


# Analysis of genetic variants of frequently mutated genes in human papillomavirus-negative primary head and neck squamous cell carcinoma, resection margins, local recurrences and corresponding circulating cell-free DNA

Susanne Flach<sup>1,2</sup>  | Jörg Kumbrink<sup>2,3</sup> | Christoph Walz<sup>3</sup> | Julia Hess<sup>4,5,6</sup> | Guido Drexler<sup>4,6</sup> | Claus Belka<sup>2,4,6</sup> | Martin Canis<sup>1,4</sup> | Andreas Jung<sup>2,3</sup> | Philipp Baumeister<sup>1,4</sup>

<sup>1</sup>Department of Otorhinolaryngology, Head and Neck Surgery, LMU Klinikum, Munich, Germany

<sup>2</sup>German Cancer Consortium (DKTK), Partner Site Munich, Munich, Germany

<sup>3</sup>Institute of Pathology, Faculty of Medicine, LMU Munich, Munich, Germany

<sup>4</sup>Clinical Cooperation Group 'Personalised Radiotherapy in Head and Neck Cancer', German Research Center for Environmental Health GmbH, Neuherberg, Germany

<sup>5</sup>Research Unit Radiation Cytogenetics, Helmholtz Zentrum München, Neuherberg, Germany

<sup>6</sup>Department of Radiation Oncology, University Hospital, LMU Munich, Munich, Germany

## Correspondence

Susanne Flach, Department of Otorhinolaryngology, Head and Neck Surgery, LMU Klinikum, Marchioninistrasse 15, 81377 Munich, Germany.  
Email: [susanne.flach@med.uni-muenchen.de](mailto:susanne.flach@med.uni-muenchen.de)

## Funding information

Munich Clinician Scientist Programme; Verein zur Förderung von Wissenschaft und Forschung an der Medizinischen Fakultät der LMU München e.V.

## Abstract

**Background:** Head and neck squamous cell carcinoma remains a substantial burden to global health. Despite evolving therapies, 5-year survival is <50% and unlike in other cancers, reliable molecular biomarkers to guide treatment do not exist.

**Methods:** We performed targeted panel next-generation sequencing to analyse somatic variants from primary and recurrent tumour tissue, corresponding resection margins and cell-free DNA from intra-operatively collected plasma samples from eight patients with human papillomavirus-negative head and neck squamous cell carcinoma. Patients were primarily treated with curative-intent surgery and received subsequent adjuvant treatment.

**Results:** The most frequently mutated gene was *TP53*. Other mutated genes included *NOTCH1*, *NF1* and *CDKN2A* among others. A total of 20.8% of variants were shared between primary tumour and resection margin. Out of all the variants detected, 37.5% were shared between cell-free DNA and primary tumour, whereas 12.5% were commonly found in cell-free DNA, primary tumour and resection margin. Mutational profiling was able to distinguish between a locoregional recurrence and a second primary tumour by identifying a different *TP53* mutation in the primary tumour compared to the recurrent tumour in addition to private *FBXW7* and *CTNNB1* mutations. We also identified identical *TP53* and *PIK3CA* mutations in another primary tumour and corresponding recurrence.

**Conclusion:** Molecular profiling of cell-free DNA and resection margins has potential applications in clinical practice to guide future treatment decisions.

## KEYWORDS

cell-free DNA, HNSCC, liquid biopsy, next-generation sequencing, resection margins

This is an open access article under the terms of the [Creative Commons Attribution-NonCommercial-NoDerivs](https://creativecommons.org/licenses/by-nc-nd/4.0/) License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2022 The Authors. *Journal of Oral Pathology & Medicine* published by John Wiley & Sons Ltd.

## 1 | INTRODUCTION

Head and neck cancer is a heterogenous disease with 5-year survival rates of patients with head and neck squamous cell carcinoma (HNSCC) remaining below 50%.<sup>1</sup> Despite evolving therapies and improved treatment options, many patients develop recurrences, metastases or a second primary tumour. Treatment of HNSCC is mainly multimodal and surgical tumour excision is a crucial pillar of treatment in many cases. Complete removal (R0 resection) is the main aim, and positive (tumour-bearing) resection margins (R1) are associated with reduced overall survival and an increased rate of locoregional recurrences. However, even a histomorphologically tumour-free surgical margin (R0) is no guarantee for recurrence-free survival. Lack of guidelines regarding uniform classification as 'negative' resection margins and the fact that premalignant lesions in close vicinity to the tumour cannot always be reliably detected using conventional histopathology impedes the assessment of the resection status, thus, affecting prognosis and further therapy planning.<sup>2-7</sup>

