Targeted next-generation sequencing of locally advanced squamous cell carcinomas of the head and neck reveals druggable targets for improving adjuvant chemoradiation

Tinhofer I[1,9], Budach V[1,9], Saki M[1,9], Konschak R[1,9], Niehr F[1,9], Jöhrens K[10], Weichert W[7,11,12], Linge A[2,13,14], Lohaus F[2,13,14], Krause M[2,13,14,24], Neumann K[15], Endris V[7,11], Sak A[3,16], Stuschke M[3,16], Balermpas P[4,17], Rödel C[4,17], Avlar M[5,18], Grosu AL[5,18], Abdollahi A[6,19], Debus J[6,19], Belka C[7,20], Pigorsch S[7,21], Combs SE[7,21,22], Mönnich D[8,23], Zips D[8,23] and Baumann M[2,13,14,24], for the DKTK-ROG.

German Cancer Research Center (DKFZ), Heidelberg, Germany and German Cancer Consortium (DKTK) partner sites: [1]Berlin, [2]Dresden, [3]Essen, [4]Frankfurt, [5]Freiburg, [6]Heidelberg, [7]Munich, [8]Tübingen; Germany; [9]Department of Radiooncology and Radiotherapy, Charité University Hospital Berlin; [10]Institute of Pathology, Charité-University Hospital Berlin; [11]Institute of Pathology, University Hospital Heidelberg and National Center for Tumor Disease; [12]Institute of Pathology, Technical University Munich; [13]Department of Radiation Oncology, Faculty of Medicine and University Hospital Carl Gustav Carus, Technische Universität Dresden; [14]OncoRay – National Center for Radiation Research in Oncology (NCRO), Faculty of Medicine and University Hospital Carl Gustav Carus, Technische Universität Dresden, Germany; [15]Institute of Biometry and Clinical Epidemiology, Charité University Hospital Berlin; [16]Department of Radiotherapy, Medical Faculty, University of Duisburg-Essen, Essen; [17]Department of Radiotherapy and Oncology, Goethe-University Frankfurt; [18]Department of Radiation Oncology, University of Freiburg; [19]Department of Radiation Oncology, Heidelberg Ion Therapy Center (HIT), Heidelberg Institute of Radiation Oncology (HIRO) and National Center for Radiation Research in Oncology (NCRO) [20]Department of Radiotherapy and Radiation Oncology, Ludwig-Maximilians-Universität, Munich; [21]Department of Radiation Oncology, Technical University Munich; [22]Institute of Innovative Radiotherapy, Helmholtz Center Munich; [23]Department of Radiation Oncology, Faculty of Medicine and University Hospital Tübingen, Eberhard Karls Universität Tübingen; [24]Helmholtz-Zentrum Dresden - Rossendorf, Dresden, Germany.

Corresponding author: Ingeborg Tinhofer, PhD, Charité University Hospital Berlin, Department of Radiooncology and Radiotherapy, Translational Radiation Oncology Research Laboratory, Charitéplatz 1, 10117 Berlin, Germany; e-mail: ingeborg.tinhofer@charite.de

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Abstract

Background

Despite clear differences in clinical presentation and outcome, squamous cell carcinomas of the head and neck (SCCHN) arising from human papilloma virus (HPV) infection or heavy tobacco/alcohol consumption are treated equally. Next-generation sequencing is expected to reveal novel targets for more individualized treatment.

Patients and methods

Tumor specimens from 208 patients with locally advanced squamous cell carcinoma of the hypopharynx, oropharynx or oral cavity, all uniformly treated with adjuvant cisplatin-based chemoradiation were included. A customized panel covering 211 exons from 45 genes frequently altered in SCCHN was used for detection of non-synonymous point and frameshift mutations. Mutations were correlated with HPV status and treatment outcome.

Results

Mutational profiles and HPV status were successfully established for 179 cases. HPV- tumors showed an increased frequency of alterations in tumor suppressor genes compared to HPV+ cases (*TP53* 67% vs. 4%, *CDKN2A* 18% vs. 0%). Conversely, HPV+ carcinomas were enriched for activating mutations in driver genes compared to HPV- cases (*PIK3CA* 30% vs. 12%, *KRAS* 6% vs. 1%, *NRAS* 4% vs. 0%). Hotspot *TP53* missense mutations in HPV- carcinomas correlated with an increased risk of locoregional recurrence (HR 4.3, 95%-CI 1.5-12.1, *P*=0.006) and death (HR 2.2, 95%-CI 1.1-4.4, *P*=0.021). In HPV+ SCCHN driver gene mutations were associated per trend with a higher risk of death (HR 3.9, 95%-CI 0.7-21.1, *P*=0.11).

Conclusions

Distinct mutation profiles in HPV- and HPV+ SCCHN identify subgroups with poor outcome after adjuvant chemoradiation. Mutant p53 and the PI3K pathway were identified as potential druggable targets for subgroup-specific treatment optimization.

