 7.360 | 0.071870 |
| 24 | Journal of Hematology & Oncology | 4,098 | 7.333 | 0.009750 |
| 25 | EUROPEAN JOURNAL OF CANCER | 29,883 | 7.191 | 0.050170 |
| 26 | ONCOGENE | 66,411 | 6.854 | 0.075960 |
| 27 | CANCER | 68,221 | 6.537 | 0.074740 |
| 28 | CANCER LETTERS | 29,311 | 6.491 | 0.042280 |
| 29 | Journal of the National Comprehensive Cancer Network | 5,143 | 6.471 | 0.017530 |
| 30 | Advances in Cancer Research | 2,343 | 6.422 | 0.003690 |
| 31 | JOURNAL OF PATHOLOGY | 16,156 | 6.253 | 0.024060 |

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

# 10. Lebenslauf

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

# 12. Danksagung

Zuallererst möchte ich mich bei Frau Prof. Dr. Ingeborg Tinhofer-Keilholz bedanken, für die Möglichkeit meine Doktorarbeit in ihrer Arbeitsgruppe durchzuführen und für die Unterstützung in den letzten Jahren. Ganz besonders möchte ich mich auch bei Robert bedanken, der mich immer in vielerlei Hinsicht während meiner Zeit im Labor unterstützt hat. Danke auch an das ganze Team der Strahlenbiologie für eine tolle gemeinsame Zeit. Von ganzen Herzen möchte ich meiner Familie und meinem Freund für die Unterstützung danken.