Oral Oncology 93 (2019) 59–65

Contents lists available at ScienceDirect

Oral Oncology

journal homepage: www.elsevier.com/locate/oraloncology

# Genetic alterations in human papillomavirus-associated oropharyngeal squamous cell carcinoma of patients with treatment failure

Henrike Reder[a,*,1], Steffen Wagner[a,1], Ulrike Gamerdinger[b], Sarah Sandmann[c], Nora Wuerdemann[a,d], Andreas Braeuninger[b], Martin Dugas[c], Stefan Gattenloehner[b], Jens Peter Klussmann[a,d,2], Claus Wittekindt[a,2]

[a] Department of Otorhinolaryngology, Head and Neck Surgery, Justus-Liebig University Giessen, 35392 Giessen, Germany
[b] Department of Pathology, Justus-Liebig University Giessen, 35392 Giessen, Germany
[c] Institute of Medical Informatics, Westphalian Wilhelms University Muenster, 48149 Muenster, Germany
[d] Department of Otorhinolaryngology, Head and Neck Surgery, Medical Faculty, University Hospital Cologne, 50931 Cologne, Germany

ARTICLE INFO

*Keywords:*
Human papillomavirus
Oropharyngeal squamous cell carcinoma
Next generation sequencing
Chromosomal aberration
Recurrence

ABSTRACT

*Objectives:* Despite improved survival rates of patients with HPV-associated OPSCC, a subset has distant metastasis or develops local recurrence during follow-up. To investigate potential underlying genetic alterations, we analyzed patients with HPV-driven OPSCC who suffered from recurrence in comparison to matching pairs with successful tumor control.

*Materials and methods:* We performed chromosomal copy number analyses and targeted next generation sequencing using a custom panel comprising genes that are frequently mutated in HPV-associated OPSCC.

*Results:* Specific differences regarding chromosomal aberrations were not observed between both groups. In HPV-driven OPSCC from patients with recurrence we found higher mutation rates compared to patients with successful tumor control. Especially mutation rates of *HRAS* ($p \leq 0.05$) *PIK3R1*, *STK11* and *TP63* ($p \leq 0.1$ each) were statistically significant or trending towards significance. The respective genes can be linked to transcription factors and signaling pathways involved in cell cycle regulation, proliferation and survival. Additionally, combinations of alterations were observed on chromosomes 16 and 19, which might also influence outcome.

*Conclusion:* Patients with HPV-driven OPSCC who develop recurrence or have metastasis may be defined by genetic alterations that might be responsible for poor outcome after standard therapy. This might be of importance for stratification in future de-escalation and targeted therapy.

## Introduction

Besides overall decreasing incidences in head and neck squamous cell carcinoma, a growing number of oropharyngeal squamous cell carcinoma (OPSCC) are associated with human papillomavirus (HPV) high risk type 16 infections [1]. Despite differences regarding biological and clinical characteristics and improved overall survival (OS), patients with HPV-associated and non-HPV-associated (HPV-negative) OPSCC are treated equally. Hence, de-escalating treatment strategies for patients with HPV-driven OPSCC are widely discussed and examined in various clinical trials [2,3]. However, a subset of patients with HPV-associated OPSCC exhibits an increased risk of recurrence and death [4]. Those patients are characterized by local recurrence or distant metastasis (LDR). Especially mutations in driver genes (*PIK3CA*, *KRAS*, and *NRAS*) are associated with worse survival prognosis [4]. Furthermore, subtypes of HPV-associated head and neck cancers with different responses to treatment and prognosis were identified; they are distinguished by grade of differentiation, gene expression patterns and genomic aberrations [5,6]. Therefore, patients with an increased risk towards the outcome might not be treated sufficiently by de-escalated treatment protocols and may later develop local recurrence or distant metastasis.

*Abbreviations:* DFS, disease-free survival; HPV, human papillomavirus; LDR, local/distant recurrence; OPSCC, oropharyngeal squamous cell carcinoma; OS, overall survival; SNP, short nucleotide polymorphism

* Corresponding author at: Aulweg 128 (ForMED), 35392 Giessen, Germany.
*E-mail address:* henrike.reder@hno.med.uni-giessen.de (H. Reder).

[1] These authors contributed equally as first authors.
[2] These authors contributed equally as last authors.

https://doi.org/10.1016/j.oraloncology.2019.04.013
Received 6 February 2019; Received in revised form 15 April 2019; Accepted 22 April 2019
Available online 28 April 2019
1368-8375/ 

We hypothesize that patients with HPV-driven OPSCC and LDR have a more aggressive kind of the disease based on a higher mutational burden and mutational profile distinct from non-LDR patients. Therefore, genetic aberrations in primary tumor tissue of patients with HPV-driven OPSCC who developed relapse were compared to matching patients without treatment failure. Hence, primary tumor tissue of the selected patients was analyzed for chromosomal aberrations and short nucleotide variants. A custom panel for targeted next generation sequencing (tNGS), with gene regions frequently described to be mutated in HPV-associated OPSCC, had been developed. Identification of patients with an increased risk for treatment failure and implementation of monitoring at close intervals post-treatment might be especially important regarding the current debate on treatment de-escalation for patients with HPV-associated OPSCC. Based on the observation of patients with HPV-associated OPSCC with different outcomes, we aimed to identify patterns of specific mutations and genetic alterations that can be associated with treatment failure and development of local or distant relapse.

## Materials and methods

### *Patient selection*

Characteristics and survival of all patients treated for a primary OPSCC at our hospital between 2000 and 2013 were investigated. Primary tumor tissue of patients with LDR during follow-up until May 2016 was examined. For comparison, primary tumor tissue of best matching counterparts of patients with HPV-associated OPSCC without treatment failure (non-LDR) was analyzed correspondingly. These counterparts were matched for N-stage, risk factors (nicotine, alcohol) and T-stage (Table 1 and Supp. Table 1). HPV-association was defined by expression of CDKN2A (p16INK4A) and detection of high-risk HPV type 16 DNA as previously described [7–10]. All patients were treated following approved guidelines, receiving surgery with or without adjuvant radio-/radiochemotherapy or definitive radio-/radiochemotherapy. The study was approved by the local ethics committee (reference number: 95/15).