It is well established that the process of tumour evolution progresses through stages of premalignancy wherein cells acquire abnormal genotypes and phenotypes that increase their propensity for malignant transformation. Such clonally unrelated premalignant cells can often be found distant from each other and contribute to local disease recurrence or the development of a second primary tumour in the upper aerodigestive tract.<sup>8-10</sup> Based on our understanding of the molecular pathogenesis of HNSCC, two major theories might explain the high local recurrence rate in this tumour entity. On the one hand, molecular characterisation of resection margins has shown that conventional histopathological assessments cannot detect all precancerous and malignant cells, which would implicate residual tumour cells as the culprit of the recurrence.<sup>11-13</sup> On the other hand, since Slaughter et al.<sup>10</sup> first described the field cancerisation theory, primary carcinomas and local recurrences can originate from large fields composed of morphologically normal but genetically altered mucosal cells following long-term exposure to carcinogens such as tobacco and alcohol.<sup>3,10,13,14</sup>

Extraordinary progress has been made advancing sequencing technologies, thereby increasing the number of potential variant discovery and their implementation in clinical applications. Circulating cell-free tumour DNA (ctDNA) is just one emerging tumour biomarker that has not yet been sufficiently studied in HNSCC.<sup>15-20</sup> The main advantage of ctDNA is its availability from liquid biopsies, for example, blood or saliva samples, that are minimally invasive.<sup>21-23</sup> With recent technological improvement and increasing cost-effectiveness of next-generation sequencing (NGS), even small amounts of ctDNA can potentially be detected and quantified.

For this study, we retrospectively analysed plasma samples, archived tissues from primary and recurrent tumours as well as adjacent resection margins. We used targeted panel NGS to profile the mutational landscape of multiregionally and sequentially collected samples from eight patients with human papillomavirus (HPV)-negative HNSCC primarily treated with curative-intent surgery. The aims of this study were firstly to provide a characterisation of the pre-conditioned mucosa of histopathologically negative resection margins

and their corresponding primary and, if applicable, recurrent tumours to shed light into their genomic and evolutionary relationship. Secondly, we wanted to assess the role of ctDNA analysis as an additional tool for therapy planning. Here we confirm that HNSCC is characterised by a heterogenous mutation profile and that alterations in various cancer genes can be detected in negative resection margins as well as in cell-free DNA, emphasising the potential of molecular analysis in determining the resection status and its role in characterising tumour evolution.

## 2 | MATERIALS AND METHODS

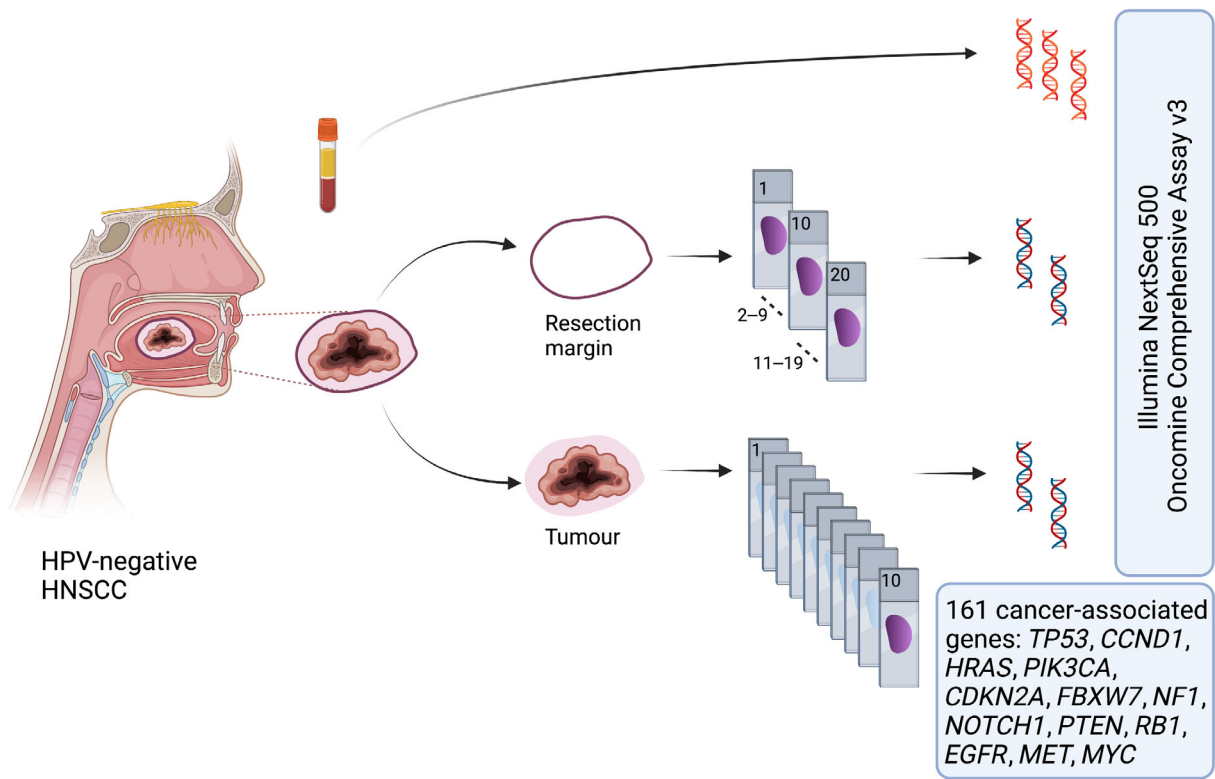
### 2.1 | Study design and patient cohort