Key words: head and neck cancer, human papilloma virus, mutation profiles, adjuvant chemoradiation, cisplatin.

Introduction

It is generally acknowledged that human papilloma virus-negative (HPV-) and HPV+ squamous cell carcinomas of the head and neck (SCCHN) represent two distinct subgroups with significant differences in treatment outcome [1]. The dissimilarities are thought to result from distinct molecular patterns which have evolved during the pathogenesis of HPV- and HPV+ SCCHN. Recent studies designed to unravel the genomic landscape of SCCHN by next-generation sequencing (NGS) have discovered large differences between HPV- and HPV+ tumors [2-6]. Smoking-related patterns which mainly comprised loss-of-function mutations in tumor suppressor genes were predominantly detected in HPV- carcinomas. In contrast, a mutation signature associated with viral infection and resembling mutation patterns of HPV-associated cervical cancer [4, 7] were described for HPV+ tumors [2-6].

Although distinct mutation patterns were identified by the above-mentioned studies, their prognostic value and their interference with the efficacy of current state-of-the art treatment regimens remain largely unresolved. Since many of the affected genes have a role in cell cycle, DNA repair and cell survival under stress conditions we hypothesized that the mutational profiles might influence the tumor cell response to chemoradiation. This multicentre study of the German Cancer Consortium Radiation Oncology Group (DKTK-ROG) was designed to test this hypothesis in a uniformly treated and well-characterized cohort of locally advanced SCCHN.

Patients and methods

Patients

Ethical approval for this retrospective biomarker study was obtained by the local Ethics Committees of all DKTK partner sites. Patients with histologically proven squamous cell carcinoma of the hypopharynx, oropharynx or oral cavity were eligible for this study; all patients had undergone complete curative surgical procedure and, due to high risk of locoregional recurrence (i.e. stage pT4, >3 affected lymph nodes, positive microscopic resection margins and/or extracapsular spread) had received adjuvant chemoradiation between 2004 and 2012. Radiotherapy had been delivered according to standard protocols and consisted of elective irradiation of cervical nodes with a dose of ≥50 Gy and a boost to the former tumor region and/or residual disease up to a total dose of 60-66 Gy. The cumulative dose of cisplatin was ≥200 mg/m². Follow-up of the patients without evidence of disease was at least 24 months. Additionally, archival formalin-fixed paraffin-embedded tumor tissue, radiotherapy treatment plans, follow-up images as well as clinical follow-up data of patients were available as centrally collected dataset.

Overall, 221 cases were included in the multicentre biomarker study. From 208 cases, sufficient DNA was available for library preparation for targeted NGS. Of those, 185 samples passed quality control and were successfully sequenced. One sample being an extreme outlier (>100 genetic alterations) was excluded from further analysis. For 5 cases the HPV status could not be determined due to technical reasons. The final cohort for analysis thus comprised 179 cases.

Sequencing analysis

Using the multiplex PCR-based Ion Torrent approach a gene panel including 45 SCCHN-related genes was designed based on the COSMIC database and NGS data from SCCHN tumors [2, 3] available at the time of panel design. Information on the panel is given in the supplementary Table 1. Detailed description of genomic DNA isolation, library preparation, quality control, sequencing and data analysis of sequencing results is provided in the supplementary information.

Classification of TP53 mutations

We first applied a model which is based on the assumption that missense mutations leading to accumulation of mutant p53 protein provide a survival benefit for tumor cells and are enriched in SCCHN patient populations. Public datasets of previous NGS studies [2-4] and own unpublished NGS data were used for identification of hotspot *TP53* mutant variants in SCCHN (supplementary Figure 1). Consistent with a previous study [8] we used a cut-off of >2% of all missense mutations for defining hotspots. Missense mutations outside these positions as well as nonsense and frameshift mutations at any site were considered non-hotspot mutations.

Secondly, we applied the criteria of Poeta and colleagues [9] and classified into three groups of disruptive *TP53* mutations, non-disruptive mutations and wildtype (wt) *TP53*. Third, we used the Evolutionary Action score of *TP53* coding variants (EAp53) recently developed by Neskey et al. [10] for stratifying patients into four groups of high-risk and low-risk missense *TP53* mutations, any other type of *TP53* alterations or wt *TP53*.

Assessment of HPV/p16Ink4a status and p53 expression

HPV status was determined by p16Ink4a immunohistochemistry and detection of HPV DNA. Immunohistochemical staining of p53 was performed as previously described [11]. The detailed protocols are described in the supplementary information (online only).