**Table 1**
Patient characteristics of LDR (n = 12) and non-LDR (n = 12) patient cohort.

| | | LDR | | non-LDR | |
|---|---|---|---|---|---|
| Age | Young (< 60) | 5 | 41.7% | 8 | % |
| | Old (≥60) | 7 | 58.3% | 4 | % |
| | Median | 65.8 years | | 57.4 years | |
| | Mean | 67.0 years | | 59.4 years | |
| | | n | [%] | n | [%] |
| Gender | Female | 3 | 25.0 | 4 | 33.3 |
| | Male | 9 | 75.0 | 8 | 66.7 |
| T-stage | T1 | 0 | 0.0 | 3 | 25.0 |
| | T2 | 3 | 25.0 | 5 | 41.7 |
| | T3 | 4 | 33.3 | 1 | 8.3 |
| | T4 | 5 | 41.7 | 3 | 25.0 |
| N-stage | N0 | 2 | 16.7 | 1 | 8.3 |
| | N+ | 10 | 83.3 | 11 | 91.7 |
| M-stage | M0 | 7 | 58.3 | 12 | 100.0 |
| | M+ | 5 | 41.7 | 0 | 0.0 |
| Smoking | Yes | 8 | 72.7 | 8 | 66.7 |
| | No | 3 | 27.3 | 4 | 33.3 |
| | Unknown | 1 | – | – | – |
| Alcohol | Yes | 2 | 18.2 | 1 | 9.1 |
| | No | 9 | 81.8 | 10 | 90.9 |
| | Unknown | 1 | – | 1 | – |
| Surgery | Yes | 6 | 50.0 | 10 | 83.3 |
| | No | 6 | 50.0 | 2 | 16.7 |
| Radiotherapy | Yes | 9 | 90.0 | 10 | 90.9 |
| | No | 1 | 10.0 | 1 | 9.1 |
| | Unknown | 2 | – | 1 | – |
| Chemotherapy | Yes | 9 | 90.0 | 6 | 50.0 |
| | No | 1 | 10.0 | 6 | 50.0 |
| | Unknown | 2 | – | – | – |

### *DNA isolation and sequencing library preparation*

Formalin-fixed paraffin-embedded (FFPE) primary tumor tissue was stained with hematoxylin and eosin to define tumor regions. DNA was isolated from macrodissected FFPE tumor tissue with the Maxwell® 16 FFPE Plus LEV DNA purification kit (Promega, Madison, Wisconsin, USA) following manufacturer's protocol. DNA concentration was determined with the Qubit™ 1.0 fluorometer (Thermo Fisher Scientific) and the High Sensitivity DNA Kit (Agilent 2100 Bioanalyzer). DNA was amplified and prepared as described in the protocol for the Ion AmpliSeq™ DNA and RNA Library Preparation (Thermo Fisher Scientific) kit. Approximately 10 ng DNA from the primary tumor tissue were used to prepare the tNGS library. Sequencing was performed on the Ion Torrent PGM (Thermo Fisher Scientific).

### *Targeted next generation (tNGS) panel and variant calling*

90 target regions in 22 genes that are frequently described to be altered in HPV-associated OPSCC [11–17] were selected for the tNGS panel (Supp. Table 2). Primers were designed with the Ion AmpliSeq Designer v5.4.2 (Thermo Fisher Scientific). Additionally, the coding sequence of *TP53* was included (exon 2 - exon 11). Sequencing data were analyzed using the pipeline appreci8 [18] and aligned to GRCh37 (hg19). Read depth was set to a minimum of 50 and the minimum number for variant allele reads to 20. The alternative's average base quality of reads must be at least 15 and the difference of the average base quality between reference and alternative must not be greater than 7. Only protein altering mutations were taken into consideration for further analysis.

### *Genome-wide SNP-array analysis*

100–200 ng DNA derived from primary tumor tissue samples containing at least 70% tumor cells in a final volume of 4 µL were prepared for the Infinium® HD FFPE Assay (Illumina, San Diego, California, USA) following protocol. Prior to this, samples were analyzed and prepared with the Infinium HD FFPE QC Assay protocol (Illumina, San Diego, California, USA) and the FFPE Restore Protocol (Illumina, San Diego, California, USA) following manufacturer's protocols. Signals were analyzed with KaryoStudio v1.4 (Illumina, San Diego, California, USA) by two independent researchers and uncertain results were reviewed collectively for agreement. Data from the B allele frequency were weighted higher than from the log R ratio. Only alterations with agreement of both researchers were taken into consideration and counted as true aberration. Fig. 4 was visualized using Circos [19].

### *Statistical analysis*

Statistics were performed using the SPSS statistical software (IBM SPSS 24.0, Chicago, Illinois, USA). Overall survival (OS) was calculated from initial date (date of routine biopsy confirmed by histology) to date of death. Disease-free survival (DFS) was calculated from end of treatment of the primary tumor to date of diagnosis of relapse. OS and DFS rates were calculated by the Kaplan-Meier method and the significance of differences was calculated by log-rank test. Qualitative and quantitative data were compared using Fisher's Exact test and *t*-test for independent samples, two-sided each. Significance of results was expected for p-values ≤ 0.05.