A retrospective subcohort study was conducted to genetically profile cell-free DNA from intra-operatively collected plasma samples, as well as DNA from resected tumour tissue and adjacent resection margins taken from the tumour bed (Figure 1). All samples were previously obtained and archived from a subcohort of eight patients with HNSCC, enrolled between May 2014 and February 2016 into a prospective cohort study conducted by the Clinical Cooperation Group at the LMU Klinikum Munich.<sup>24</sup> Patients with HPV-negative, resectable HNSCC of the oral cavity, pharynx or larynx with Stages II-IV (American Joint Committee on Cancer 8th edition) were included. Patients with distant metastasis (cM1) or other active malignancies at the time of enrolment as well as HPV-positive cases were excluded. All patients were staged to exclude distant metastasis with computed tomography and/or magnetic resonance imaging. Patients received adjuvant radio(chemo)therapy according to the National Comprehensive Cancer Network guidelines,<sup>25</sup> if necessary and following recommendation by the local multidisciplinary tumour board. HPV status was confirmed by quantitative polymerase chain reaction in combination with p16 immunohistochemistry as described previously.<sup>24</sup>

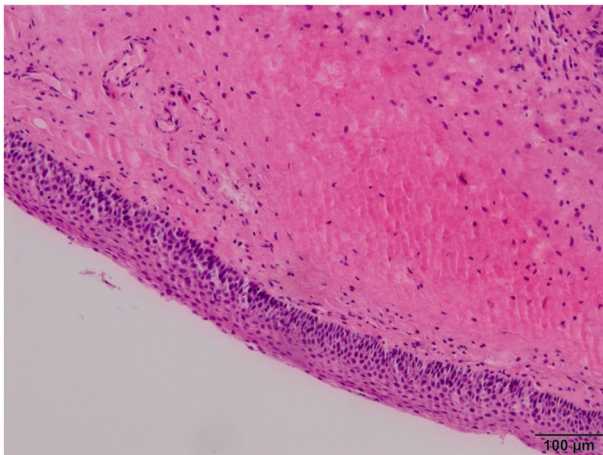
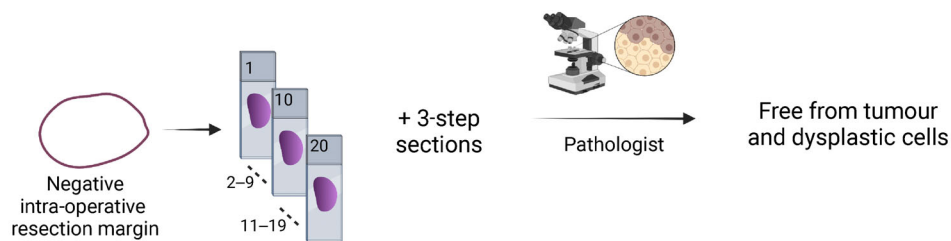
### 2.2 | Sample collection and nucleic acid extraction

Peripheral blood was collected intra-operatively, immediately after resection of the primary tumour, in EDTA Vacutainer tubes (Monovette, Sarstedt, Germany) and processed within 4 h. Plasma was separated by centrifugation at 350 g for 10 min, transferred to microcentrifuge tubes and centrifuged twice again at 1200 g for 3 min and at 14 000 g for 10 min, respectively. Plasma cell-free DNA was isolated using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) and stored at -80°C until further analysis.

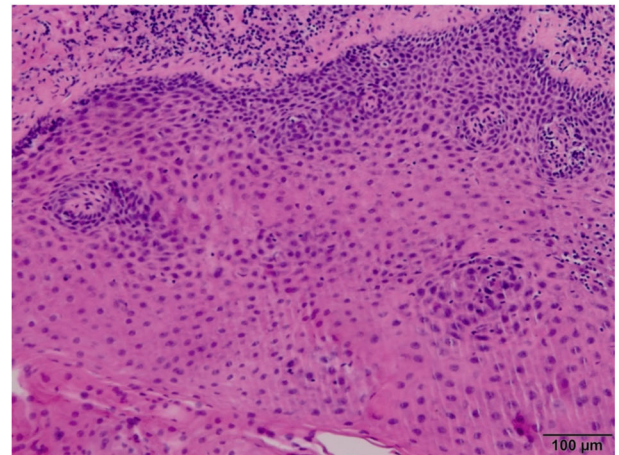
Formalin-fixed paraffin-embedded (FFPE) tumour blocks of the resected specimen as well as the corresponding resection margins taken during intraoperative frozen section analysis (subsequently formalin-fixed and paraffin-embedded) and initially deemed histomorphologically tumour-free, that is, negative, were obtained from the archives. Sections from FFPE tumour tissue samples were prepared followed by haematoxylin-eosin (H&E) staining of one slide. Tumour