Statistical analysis

Since HPV-driven oropharyngeal carcinomas (OPC) are considered a biologically and clinically distinct group, mutational patterns were compared between HPV DNA+/p16+ OPC and the combined group of HPV- SCCHN and HPV+ non-OPC (hereinafter referred to as non-HPV-driven SCCHN). Differences in mutation frequencies between the two groups were analyzed with the Fisher's Exact test. The influence of mutations on overall survival (OS), in which failure was defined as death due to any cause, and locoregional tumor control (LRC), in which failure was defined as local, regional or locoregional recurrence was determined. Event times were measured from the date of diagnosis (OS) or the date of start of adjuvant chemoradiation (LRC) to the date of event occurrence or the last follow-up. OS and LRC curves were estimated by the Kaplan-Meier method and compared between molecular groups with the log-rank test. Hazard ratios and interactions between risk parameters were estimated using Cox regression models. Statistical analyses were carried out using the SPSS Statistics software (version 22.0.0, IBM, Armonk, NY, USA). For all analyses, two-sided tests were performed. The level of significance was set at $P < 0.05$.

Results

Patient characteristics and genomic profiles

Patient characteristics according to the HPV status are presented in supplementary Table 2. Median follow-up of patients for OS and LRC was 55 and 47 months, respectively. As reported previously [11], patients with HPV+ OPC (N=47) had a significantly better OS (HR 0.3, 95% CI 0.14-0.69, *P*=0.004) and LRC (HR 0.09, 95% CI 0.01-0.65, *P*=0.018) compared to the group of patients with non-HPV-driven SCCHN (N=132).

After filtering against the NCBI dbSNP and COSMIC databases, 381 non-synonymous single or small nucleotide substitutions were detected within the coding regions of 33 of the analyzed 45 genes. These alterations comprised 281 missense mutations (73.8%), 68 nonsense mutations (17.8%), 18 frameshift deletions (4.7%), 10 frameshift insertions (2.6%) and 4 in-frame deletions (1.0%). In the total cohort, the mean number of mutations per patient in the targeted regions was 2.1 (range: 0 to 7). We observed significantly higher rates in the target genes in non-HPV-driven SCCHN (mean 2.2, range 0-7) compared to HPV+ OPC (mean 1.7, range 0-4, *P*=0.01). More strikingly however was the difference in mutation patterns (supplementary Table 3): While tumor suppressor genes with known function as cell cycle checkpoints were more frequently affected in non-HPV-driven SCCHN compared to HPV+ OPC (*TP53* 67% vs. 4%, *CDKN2A* 18% vs. 0%), the reverse was observed for activating mutations in oncogenes and genes of the PI3K signaling pathway (*KRAS* 1% vs. 6%, *NRAS* 0% vs. 4%, *HRAS* 0% vs. 2%, *PIK3CA* 12% vs. 30%, *PTEN* 2% vs. 6%). While similar mutation frequencies were observed for *NOTCH1*, a trend of a higher prevalence of mutations in *FBXW7* was observed in HPV+ compared to HPV- tumors (9% vs 2%). No differences in the occurrence of

mutations in receptor tyrosine kinases were observed between the two subgroups (Table 1, supplementary Table 3).

TP53 mutations and the efficacy of adjuvant chemoradiation in non-HPV-driven SCCHN

The effects of *TP53* mutations on treatment efficacy were addressed in the group of non-HPV-driven SCCHN (N=132) in order to avoid a confounding effect of the HPV status itself. Patient classification based on 'hotspot' criteria [8] (Figure 1A) or the 'Poeta' criteria [9] (Figure 1B) each identified a subgroup of patients with significantly increased risk of locoregional recurrence. Classification according to the Eap53 score [10] (Figure 1C) or independent of the type of *TP53* mutation (Figure 1D) had a less pronounced discriminative potential.

We were then interested whether a high-risk *TP53* genotype was also associated with elevated p53 expression and whether the two parameters (genotype and expression) had independent prognostic influence. Overexpression of p53 was significantly enriched in the respective high-risk genotype group of each model (supplementary Table 4) and was associated per trend with poor locoregional control (Figure 1E). Stepwise multivariate analysis using conditional backward elimination of variables indicated that tumor site, pathologic nodal stage, extracapsular spread (ECE), smoking and p53 expression levels had no confounding effects and that hotspot missense mutations represented an independent prognostic variable for LRC and OS (Table 2). An independent prognostic value for LRC but not for OS was also observed for non-disruptive mutations according to the 'Poeta' criteria (Table 2).

Independent prognostic role of CDKN2A mutations in non-HPV-driven SCCHN

Mutations in *CDKN2A* were exclusively detected in non-HPV-driven SCCHN but occurred independently of *TP53* alterations. We therefore asked whether *CDKN2A* mutations had also an independent prognostic impact and as a consequence, whether combined assessment of *CDKN2A* and *TP53* would improve risk stratification of these patients. Patients were grouped according to the presence or absence of *TP53* hotspot missense and *CDKN2A* mutations. Kaplan-Meier analysis of LRC (not shown) and OS (Figure 2A) revealed a negative impact of *TP53* hotspot mutations alone whereas no such effect was not observed for *CDKN2A* mutations (Figure 2A). The co-occurrence of hotspot missense *TP53* and *CDKN2A* mutations significantly increased the risk of locoregional recurrence (HR 11.5, 95%-CI 4.1-31.6) $P<0.001$) and death (HR 5.9, 95%-CI 2.4-14.6, $P<0.001$, Figure 2A).