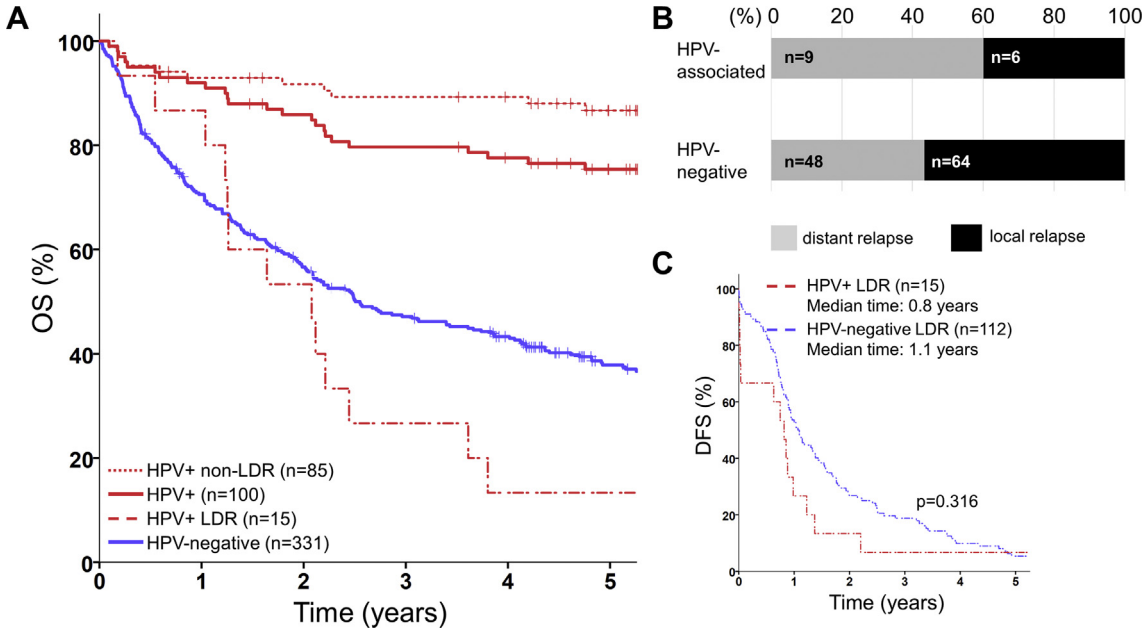



**Fig. 1.** Survival of patients with OPSCC (n = 431). A: Overall survival (OS) of patients with HPV-associated (HPV+, red, n = 100) and HPV-negative (blue, n = 331) OPSCC (solid lines). Dotted and dashed lines show patients with LDR (dashed; n = 15) and non-LDR (dotted; n = 85) in HPV-associated OPSCC. B: Ratio of distant (grey) and local relapse (black) in HPV-associated (n = 15) and HPV-negative patients (n = 112). C: Disease-free survival (DFS) of patients with LDR according to HPV-status (blue: HPV-negative, n = 112; red: HPV+, n = 15). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

## Results

### *Identification and characterization of patients with HPV-associated OPSCC and treatment failure*

In survival analyses for all OPSCC treated between 2000 and 2013 in our hospital, we observed that patients with HPV-driven OPSCC showed significantly better OS (n = 100; 5y-OS: 75.4%; $p \leq 0.001$) compared to HPV-negative OPSCC patients (n = 331; 5y-OS: 37.8%). In the LDR subgroup of HPV-driven OPSCC, 5-year OS rates were 13.3% (n = 15) compared to 86.7% in patients without relapse (n = 85) (Fig. 1A). Patients with HPV-driven OPSCC and relapse had decreased OS rates compared to HPV-negative patients ($p \leq 0.001$). In the case of LDR in HPV-driven OPSCC, more patients developed distant metastasis than local recurrence (60% vs. 40%) compared to HPV-negative LDR patients (Fig. 1B). Median time of disease-free survival (DFS) did not differ significantly between patients with HPV-associated and HPV-negative OPSCC (0.8 vs. 1.1 years; p = 0.316, Fig. 1C).

Primary tumor tissue of all patients with HPV-associated OPSCC and LDR with sufficient material, which passed DNA quality control (n = 12) were sequenced by tNGS. Matching counterparts (n = 12, non-LDR) were also included in the study (see Methods section). 5/12 (41.7%) patients with HPV-associated OPSCC suffered local relapse, the remaining 7 patients (58.3%) developed distant metastases (2 liver, 4 lung, 1 bone). The median age of the HPV-associated LDR group was 65.8 years and 75% were male. Cohort and individual patient characteristics are listed in Table 1 and Supp. Table 1, respectively.

### *Targeted next generation sequencing (tNGS)*

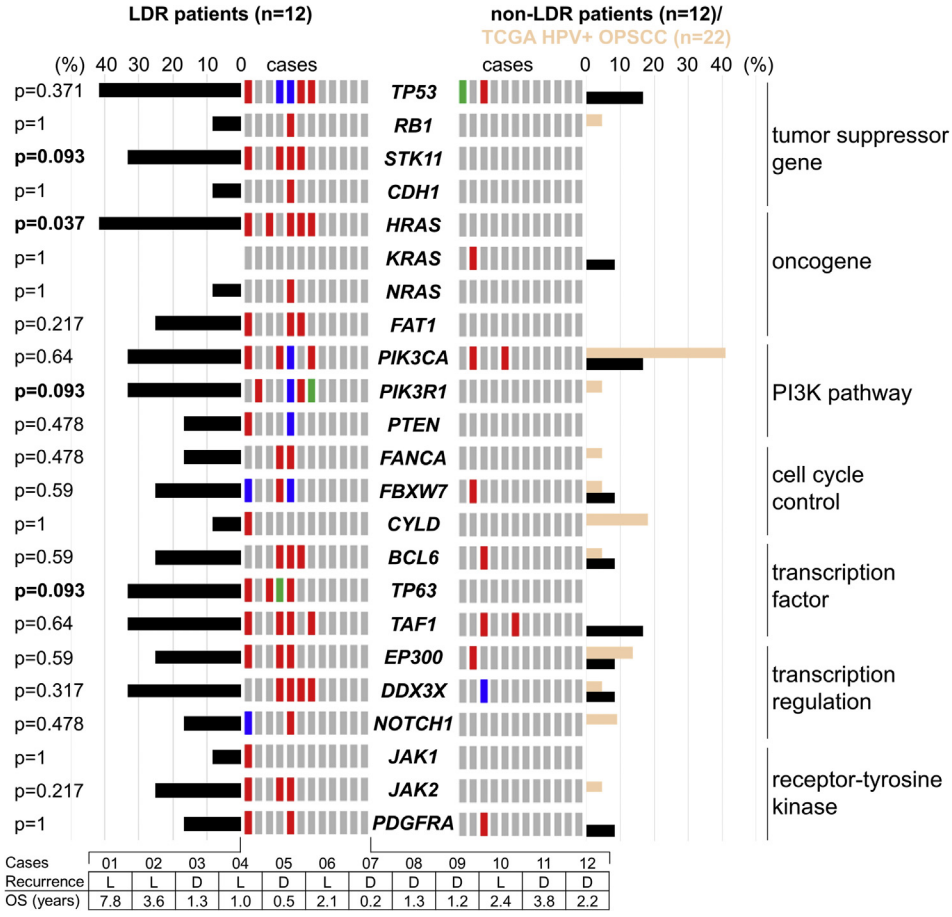
The average depth of base coverage was 3289 reads and over 97% of bases had more than 100 reads in the sequenced gene regions. Overall, mutations were detectable in 7/12 (58.3%) primary tumor tissue samples of patients with LDR and in 5/12 (41.7%) of non-LDR patients (Fig. 2). Only one of the selected genes (*KRAS*) was not mutated in patients with LDR, while 14 genes were not mutated in non-LDR patients. *HRAS* and *TP53* were the most frequently mutated genes in our cohort with LDR (41.7%). In non-LDR patients, the most frequently affected genes were *PIK3CA*, *TAF1* and *TP53* (16.7%). Altogether, tumor tissue from patients with LDR harbored 164 distinct mutations (13.7 mutations/tumor) versus 18 mutations (1.5 mutations/tumor) in non-LDR patients (p = 0.097, *t*-test; Supp. Tables 3 and 4). Taking into consideration whether LDR patients developed local or distant recurrence, a trend for a higher mutation rate in patients with local recurrence was detected (p = 0.167, *t*-test). A significantly higher mutation rate was detected in *HRAS* ($p \leq 0.05$, Fisher's Exact test) in tissue of LDR-patients compared to non-LDR patients (Fig. 2). Moreover, the mutation frequency of *STK11*, *TP63*, and *PIK3R1* ($p \leq 0.1$ each, Fisher's Exact test) showed a trend towards significance for tissue of LDR patients compared to non-LDR patients. Six variants in patients with LDR and only one in a non-LDR patient resulted in a truncated and therefore putatively non-functional protein (Fig. 2). All detected single mutations can be found in Supp. Table 4.