**FIGURE 1** Illustration of the workflow. Figure generated with [BioRender.com](https://www.biorender.com). HPV, human papillomavirus; HNSCC, head and neck squamous cell carcinoma



Case 1: H&E stain resection margin



Case 4: H&E stain resection margin

**FIGURE 2** Histopathological confirmation of negative resection margins. Top panel: illustration of the workflow. Bottom panel: Haematoxylin and eosin-stained (H&E) formalin-fixed paraffin-embedded tissues from three-step sections confirmed that no tumour or dysplastic cells were present. Figure generated with [BioRender.com](https://www.biorender.com)

areas with a minimum percentage of 40% tumour cells were microdissected from subsequent unstained sections and used for nucleic acid preparation. For resection margin analysis, 20 sections were cut including three slides for H&E staining (first, middle and last slides) and the residual ones for further analysis. In addition to the extra H&E-stained slides, three-step sections were cut to ensure that resection margins were free from tumour and dysplastic cells. This was confirmed by an experienced head and neck pathologist (Figure 2). DNA was extracted from the FFPE tumour and resection margin tissue samples using the GeneRead DNA FFPE Kit (Qiagen, Hilden, Germany), respectively. Nucleic acid concentrations were measured using a Qubit 2.0 fluorometer (Invitrogen, CA, USA) and the Qubit dsDNA HS (High Sensitivity) Assay Kit (ThermoFisher, MA, USA).

### 2.3 | Sequencing library preparation, NGS and variant interpretation

Library preparation with the AmpliSeq for Illumina OncoPrint Comprehensive Assay v3 (Illumina, CA, USA), targeting 161 cancer-associated genes (Table S1), subsequent sequencing and variant calling were performed as described in detail previously.<sup>26</sup> Briefly, sequencing was performed on an Illumina NextSeq 500 system using NextSeq 500/550 High Output Kits v2.5 according to the manufacturer's protocols. Analysis of the results was performed with the Illumina Local Run Manager, subsequent annotation of variant call format files using wANNOVAR<sup>27</sup> and an in-house python script filtering for relevant mutations. A high-sensitivity approach (>10 000 average coverage) was used for liquid biopsy analyses. Sequencing quality parameters are presented for tissue and liquid analyses in Table S2. Alterations were confirmed with the integrative genomics viewer (Broad Institute, MA, USA). Variants were judged as relevant based on the interpretation criteria utilised in ClinVar.<sup>28</sup> Moreover, pathogenicity prediction algorithms and other publicly available databases were used for variant interpretation (VarSome,<sup>29</sup> dbSNP [available from: <https://www.ncbi.nlm.nih.gov/snp/>]). Only likely pathogenic and pathogenic mutations as well as VUS (variant of unknown significance or not evaluated in ClinVar with a prediction trend of being likely pathogenic) were reported. Single-nucleotide variants, multi-nucleotide variants, small insertions, deletions, indels and copy number variation were analysed.

## 3 | RESULTS

### 3.1 | Patient characteristics

Eight patients who were diagnosed with HPV-negative HNSCC and treated by surgical tumour resection were included into this subcohort study. The median age of the study participants was 55 years (range 41–74 years), and the majority (87.5%) were male. All patients were scheduled for curative-intent surgery and received adjuvant treatment according to the recommendations of the multidisciplinary tumour board. Median follow-up time was 16.1 months (range 7.0–71.6 months)