Activating driver mutations in HPV+ OPC are associated with poor prognosis

Growing evidence points to a negative influence of mutations and gene amplifications within the PI3K/AKT and MAPK pathways on the efficacy of radiotherapy [12, 13]. Since activating mutations in known master regulators of these pathways were observed in approximately one third of HPV+ OPC, we evaluated their prognostic value for outcome after adjuvant chemoradiation. Event numbers of locoregional recurrences in HPV+ OPC were low and OS was therefore used as endpoint in this analysis. Univariate Cox regression analysis revealed a trend to an increased risk of death for the group with driver gene mutations vs. the wt group (HR 3.9, 95%-CI 0.7-21.1, $P=0.11$, Figure 2B). No such trend, neither for OS nor for LRC was found in the group of HPV- SCCHN or HPV+ non-OPC (OS: HR 0.8, 95%-CI 0.3-2.3, $P=0.71$; LRC: HR 0.7, 95%-CI 0.2-3.0, $P=0.65$).

Discussion

Our study provides first evidence that targeted NGS may be of clinical use for defining molecular subgroups of SCCHN patients with distinct outcome after adjuvant cisplatin-based chemoradiation. We also confirmed previous data [4-6] that non-HPV-driven SCCHN and HPV+ OPC have distinct mutation profiles, with *TP53* and *CDKN2A* mutations exclusively detected at high frequency in the former and activating driver mutations in *PIK3CA* accumulating in the latter group.

After recognition of *TP53* mutations as one of the most frequent alterations in human cancer it was rapidly discovered that *TP53* variants can differ in the functional consequences of distinct mutation sites [14]. Crystal structure analysis provided the basis for understanding why some mutations had prognostic impact while others did not [15]. In line with previous studies [8-10, 15-17], we provide additional evidence that the association between the presence of *TP53* mutations and outcome significantly depends on the mutation type. Classification according to any type of *TP53* mutations or p53 immunoreactivity had poor discriminative power, an observation already made by others also for surgically treated SCCHN patients [9, 18]. Although we observed a significant difference in outcome between genetic subgroups classified by the ‘Poeta’ criteria, the most unfavorable group in our study had non-disruptive mutations whereas the poor-outcome group in the study of Poeta et al. displayed disruptive mutations [9]. The reason for this discrepancy remains unclear but differences in treatment might represent one possible explanation, considering that only 48% of patients received surgery and adjuvant treatment in the study of Poeta et al. [9] compared to 100% in our study. It could be reasoned that the poor prognostic value of disruptive mutations as defined by the ‘Poeta’ criteria vanished because this group of patients preferentially benefited from the addition of cisplatin to adjuvant radiotherapy. Alternatively, the different prognostic value of

disruptive TP53 mutations might result from differences in the exon coverage between our and the study of Poeta et al. [9], potentially leading to an underestimation of disruptive mutations in our study. As shown in the supplementary Table 1, our targeted NGS panel included exons 4 to 10 of *TP53* whereas in the study of Poeta et al. *TP53* mutation analysis was directed to exons 2 to 11. According to the mutation data from the TCGA cohort [4] and the results from the study of Agrawal et al. [2] and Stranksky et al. [3], 96% of all reported *TP53* missense/nonsense mutations would have been detected by our panel. The very low incidence of mutations outside exons 4-10 speak against the difference in exon coverage as possible explanation for the different results on the prognostic value of disruptive mutations in the two studies.

Accumulation of hotspot variants could result from positive selection of mutations that render cells with growth and survival advantages during tumorigenesis [19], and that are potentially also protective during chemoradiation. A causal relationship between gain-of-function (GOF) p53 variants [20, 21] and radio-/chemoresistance has previously been reported in ovarian [22] and lung cancer [23]. The results presented here and own unpublished data from preclinical models (Niehr et al., manuscript submitted for publication) support the existence of such a relationship in SCCHN as well. Based on its tumor-promoting activities, significant effort has been made to identify means to counteract mutant p53 in tumors. Current strategies include the use of small molecules that either revert mutant p53 into its wt conformation [24], destabilize mutant p53 [25], or target the crosstalk of mutant p53 with other oncogenic pathways [21].