### *In silico analysis of HPV-driven OPSCC*

For comparison, we investigated published data from 22 HPV-driven OPSCC patients sequenced by The Cancer Genome Atlas (TCGA) research group [13]. The relative number of mutations in all genes is comparable to our group of non-LDR patients (Fig. 2). On one hand, in our non-LDR patients we found mutations in *TP53*, *KRAS*, *TAF1* and *PDGFRA*, which were not detected in the HPV-driven TCGA cohort. On the other hand, patients sequenced by TCGA had mutations in *RB1*, *FANCA*, *CYLD*, *PIK3R1*, *NOTCH1* and *JAK1*, which were not mutated in samples from our non-LDR patients, but in our cohort with LDR. The genes *FBXW7*, *BCL6*, *DDX3X* (*DDX3*) and *EP300* were mutated to a similar extent in non-LDR patients of our group and samples analyzed by TCGA. *PIK3CA* was mutated in both cohorts. However in approximately 40% of the cases analyzed by TCGA, while it was only mutated in 15% of our non-LDR patients.

### *Variants in the tumor suppressor gene TP53*

Altogether, we detected 25 *TP53* variants in primary tumor tissue of



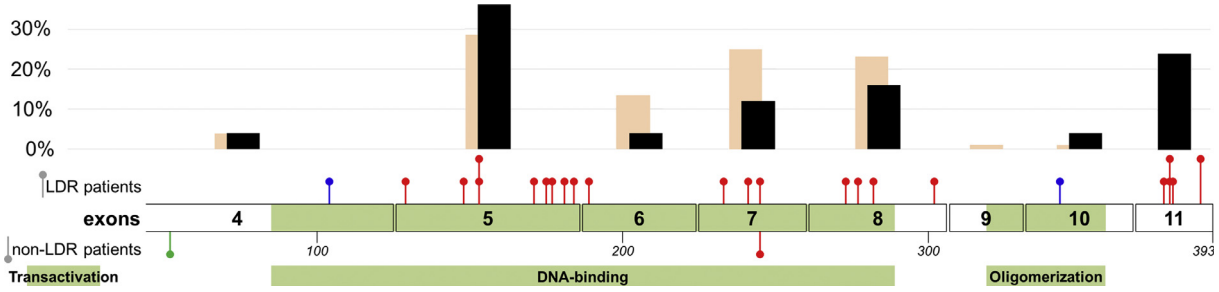




**Fig. 2.** Mutation pattern of HPV-associated OPSCC with local/distant recurrence (left) and non-recurrence (right). Shown are 12 patients each in the group with LDR (left columns) and non-LDR (right columns). Each row corresponds to one or more regions of the indicated gene analyzed by targeted sequencing. Black bars indicate the percentage of patients with a variant in that group. Type of recurrence and overall survival data are shown for LDR patients below. Data from HPV-driven OPSCC (n = 22) analyzed by TCGA are added in beige for *in silico* analysis. Red: missense mutation, blue: truncating mutation, green: frameshift mutation, OS: overall survival, L: local recurrence, D: distant recurrence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Fig. 3.** *TP53* mutations in patients with local/distant recurrence (top) and non-recurrence (bottom). The diagram shows the relative frequency of somatic mutations in the indicated exon of our patients with LDR (n = 12; black) and of the IARC TP53 database (n = 28,869; beige; IARC, Lyon, France [20]). Exons are represented in black boxes labeled 4–11; domains are highlighted in green with their respective functions indicated. Exons 2 and 3 containing the transactivation domain are left out because no variants were detected. Length of each dot represents the number of patients with a mutation in the respective position. Italic numbers: amino acid residues; red: missense mutation, black: truncating mutation, green: frameshift mutation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

LDR-patients and only in two of non-LDR patients (Supp. Table 4). 17/25 variants found in OPSCC of LDR-patients are located in the DNA-binding domain: one in the oligomerization domain, six in the C-terminus and one in exon 8 between the DNA-binding and the oligomerization domain. The two variants in tissue of non-LDR patients are located in exon 4 (between the transactivation and the DNA-binding domain) and in exon 7 (DNA-binding domain). Comparing the distribution of *TP53* mutations in our study with published data from the international agency for research on cancer (IARC, release R19) [20], differences exist in exon 6, exon 7 and exon 11. While the IARC counts 13.4% of all mutations in exon 6, 24.9% in exon 7 and 0.1% in exon 11, we detected 4% in exon 6 12% in exon 7 and 24% in exon 11 in our cohort with LDR (Fig. 3).