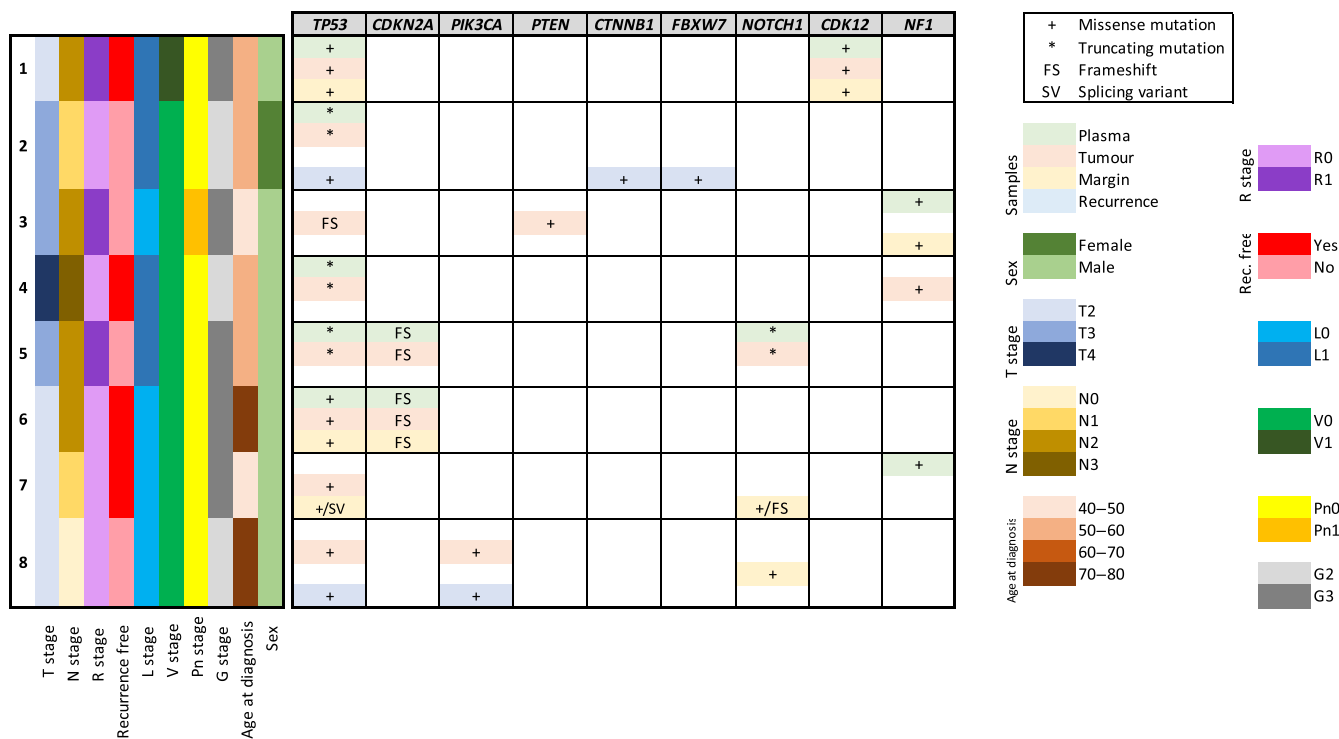
**TABLE 1** Patient demographics

	Absolute number, <i>n</i>	%
Age (years)		
Stage II ( <i>n</i> = 1)	74	
Stage III ( <i>n</i> = 2)	Median: 65 (range: 55–75)	
Stage IV ( <i>n</i> = 5)	Median: 52 (range: 41–58)	
Sex		
Male	7	87.5
Female	1	12.5
Location		
Oral cavity	3	37.5
Oropharynx	3	37.5
Larynx	1	12.5
Hypopharynx	1	12.5
pT stage		
pT1	0	0
pT2	4	50.0
pT3	3	37.5
pT4	1	12.5
pN stage		
pN0	1	12.5
pN1	2	25.0
pN2	4	50.0
pN3	1	12.5
Grade		
G1	0	0
G2	3	37.5
G3	5	62.5
Resection status		
R0	5	62.5
R1	3	37.5
Adjuvant therapy		
Radiotherapy	2	25.0
Radiochemotherapy	6	75.0

and four of eight patients developed a histologically confirmed recurrence after completing their treatment. FFPE tumour tissue from a biopsy of the recurrence was available and could be retrieved from the archives from two of the four patients (Patients 2 and 8). A total of 26 retrospectively collected plasma, primary and recurrent tumour tissue and resection margin samples from eight patients with HNSCC were selected for further analysis. The demographics and clinical characteristics of the patient cohort are shown in Table 1 and listed in more detail for each patient in Table S3.

### 3.2 | Mutations in primary and recurrent tumours

DNA sequencing from primary and recurrent tumour tissue was successful in all samples and a mean read depth of 3453.6 was



**FIGURE 3** Overview of somatic variants identified in primary tumour, recurrence, plasma and resection margin. Histopathological and demographic data are from the time of the initial primary tumour resection. V stage, invasion into vein; L stage, invasion into lymphatic vessels; Pn stage, perineural invasion; G stage, grade of tumour

achieved to detect somatic DNA variants (Table S2). The average identified number of somatic variants per primary tumour after applying our filtering criteria was 1.875 (range 1–3). Both recurrent tumours, Samples 2 and 8, had two or three somatic variants each, respectively. Somatic variants were identified in nine genes, four of which were recurrently mutated (Figure 3). The most frequently mutated gene was *TP53* (8/8) with predominantly missense and truncating mutations identified (Figure 4). Other mutated genes included *NOTCH1* (3/8), *NF1* (3/8) and *CDKN2A* (2/8). *CDK12*, *CTNNB1*, *FBXW7*, *PTEN* and *PIK3CA* were each mutated once (Tables S4 and S5). In total 8 of 24 variants were private to either primary tumour or recurrence and not detected in plasma or resection margin. The median VAF of *TP53* in all primary tumours was 34% (range 19–48.5) and the median VAF of all detected somatic variants was 35.2% (range 11.2%–93.9%), respectively.