Inactivation of the *CDKN2A* locus is an efficient means of cancer cells to abrogate both the retinoblastoma (Rb) and p53 pathways as the *CDKN2A* protein products, p16INK4 and p14ARF, serve to maintain the functional activities of Rb and p53,

respectively [26]. Our data, though preliminary due to the small sample size of subgroups, suggest that the negative effect of *TP53* mutations on the efficacy of chemoradiation may be aggravated by *CDKN2A* inactivation. Such cooperative effect has previously also been reported for squamous cell carcinoma of the skin [27] where sequential acquisition of *TP53* and *CDKN2A* alterations was shown to be responsible for tumor initiation and progression, respectively. In addition to mutations *CDKN2A* has been shown to be frequently inactivated in HPV- SCCHN by methylation or chromosomal loss [4-6]. Future studies will thus have to include a more comprehensive analysis of *CDKN2A* or its encoded proteins p16INK4 and p14ARF before a definite conclusion on a cooperative negative effect of loss-of-function alterations in *CDKN2A* and *TP53* for the efficacy of cisplatin-based chemoradiation in HPV- SCCHN can be drawn.

For the first time we provide evidence that activating mutations in *PIK3CA, KRAS, NRAS* and *HRAS* identify a subgroup of patients with HPV+ OPC with reduced OS after adjuvant chemoradiation. In support of our findings, mutations in *PIK3CA* have already been described as predictor of poor response to chemoradiation in locally advanced cervical [12, 28] and rectal cancer [29]. Although Ras activation can induce radioresistance [30] the predictive role of individual *KRAS* mutations for outcome after chemoradiation remains controversial [31, 32]. This might be explained by the fact that the influence on radio- and chemosensitivity can differ between different mutation types [33]. Importantly, among downstream pathways of Ras, the dominant mediator of radioresistance was shown to be the PI3K pathway [34], suggestive of a common therapeutic target for treatment optimization in HPV+ OPC carrying mutations in *PIK3CA* or members of the Ras gene family.

Recently, increased cellular radiosensitivity due to compromised DNA repair capacity has been reported for HPV+ tumor cells [35]. However, such a relationship could not

be confirmed by another *in vitro* study [36]. Unfortunately, our gene panel did not include genes involved in DNA repair. Therefore, a prospective DKTK-ROG study has been started in which a larger NGS panel will be used to clarify whether mutations in genes from the DNA repair machinery contribute to the different efficacy of chemoradiation in HPV+ and HPV- carcinomas. Future studies will also include assessment of 3p deletions associated with the loss of *FHIT* which has recently been reported to frequently co-occur with *TP53* mutations [17] and to be linked with reduced radiosensitivity of SCCHN cells [37].

In conclusion, this is the first NGS study in a uniformly treated patient cohort which identified patient subgroups with unfavorable prognosis after standard adjuvant chemoradiation and established potential targets for optimized treatment of HPV+ and HPV- disease. The strength of our study was that associations between mutations and outcome were studied in a uniform and well-characterized patient cohort. Limitations were the sample size as well as the retrospective nature of the study. Prospective validation of our findings will therefore be important to further substantiate our findings. Meanwhile, evaluating inhibitors of mutant p53 and the PI3K pathway in preclinical models may help to develop effective treatments for patients carrying these mutations.

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The sponsors of the study had no role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

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Conflict of interest statement

All authors have declared no conflict of interest.

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Table 1: Mutation profile of SCCHN cases (N=179), according to the HPV status

Affected genes / pathways	**non-HPV-driven SCCHN (N=132)** **No. of patients (%)**	**HPV+ OPC (N=47)** **No. of patients (%)**	***P* value**
Cell cycle checkpoints (CDKN2A, TP53)	96 (73)	2 (4)	<0.001
Receptor tyrosine kinases (*EGFR, ERBB4, FGFR1, FGFR3, MET*)	14 (11)	8 (17)	0.30
Oncogenes (*KRAS, NRAS, HRAS, MYC*)	16 (12)	11 (23)	0.09
NOTCH pathway (*NOTCH1*, *FBXW7*)	19 (14)	9 (19)	0.48
PI3K pathway (*PIK3CA, PTEN*)	16 (12)	16 (34)	0.002

Table 2: Hazard ratios for LRC and OS in HPV- SCCHN (N=132), according to *TP53* mutation classification.

	Locoregional tumor control		Overall survival	
***TP53* alterations**	**HR (95% CI)**	***P* value**	**HR (95% CI)**	***P* value**
a) Hotspot criteria				
wt (n=45)	1.0		1.0	
hotspot missense (n=34)	4.4 (1.3-14.8)	**0.016**	2.3 (1.0-5.0)	**0.048**
any other alteration (n=53)	1.2 (0.4-3.9)	0.762	0.9 (0.4-2.0)	0.847
b) 'Poeta' criteria				
wt (n=45)	1.0		1.0	
disruptive (n=49)	1.3 (0.4-4.3)	0.680	1.0 (0.5-2.3)	0.913
non-disruptive (n=38)	3.4 (1.0-11.6)	**0.051**	1.7 (0.8-3.9)	0.202
*p53 expression (IHC)**				
negative (n=64)	1.0		1.0	
positive (n=68)	0.9 (0.3-2.5)	0.877	1.0 (0.5-1.9)	0.989
Pathological nodal stage				
N0 (n=15)	1.0		1.0	
N1-N3 (n=117)	0.4 (0.1-1.6)	0.192	0.6 (0.2-1.9)	0.422
Extracapsular spread				
ECE- (n=63)	1.0		1.0	
ECE+ (n=69)	1.6 (0.6-4.5)	0.371	1.9 (0.9-3.8)	0.089
Localization				
Oropharynx (n=56)	1.0		1.0	
oral cavity (n=52)	2.1 (0.8-5.7)	0.120	1.3 (0.7-2.5)	0.435
hypopharynx (n=24)	0.8 (0.2-3.3)	0.779	0.7 (0.3-1.7)	0.441
Smoking status[#]				
never-smoker (n=7)	1.0		1.0	
ex/current smoker (n=104)	0.4 (0.1-2.1)	0.315	1.5 (0.3-6.7)	0.590