*Chromosomal aberrations in relation to mutations*

DNA with sufficient quality was isolated from 18/24 (9/12 of each group of patients) primary tumor tissue samples and further analyzed for chromosomal aberrations. Three tumors showed no imbalances: two from non-LDR patients and one from a patient with LDR. The most common gains were observed on chromosome 3q. Gain on chromosome 3q26–q29 was observed in 5 samples from patients with LDR and non-LDR, each (Fig. 4). Amongst others, this region covers the genes *PIK3CA*, *TP63* and *BCL6*, which are also mutated in primary tumor tissue of some patients with LDR. The most common losses were seen on chromosome 11q and 14q (Fig. 4). Loss on chromosome 11q22.3–q24.2 was seen in 4/9 tumors tissues of patients with LDR and non-LDR each. Loss on chromosome 14q23.3–q32.33 was observed in 4/9 (44.4%)

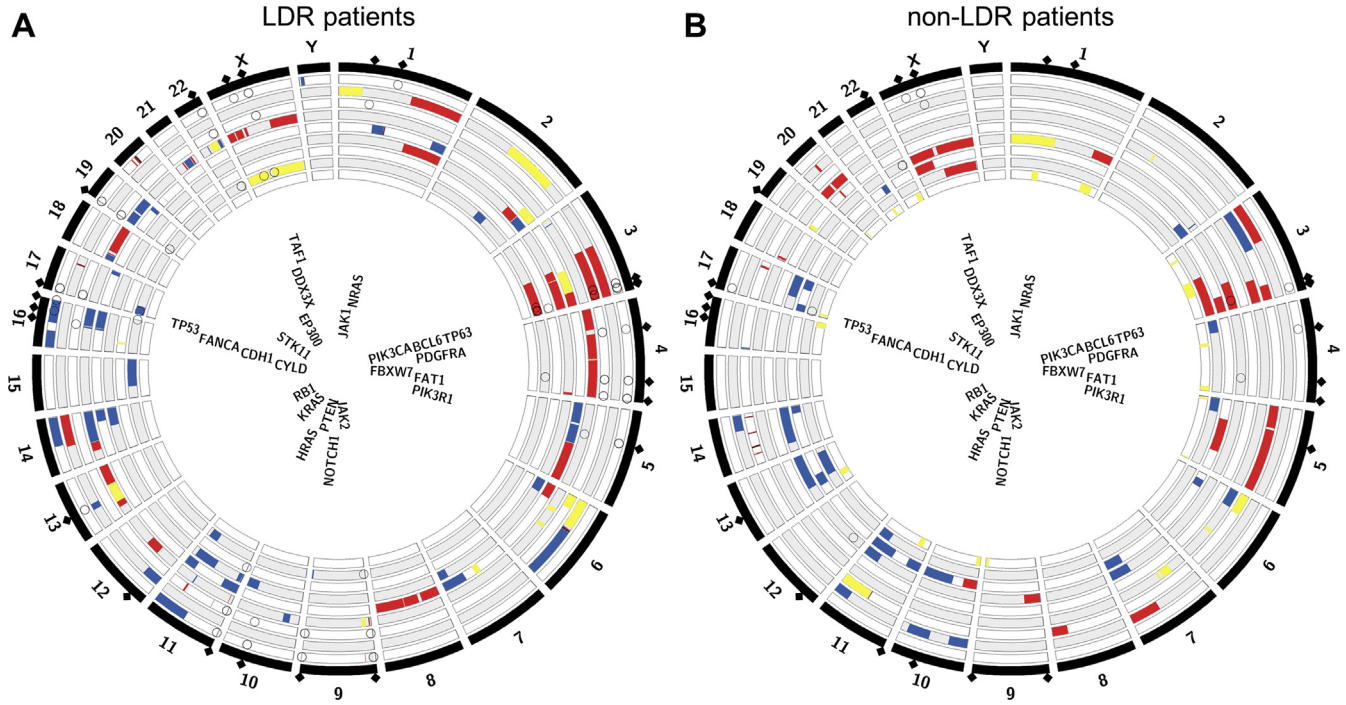



**Fig. 4.** Chromosomal aberrations for patients with HPV-associated OPSCC. Chromosomal aberrations are shown for 9 patients with local/distant recurrence (A) and non-recurrence (B), each. The outer circle (black) represents each chromosome of the entire genome, labeled with the respective number. Chromosomes are shown clockwise from short (p) to long (q) arm. Each inner circle (grey or white) represents one patient. Centromere regions are not sufficiently covered with SNPs and were therefore not analyzed. Black diamonds on the outer circle indicate the location of genes examined by tNGS (Fig. 2). Blank (small) circles indicate if a mutation was detected in the respective gene in the particular patient. Red: gain; dark red: amplification; blue: loss; yellow: loss of heterozygosity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

OPSCC with LDR and 3/9 (33.3%) non-LDR. Tumor tissue of one patient with LDR had a gain on chromosome 14q23.3–q32.33.

Considerable differences could be observed on chromosomes 16 and 19. Tumor tissues from four patients with LDR had alterations on chromosome 19p (1x deletion, 3x mutations in *STK11*) while the tumor sample of one non-LDR patient had a loss of heterozygosity on chromosome 19p13.3 (Fig. 4). Chromosome 16q12.1–q24.3 was lost in 3/9 (33.3%) OPSCC of patients with LDR but in none with non-LDR. 5/9 (55.5%) samples of patients with LDR who were analyzed for both, mutations and chromosomal aberrations, had an alteration on chromosome 16q: two patients had only deletions, two patients had only mutations and one patient had both, mutations and deletions. The chromosome regions carrying *CYLD*, *CDH1* and *FANCA* were deleted in OPSCC of 3 patients with LDR. Additionally, *FANCA* was mutated in tumor tissue of one patient with LDR; *CDH1* and *CYLD* were mutated each in one OPSCC with LDR. In line with this, from non-LDR patients only one tissue sample showed loss of heterozygosity on chromosome 16q23.1–q26.3.

## Discussion

### *Alterations in patients with local/distant recurrence are associated with tumor progression*

By comparing HPV-driven OPSCC with LDR and non-LDR, we detected different mutation frequencies in several genes. Primary tumor tissue of patients with LDR had mutations in more genes analyzed by the custom designed panel and more distinct mutations in single genes than non-LDR patients (Fig. 4, Supp. Tables 3 and 4). Especially *STK11*, *HRAS*, *PIK3R1* and *TP63* were more frequently mutated. In line with previous findings, we did not detect any mutation in codons 12, 13 or 15 of *KRAS* [21]. However, one mutation in KRAS was found in non-LDR patients but not in patients with LDR. The detected missense mutation in codon 17 (S17N) had been shown to completely block KRAS activation [22].