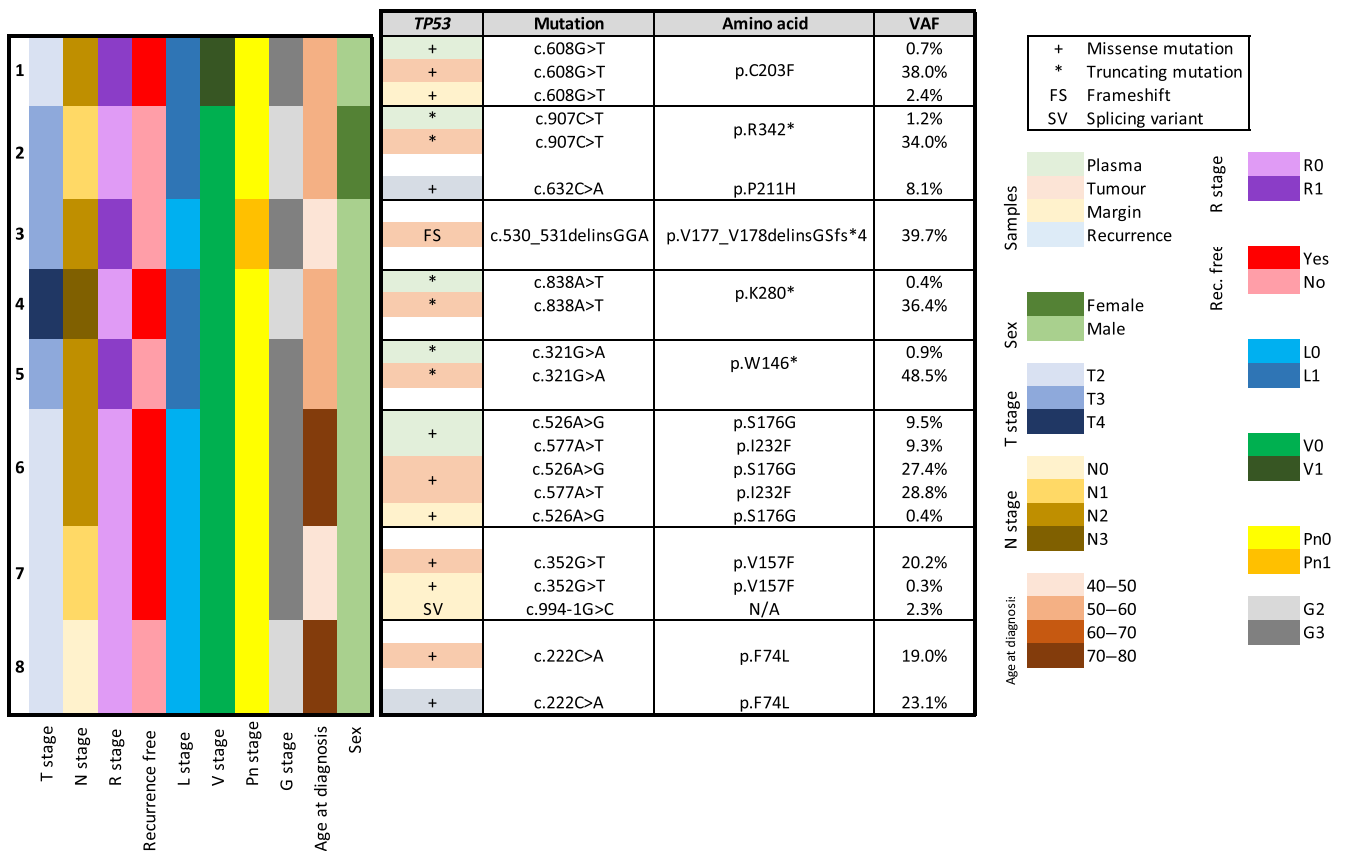
Patient 2 had a pT3 lateral tongue squamous cell carcinoma (SCC) (pN0, Stage III), which was resected in addition to removing the cervical lymph nodes. Three years later the patient developed a local recurrence in the oral cavity which was histomorphologically believed to be a second primary tumour due to differences in the grade of differentiation between the tumour pairs. Targeted panel NGS identified a different *TP53* mutation in the primary tumour (R342\*) compared to the recurrent tumour (P211H) which also showed additional *FBXW7* (R339G) and *CTNNB1* (S33Y) mutations that had not been present in the original tumour of the tongue.

Patient 8 also developed a locoregional recurrence 10 months after resection of a pT2 oropharyngeal SCC and bilateral neck

dissection (pN0, Stage II). Here, an identical *TP53* mutation (F74L) and *PIK3CA* mutation (N345K) were found to be present in the primary tumour and in the recurrence.

### 3.3 | Mutations in resection margins

We sequenced DNA isolated from one representative intraoperative resection margin that was sent for frozen section analysis for each of the eight resected primary tumours. Pathological review ensured that selected margins did not contain any dysplastic or tumour cells (Figure 2). DNA sequencing was successful in all samples with a mean DNA sequencing depth of 3622.8. The average identified number of somatic variants per resection margin tissue after applying our filtering criteria was 1.0 (range 0–2). Somatic variants were identified in five genes; two genes were recurrently mutated in at least two resection margins, including *TP53* (3/8) and *NOTCH1* (2/8). *CDKN2A*, *NF1* and *CDK12* were mutated once. However, the *CDK12* variant was detected in the primary tumour and tumour margin as well as in the liquid sample at around 50% VAF, suggesting it is a germline variant/polymorphism. Two of the patients who experienced disease recurrence (Patients 3 and 8) had mutations in *NF1* and *NOTCH1*. Three of four cases without recurrence had each 2 genes mutated. The median VAF of *TP53* in all resection margins was 1.35% (range 0.3–2.4) and the median VAF of all detected somatic variants was 2.3% (range 0.3% – 50.5%) in resection margins, which was notably lower compared to what we detected in primary tumours (Figure S1).