*Tumors with moderate (++) or strong (+++) staining in ≥70% of nuclei were classified as p53 positive.
[#]Information on smoking habits was missing for 21 patients.

Figure Legends

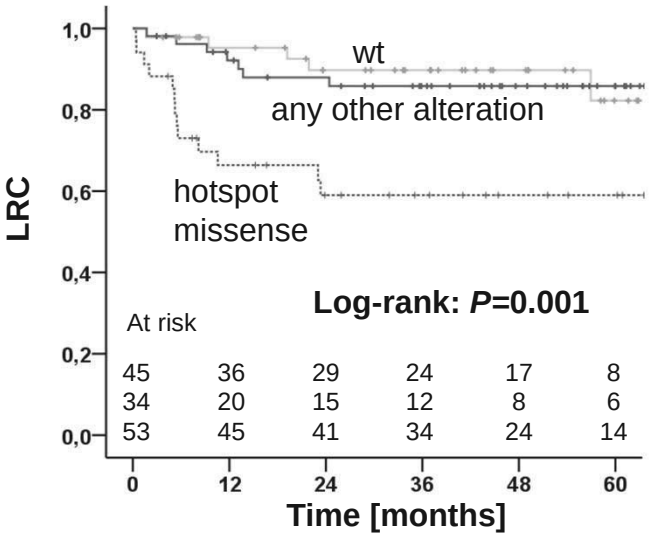
Figure 1: Kaplan-Meier survival estimates of LRC according to *TP53* alterations in HPV- SCCHN (N=132). Patients were stratified using different classifiers (hotspot criteria (A), 'Poeta' criteria (B), Eap53 score (C), any *TP53* alteration (D), p53 expression (E)), as described in detail in the Methods section. Numbers of patients at risk for the respective subgroups are given in the plot (from top to bottom: groups 1 to 3).

Figure 2: Kaplan-Meier survival estimates of OS for patients with HPV- SCCHN (A) and HPV+ OPC (B), according to mutational patterns. (A) Patients with HPV- SCCHN were stratified into four subgroups according to the absence or presence of hotspot *TP53* and *CDKN2A* mutations. (B) Patients were stratified according to the absence of presence of activating driver gene mutations. The types of the individual driver mutations in the HPV+ OPC are listed.

Figure 1

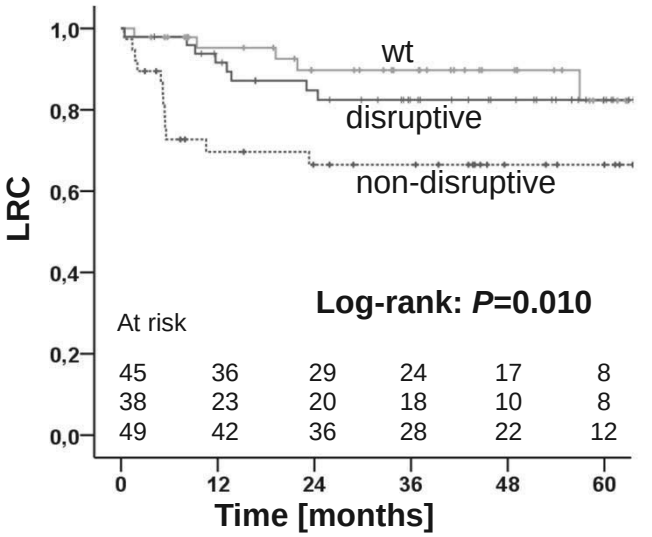
A - Hotspot criteria

1 wt (ref)
2 hotspot missense: HR 4.3 (1.5-12.1) *P*=0.006
3 any other: HR 1.3 (0.4-3.9), *P*=0.67