A weakening of our study is the low number of primary OPSCC specimen from patients developing LDR. Respective cases are rare and we analyzed all samples that had been available at our site. Only multicenter studies can overcome this issue, which might be initiated based on our findings in future. A second weakening is the application of tNGS instead of genome wide methods, which is related to the foregoing point. We were dependent on FFPE OPSCC samples from our archive. Therefore, only tNGS based methods are currently suitable, but progress in sequencing technologies might overcome this issue. Nevertheless, with our limited panel of sequenced regions, this study yielded some interesting and (regarding *TP53*) unexpected findings.

TCGA data does not contain information on treatment failure in patients with HPV-driven OPSCC. Nevertheless, considering the sample size of the TCGA data and the recurrence rate in patients with HPV-driven OPSCC, we assume that all 22 patients are non-LDR patients. Mutations in *TP53* are absent in TCGA data of HPV-driven OPSCC [13], while we found *TP53* variants in five OPSCC patients with LDR (41.7%) and two (16.7%) with non-LDR. Apart from exon 6, exon 7 and exon 11, the frequency and distribution of *TP53* mutations across all exons are similar to published databases (Fig. 3). Exon 6 and exon 7 (lower mutational frequency found in our cohort with LDR) are part of the DNA-binding domain, while no functional domain is described for exon 11. However, exon 11 (higher mutational frequency in our cohort with LDR) is known to contain residues important for the biological function of p53 (like variant p.G389E) or for post-translational modifications influencing transcriptional activity [23–25]. It was reported that metachronous recurrent OPSCC share a genomic landscape with HPV-negative OPSCC and also exhibit higher frequencies of *TP53* mutations [26]. Although absent in HPV-driven cancers in general, *TP53* mutations seem to be an important factor for the development of recurrence in our cohort. It remains open, whether LDR in patients with HPV-

driven OPSCC is causally related to individual TP53 mutations or whether additional lifestyle-risk factors (e.g. smoking) and corresponding (pre-cancerous) genetic alterations in patient's history have been superimposed by carcinogenic mechanisms due to HPV-infection.

Evidence strongly supports the hypothesis that the N-terminally truncated major isoform of the p53-related protein p63 (ΔNp63α; encoded by *TP63*) is an activator of the receptor interacting serine/threonine kinase 4 (RIPK4) [27]. RIPK4 is a protein involved in WNT/β-catenin signaling and aberrant expression was reported to be associated with progression and poor prognosis in cervical squamous cell carcinoma patients [28]. In our study, *TP63* was mutated in tumor tissue of four patients in the group with LDR but in none with non-LDR ($p < 0.1$, Fisher's Exact test). Interestingly, HPV-DNA integration within or close to *TP63* has been reported for head and neck cancers [29–32] and one of the few HPV-positive head and neck cancer cell lines (UM-SCC-47) harbors HPV-DNA integrated in *TP63* [33]. Therefore, we presume a putative relation between HPV, p63 and RIPK4, which might have an impact on cancer progression in HPV-driven OPSCC.

*HRAS* is a potent oncogene, Especially codons 12, 13 and 61 have been described as common mutational hotspots. *HRAS* was mutated in tumor tissue of five patients with LDR in our cohort but in none with non-LDR ($p < 0.05$, Fisher's Exact test). Hotspot codons 12 and 13 were affected in two cases. An additional mutation was found in codon 17 (S17N). The latter alteration leads to a dominant negative effect and inhibition of endogenous Ras-signaling [34,35]. Four detected mutations in exon 3 (p.G115R, p.C118Y, p.D119N, p.A122T) are located in the GTP-nucleotide binding domain. Residues p.C118 and p.D119 are critical elements for nucleotide sensing and nucleotide specific interactions [36]. Our results show that *HRAS* mutations might indeed play an important role in the development of relapse in HPV-associated OPSCC as it has been suggested for HPV-associated cervical and oral carcinoma [37,38].

Hotspot amino acid mutations in *PIK3CA* (residues p.E542, p.E545 and p.H1047) and mutations in *PIK3R1* have been reported in numerous human cancers [39–42] (and recently reviewed in [43]). We found *PIK3CA* hotspot mutations equally in two patients of each group with LDR and non-LDR, indicating an association with HPV rather than disease outcome. In colorectal cancer, the *PIK3CA* mutation p.G106S, we found in one LDR patient, was shown to result in weaker interactions with the regulatory subunit PIK3R1 compared to PIK3CA wild-type [44]. *PIK3R1* mutations, we detected in the LDR cohort, are located in the RhoGAP and the SH2 domain, which play a role in binding and activating downstream targets and signal transduction. Previous studies reported oncogenic effects of such mutations by weakening the inhibitory interaction between PIK3R1 and PIK3CA [43]. Additionally, it has been shown in breast cancer that mutation or loss of PIK3R1 increases PI3K/Akt signaling and oncogenic transformation, suggesting a role as tumor suppressor [45]. In a whole exome study, patients with alterations in the PI3K-pathway showed improved outcome [46]. In contrast, our findings suggest that aberrant PI3K/Akt pathway signaling by altered PIK3R1 and an instable PIK3CA/PIK3R1 complex might be involved in cancer progression and poor prognosis.

The tumor suppressor STK11 (LKB1) is a serine/threonine kinase originally identified as an activator of the AMP-activated protein kinase (AMPK) pathway. In our study, *STK11* was mutated in OPSCC of four patients with LDR and in none with non-LDR ($p < 0.1$, Fisher's Exact test). Mutated *STK11* promotes progression and cell proliferation in HPV-driven cervical carcinoma [47,48] which is in line with our findings.

Gain of chromosome 3q was detected in 10/18 (55.6%), partial loss of chromosome 11q in 8/18 (44.4%) and partial loss of chromosome 14q in 7/18 (38.9%) patients with HPV-associated OPSCC, irrespective of the outcome and in line with literature [49–51]. Synchronous deletions of *TRAF3* (14q32.32) and *CYLD* (16q12.1) was observed in tumor tissue of three patients with LDR but in none with non-LDR. TRAF3 has been suggested as tumor suppressor during pathogenesis of viral infection and tumor progression [52]. In line, defective TRAF3 promoted cancer cell survival and drug resistance in HPV-associated head and neck cancer [53], supporting our findings. Defects in *TRAF3* and *CYLD* have also been related to NF-κB signaling activation and an improved overall survival [54]. In contrast to our data, mutations in *TRAF3* and *CYLD* did not occur simultaneously in the mentioned study, which might indicate synergistic effects of alterations in both genes.