**FIGURE 4** Overview of somatic variants identified in *TP53* in primary tumour, recurrence, plasma and resection margin. VAF, variant allele frequency. Histopathological and demographic data are from the time of the initial primary tumour resection. V stage, invasion into vein; L stage, invasion into lymphatic vessels; Pn stage, perineural invasion; G stage, grade of tumour

Approximately one fifth (5/24) of the variants were shared between primary tumour and resection margin. In contrast, the *TP53* mutation (p.P211H) identified in the tumour recurrence of Patient 2, for example, was not detected in the resection margin.

in primary tumour tissue. Out of all the variants detected, 37.5% (9/24) were shared between plasma and primary tumour, whereas 12.5% (3/24) were commonly found in plasma, primary tumour and resection margin.

### 3.4 | ctDNA detection in plasma

Targeted sequencing of cell-free DNA from eight intra-operatively collected plasma samples was done. ctDNA sequencing was successful in all samples with a mean DNA sequencing depth of 11 697.1. We detected potential clinically relevant variants in the ctDNA of seven of eight samples analysed. The average identified number of somatic variants per plasma sample after applying our filtering criteria was 1.375 (range 0–3). In keeping with the results shown for tumours and resection margins, the most frequently mutated gene was *TP53* with 62.5% of samples showing either missense or truncating mutations. The median VAF of *TP53* in all plasma samples was 1.05% (range 0.4%–9.5%). Other recurrently mutated genes identified included *CDKN2A* (2/8) and *NF1* (2/8). Genes mutated once were *NOTCH1* and *CDK12*. The median VAF of all detected somatic variants in plasma was 1.3% (0.4%–72.2%), thus lower than the median VAF detected in resection margins and

### 4 | DISCUSSION

The existence of multilocal and distinct premalignant conditions in the upper aerodigestive tract presents the need and opportunity to define which are at greatest risk for progression, including if, how, and when to intervene.<sup>8,9</sup> Even though therapeutic options have improved over the past decade, the successful treatment of locally advanced tumours remains a challenge and requires a multimodal approach, with surgical resection remaining one of the most important treatment choices for this cohort of patients. Suitable biomarkers for HNSCC that can be used to guide treatment decisions are still lacking. This is particularly relevant regarding the concept of field cancerisation in the upper aerodigestive tract where fields of premalignant epithelia can be large and remain frequently undetectable. Following resection of the primary tumour the original field, or a near-by field can be the source of a local tumour recurrence or the emergence of a second primary tumour.<sup>2,3</sup> A recently published study by Wu et al. used targeted

panel sequencing to analyse primary tumours and tumour-adjacent tissue (TAT) of 27 patients with HPV-negative HNSCC. The group identified *TP53* as the most frequently mutated gene in TAT. They further showed that *TP53* mutations in TAT were associated with pre-malignant transformation and were indicative of worse survival (hazard ratio = 14.01). TAT-specific mutations were also detected in matched pre- and post-operative plasma and saliva samples.<sup>17</sup> The emergence of liquid biopsy has had a significant impact in the development of tools for detection of molecular residual disease and recurrence over the past decade. In patients with Stages I and II HNSCC ctDNA could be measured in plasma of 70% of patients, and 92% of patients with Stages III and IV disease had ctDNA detected in their blood.<sup>16</sup> High levels of ctDNA have previously been shown to correlate with reduced overall survival as well as tumour stage.<sup>18</sup> In patients with clinical N2–N3 HNSCC who were treated with definitive radio(chemo)therapy higher levels of total cell-free DNA quantified using TaqMan-based TERT amplification were detected compared to patients with clinical N0–N1 disease.<sup>20</sup> The growing number of studies on liquid biopsy thus supports a future role of ctDNA analysis in potentially guiding treatment decisions.<sup>16,19</sup> In this subcohort study we took advantage of liquid biopsy approaches and the availability of primary tumour tissue and corresponding resection margins from a well-characterised clinical HNSCC cohort.<sup>24</sup> We used targeted panel NGS to analyse tumour tissue, corresponding resection margins and cell-free DNA from intra-operatively collected plasma samples. Tissue from resection margins collected during intra-operative frozen section analysis with subsequent formalin-fixation and paraffin-embedding was used as this is closer to real world application and commonly used for intra-operative margin analysis. In agreement with results shown by Wu et al. and others, HNSCC is characterised by a heterogeneous mutation profile, affecting numerous genes and pathways, with *TP53* being the most frequently mutated gene in our cohort.