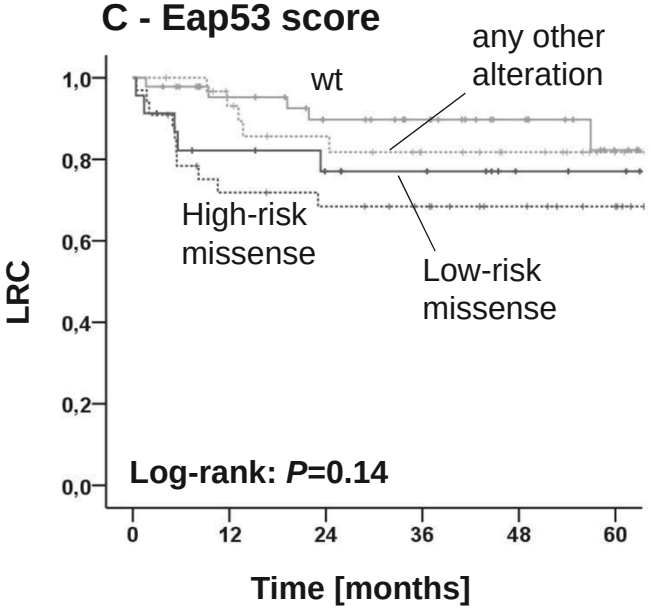


B - Poeta criteria

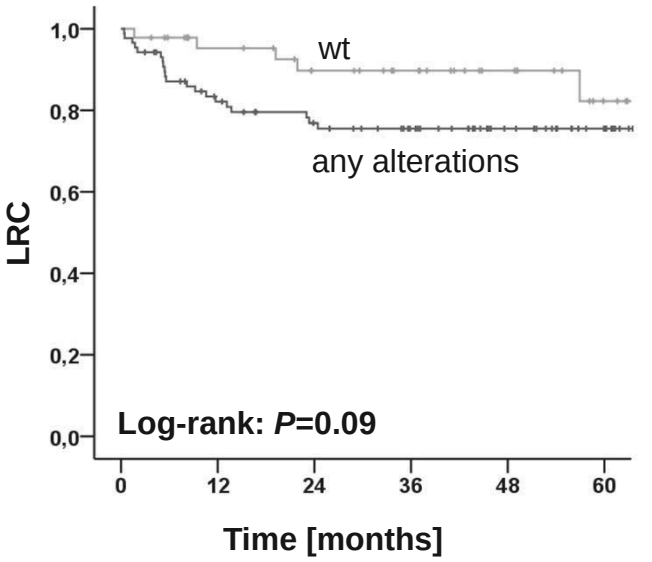
1 wt (ref)
2 non-disruptive: HR 3.7 (1.3-10.4) *P*=0.013
3 disruptive: HR 1.4 (0.5-4.3), *P*=0.56



C - Eap53 score



D - Any *TP53* alteration



E - p53 expression

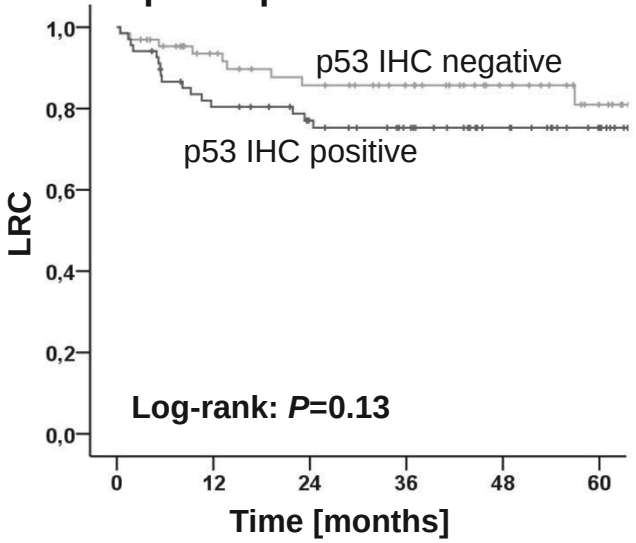
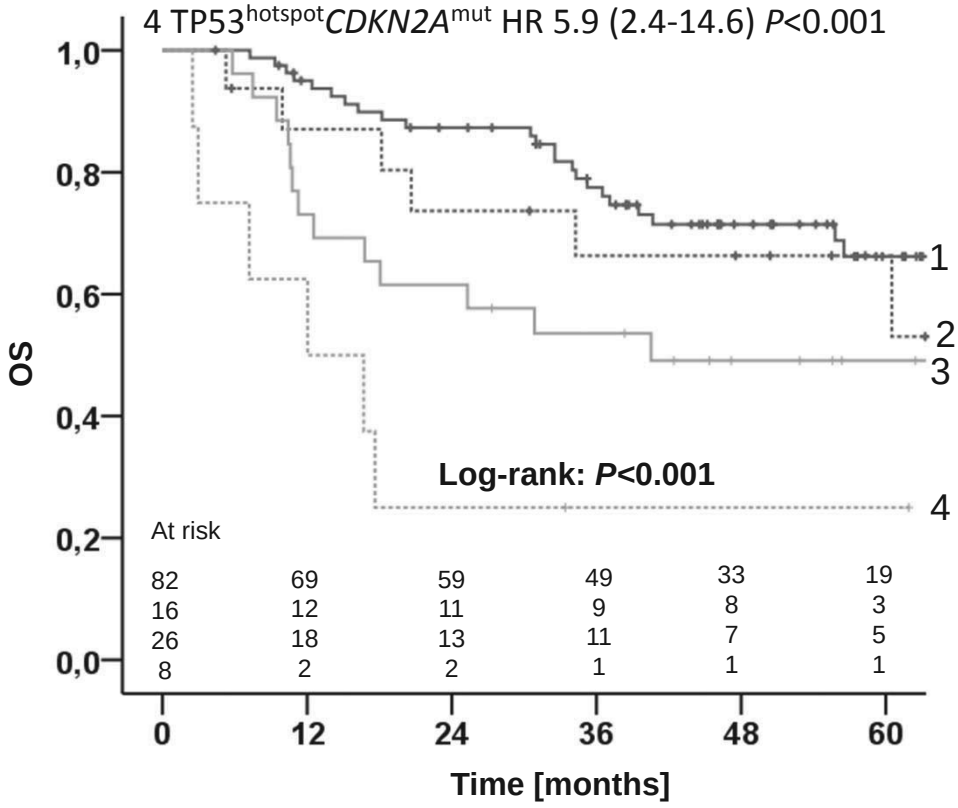