The HPV-specific set of genes we investigated by tNGS results from the technical demands regarding FFPE samples. Methodic development in sequencing techniques may help in future to yield more information from this demanding sample type. Although patients with HPV-associated OPSCC and local/distant recurrence are rare and our patient cohort was limited, we could provide new findings. We show that genes associated with tumor progression are more frequently mutated in tumor samples of patients with LDR compared to non-LDR patients. Regarding chromosomal copy aberrations, no obvious differences between tumor samples from patients with LDR and non-LDR could be detected. However, additive effects of mutations and chromosomal aberrations like synchronous deletion of *CYLD* and *TRAF3* might have an impact on disease outcome. In order to corroborate our findings, functional assays of the detected variants in cell culture models should be performed. In addition, bio-banking of fresh-frozen tumor samples is adjuvant to support future research, especially regarding rare entities.

In summary, genetic markers potentially relevant for the outcome of patients with HPV-associated OPSCC were found in a limited experimental setup. Our data demonstrates that suitable methods for detection of such markers might support patient selection, particularly in consideration of future de-escalating strategies for patients with HPV-associated OPSCC.

## Author contributions

Conceptualization: JPK, NW, CW; Methodology: SW, AB; Software: SS, MD; Verification: HR, AB; Formal Analysis: HR, SW, UG; Investigation: HR, UG; Resources: JPK, CW, AB, SG, MD; Data Curation: SS, MD; Writing – Original Draft: HR, SW; Writing – Review & Editing: CW, JPK, NW, SS, UG, MD, AB, SG; Visualization: HR, SW; Supervision: JPK, CW, AB, SG, MD; Project Administration: JPK, NW, SW; Funding Acquisition: JPK, NW, SW.

## Acknowledgements

We thank Maike Roth, Cindy Arnold, Sebastian Schaefer, Katharina Sack and Stefanie Rudloff for their excellent technical support; Magnus von Knebel-Doeberitz, Elena Sophia Prigge and colleagues from the department of applied tumor biology in Heidelberg for performing HPV diagnostics; and Shachi Jenny Sharma for her critical and helpful feedback.

## Funding

Funding: This work has been financially supported by the, Verein zur Förderung der Krebsforschung in Gießen e.V.“ and by the Rhön Klinikum AG (project: FI 46).

## Declaration of interest

Declaration of interest: none.

## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.oraloncology.2019.04.013.