Figure 1, Tinhofer et al. 2015

Figure 2

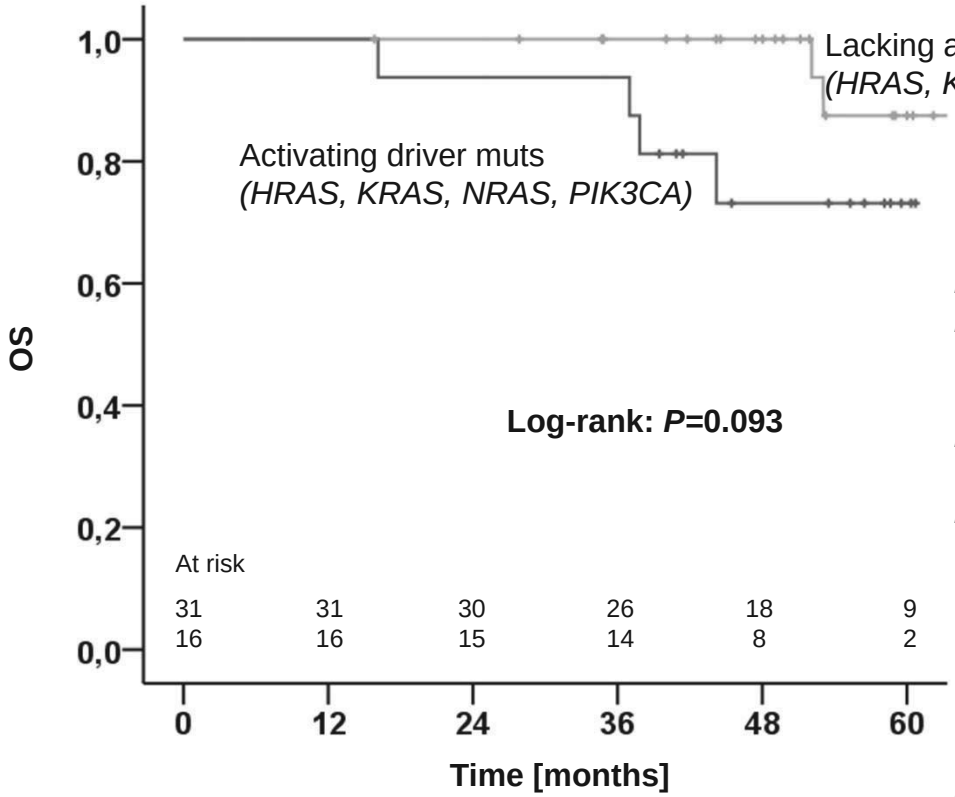
A HPV- SCCHN / HPV+ non-OPC

1 (*TP53*^{wt} or non-hotspot *TP53* alteration) & *CDKN2A*^{wt} (ref)
2 (*TP53*^{wt} or non-hotspot *TP53* alteration) & *CDKN2A*^{mut}: HR 1.6, *P*=0.26
3 TP53^{hotspot}*CDKN2A*^{wt} : HR 2.2 (1.1-4.3), *P*=0.025
4 TP53^{hotspot}*CDKN2A*^{mut} HR 5.9 (2.4-14.6) *P*<0.001



B HPV+ OPC

1 Lacking activating driver muts (ref)
2 Activating driver muts: HR 3.9 (0.7-21.1) *P*=0.11



Hotspot driver mutations

HRAS:	G13R (N=1)
KRAS:	G12V (N=1)
	G12R (N=1)
	G12D (N=1)
NRAS:	G12D (N=1)
	A59D (N=1)
PIK3CA:	E542K (N=2)
	E545K (N=10)

Figure 2, Tinhofer et al. 2015