## References


[1] Chai RC, et al. Current trends in the etiology and diagnosis of HPV-related head and neck cancers. Cancer Med 2015;4(4):596–607.
[2] Mirghani H, et al. Treatment de-escalation in HPV-positive oropharyngeal carcinoma: ongoing trials, critical issues and perspectives. Int J Cancer 2015;136(7):1494–503.
[3] Masterson L, et al. De-escalation treatment protocols for human papillomavirus-associated oropharyngeal squamous cell carcinoma: a systematic review and meta-analysis of current clinical trials. Eur J Cancer 2014;50(15):2636–48.
[4] Tinhofer I, 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.
[5] Zhang Y, et al. Subtypes of HPV-positive head and neck cancers are associated with HPV characteristics, copy number alterations, PIK3CA mutation, and pathway signatures. Clin Canc Res Off J Am Assoc Canc Res 2016;22(18):4735–45.
[6] Keck MK, et al. Integrative analysis of head and neck cancer identifies two biologically distinct HPV and three non-HPV subtypes. Clin Canc Res Off J Am Assoc Canc Res 2015;21(4):870–81.
[7] Wagner S, et al. Human papillomavirus association is the most important predictor for surgically treated patients with oropharyngeal cancer. Br J Cancer 2017;116(12):1604–11.
[8] Prigge ES, et al. Diagnostic accuracy of p16(INK4a) immunohistochemistry in oropharyngeal squamous cell carcinomas: a systematic review and meta-analysis. Int J Cancer 2017;140(5):1186–98.
[9] Schmitt M, et al. Bead-based multiplex genotyping of human papillomaviruses. J Clin Microbiol 2006;44(2):504–12.
[10] Klussmann JP, et al. Expression of p16 protein identifies a distinct entity of tonsillar carcinomas associated with human papillomavirus. Am J Pathol 2003;162(3):747–53.
[11] Seiwert TY, et al. Integrative and comparative genomic analysis of HPV-positive and HPV-negative head and neck squamous cell carcinomas. Clin Canc Res Off J Am Assoc Canc Res 2015;21(3):632–41.
[12] Lechner M, et al. Targeted next-generation sequencing of head and neck squamous cell carcinoma identifies novel genetic alterations in HPV+ and HPV- tumors. Genome Med 2013;5(5):49.
[13] The Cancer Genome Atlas Network. Comprehensive genomic characterization of head and neck squamous cell carcinomas. Nature 2015;517(7536):576–82. https://doi.org/10.1038/nature14129.
[14] Stransky N, et al. The mutational landscape of head and neck squamous cell carcinoma. Science 2011;333(6046):1157–60.
[15] Gaykalova DA, et al. Novel insight into mutational landscape of head and neck squamous cell carcinoma. PloS one 2014;9(3):e93102.
[16] Lui VW, et al. Frequent mutation of the PI3K pathway in head and neck cancer defines predictive biomarkers. Cancer Discov 2013;3(7):761–9.
[17] Hedberg ML, et al. Genetic landscape of metastatic and recurrent head and neck squamous cell carcinoma. J Clin Investig 2016;126(1):169–80.
[18] Sandmann S, et al. appreci8: a pipeline for precise variant calling integrating 8 tools. Bioinformatics 2018;34(24):4205–12. https://doi.org/10.1093/bioinformatics/bty518.
[19] Krzywinski M, et al. Circos: an information aesthetic for comparative genomics. Genome Res 2009;19(9):1639–45.
[20] Bouaoun L, et al. TP53 variations in human cancers: new lessons from the IARC TP53 database and genomics data. Hum Mutat 2016;37(9):865–76.
[21] Prigge ES, et al. No evidence of oncogenic KRAS mutations in squamous cell carcinomas of the anogenital tract and head and neck region independent of human papillomavirus and p16(INK4a) status. Hum Pathol 2014;45(11):2347–54.
[22] Matallanas D, et al. Differences on the inhibitory specificities of H-Ras, K-Ras, and N-Ras (N17) dominant negative mutants are related to their membrane microlocalization. J. Biol Chem 2003;278(7):4572–81.
[23] Pirolli D, et al. Insight into a novel p53 single point mutation (G389E) by Molecular Dynamics Simulations. Int J Mol Sci 2010;12(1):128–40.
[24] Feng L, et al. Functional analysis of the roles of posttranslational modifications at the p53 C terminus in regulating p53 stability and activity. Mol Cell Biol 2005;25(13):5389–95.
[25] Friedler A, et al. Modulation of binding of DNA to the C-terminal domain of p53 by acetylation. Structure 2005;13(4):629–36.
[26] Harbison RA, et al. The mutational landscape of recurrent versus nonrecurrent human papillomavirus-related oropharyngeal cancer. JCI Insig 2018;3(14).
[27] Mitchell K, et al. Exome sequence identifies RIPK4 as the Bartsocas-Papas syndrome locus. Am J Hum Genet 2012;90(1):69–75.
[28] Liu DQ, et al. Increased RIPK4 expression is associated with progression and poor prognosis in cervical squamous cell carcinoma patients. Sci Rep 2015;5:11955.
[29] Koneva LA, et al. HPV integration in HNSCC correlates with survival outcomes, immune response signatures, and candidate drivers. Molecul Cancer Res MCR 2018;16(1):90–102.
[30] Walline HM, et al. Genomic integration of high-risk HPV alters gene expression in oropharyngeal squamous cell carcinoma. Molecul Cancer Res MCR 2016;14(10):941–52.
[31] Bodelon C, et al. Genomic characterization of viral integration sites in HPV-related cancers. Int J Cancer 2016;139(9):2001–11.
[32] Parfenov M, et al. Characterization of HPV and host genome interactions in primary head and neck cancers. PNAS 2014;111(43):15544–9.
[33] Akagi K, et al. Genome-wide analysis of HPV integration in human cancers reveals recurrent, focal genomic instability. Genome Res 2014;24(2):185–99.
[34] Guil S, et al. Alternative splicing of the human proto-oncogene c-H-ras renders a new Ras family protein that trafficks to cytoplasm and nucleus. Cancer Res 2003;63(17):5178–87.
[35] Song C, et al. Regulation of a novel human phospholipase C, PLCepsilon, through membrane targeting by Ras. J Biol Chem 2001;276(4):2752–7.
[36] Johnson CW, et al. The small GTPases K-Ras, N-Ras, and H-Ras have distinct biochemical properties determined by allosteric effects. J Biol Chem 2017;292(31):12981–93.
[37] Cordova-Alarcon E, et al. Effects of HRAS oncogene on cell cycle progression in a cervical cancer-derived cell line. Arch Med Res 2005;36(4):311–6.
[38] Anderson JA, et al. H-ras oncogene mutation and human papillomavirus infection in oral carcinomas. Arch Otolaryngol–Head & Neck Surg 1994;120(7):755–60.
[39] Wood LD, et al. The genomic landscapes of human breast and colorectal cancers. Science 2007;318(5853):1108–13.
[40] Philp AJ, et al. The phosphatidylinositol 3'-kinase p85alpha gene is an oncogene in human ovarian and colon tumors. Cancer Res 2001;61(20):7426–9.
[41] Cheung LW, et al. High frequency of PIK3R1 and PIK3R2 mutations in endometrial cancer elucidates a novel mechanism for regulation of PTEN protein stability. Cancer Discov 2011;1(2):170–85.
[42] Parsons DW, et al. An integrated genomic analysis of human glioblastoma multiforme. Science 2008;321(5897):1807–12.
[43] Madsen RR, Vanhaesebroeck B, Semple RK. Cancer-associated PIK3CA mutations in overgrowth disorders. Trends Mol Med 2018.
[44] Ikenoue T, et al. Functional analysis of PIK3CA gene mutations in human colorectal cancer. Cancer Res 2005;65(11):4562–7.
[45] Thorpe LM, et al. PI3K-p110alpha mediates the oncogenic activity induced by loss of the novel tumor suppressor PI3K-p85alpha. PNAS 2017;114(27):7095–100.
[46] Hanna GJ, et al. Improved outcomes in PI3K-pathway-altered metastatic HPV oropharyngeal cancer. JCI insight 2018;3(17).
[47] Zeng Q, et al. LKB1 inhibits HPV-associated cancer progression by targeting cellular metabolism. Oncogene 2017;36(9):1245–55.
[48] Wingo SN, et al. Somatic LKB1 mutations promote cervical cancer progression. PloS one 2009;4(4):e5137.
[49] Hayes DN, Van Waes C, Seiwert TY. Genetic landscape of human papillomavirus-associated head and neck cancer and comparison to tobacco-related tumors. J Clin Oncol Off J Am Soc Clin Oncol 2015;33(29):3227–34.
[50] Dahlgren L, et al. Comparative genomic hybridization analysis of tonsillar cancer reveals a different pattern of genomic imbalances in human papillomavirus-positive and -negative tumors. Int J Cancer 2003;107(2):244–9.
[51] Smeets SJ, et al. Genome-wide DNA copy number alterations in head and neck squamous cell carcinomas with or without oncogene-expressing human papillomavirus. Oncogene 2006;25(17):2558–64.
[52] Chen T, et al. Genetic alterations in TRAF3 and CYLD that regulate nuclear factor kappaB and interferon signaling define head and neck cancer subsets harboring human papillomavirus. Cancer 2017;123(10):1695–8.
[53] Zhang J, et al. Defective TRAF3 modulates alternative NF-kB signaling and cytokine expression to promote cancer cell survival in HPV positive head and neck cancer (TUM10P.1049). J Immunol 2015;194(1 Suppl.). p. 211.30–211.30.
[54] Hajek M, et al. TRAF3/CYLD mutations identify a distinct subset of human papillomavirus-associated head and neck squamous cell carcinoma. Cancer 2017;123(10):1778–90.