We were able to demonstrate that mutational profiling can be used to distinguish a locoregional recurrence from a metachronous second primary tumour that may have arisen from the same or a different premalignant field. The latter was the likely case for Patient 2 where a second primary tumour in the oral cavity harboured a different *TP53* mutation compared to the initial primary tumour, in addition to missense mutations in *FBXW7* and *CTNNB1*. All together this suggests that the recurrence may have resulted from residual low numbers of single tumour cells left behind post-operatively or an extensive field cancerisation that eventually evolved into a recurrence. Wu et al. claimed that in relapsed patients with TAT-private mutations detected in post-operative plasma samples TAT may be evolutionary independent of the primary tumour during tumourigenesis when contributing to a recurrence. However, it appears more likely that mutations apparently private to TAT were not identified in the primary tumour due to sampling bias in a heterogeneous tumour and that variants identified in plasma were originating from the primary tumour.

Limitations of our study include the small sample size. We acknowledge that only very limited conclusions can be drawn from a

cohort of eight patients; however, we believe that this study contributes to the very limited number of studies to date that have looked at cell-free DNA as well as tumour tissue and adjacent mucosa in HPV-negative HNSCC. Larger cohort studies are needed to further investigate the role of molecular analysis of resection margins and cell-free DNA for therapy planning in patients with HNSCC. Furthermore, the lack of sequencing data available from matched peripheral blood lymphocytes is a limitation as this prevents filtering of variants from clonal haematopoiesis of indeterminate potential. However, we tried to address this concern using extensive filtering, including dbSNP. The strength of our subcohort study includes that the patients enrolled were clinically well characterised with long follow-up lengths and several types of samples available, including cell-free DNA, primary and recurrent tumour tissue as well as resection margins. We decided to use archived FFPE tissue as this is more common in daily clinical practice. The option of using a fixed sequencing panel appears to be a more realistic approach of trying to introduce ctDNA testing in patients with HNSCC into clinical practice. Frequently mutated genes in HNSCC include *TP53*, *PIK3CA*, *CDKN2A*, *NOTCH1*, *FBXW7* and *HRAS*,<sup>30</sup> all or most of which can be assayed by the AmpliSeq for Illumina OncoPrint Comprehensive Assay.

In summary, findings from this subcohort study support the use of NGS analysis for the identification of molecular markers in patients with HNSCC.

#### AUTHOR CONTRIBUTIONS

Susanne Flach, Philipp Baumeister and Jörg Kumbrink designed the study. Andreas Jung, Claus Belka and Martin Canis assisted with advice for realisation of the study. Guido Drexler and Julia Hess performed most of the initial sample processing. Susanne Flach performed the sample preparation for sequencing analysis. Philipp Baumeister and Martin Canis performed HNSCC treatments. Christoph Walz was responsible for assessment of the primary FFPE tumour tissue. Susanne Flach and Jörg Kumbrink performed bioinformatic analysis and interpretation of the data. Susanne Flach, Jörg Kumbrink and Philipp Baumeister contributed to the writing of the paper.

#### ACKNOWLEDGEMENTS

We thank all the patients who participated in this study. We thank Sabine Sagebiel-Kohler and Konstanze Schäfer for technical support. Open Access funding enabled and organized by Projekt DEAL.

#### FUNDING INFORMATION

The authors received funding from 'Verein zur Förderung von Wissenschaft und Forschung an der Medizinischen Fakultät der LMU München e.V.' Susanne Flach's research position was funded by the Munich Clinician Scientist Programme (LMU Klinikum).

#### CONFLICT OF INTEREST

Susanne Flach, Christoph Walz, Julia Hess, Guido Drexler, Claus Belka and Philipp Baumeister declare no conflict of interest. Andreas Jung received honoraria for talks and Consulting or Advisory Board and

reimbursement for travel, accommodation and expenses from Amgen, AstraZeneca, Bayer Pharmaceuticals, BMS, Biocartis, Boehringer Ingelheim, Merck KGaA, Lilly Oncology, MSD Sharp and Dohme, Novartis, QulP GmbH, Roche Pharma, Takeda and Thermo Fisher. Jörg Kumbrink received honoraria and reimbursement for travel and accommodation for participants in advisory boards and from the speaker's bureau from AstraZeneca, Novartis and Roche Pharma. Martin Canis received honoraria for talks from MSD Sharp and Dohme.

## PEER REVIEW

The peer review history for this article is available at <https://publons.com/publon/10.1111/jop.13338>.

## DATA AVAILABILITY STATEMENT

Data will be made available upon reasonable request to the submitting authors.

## ETHICS STATEMENT

All patients included provided written informed consent to a clinical study which has been approved by the local ethics committee of the University of Munich (EA 448-13/17-116). The study has been conducted in accordance with the Declaration of Helsinki and in keeping with the rules of good clinical practice and according to the German laws and ethical standards.

## ORCID

Susanne Flach  <https://orcid.org/0000-0001-6196-3706>

## REFERENCES

- Gatta G, Botta L, Sánchez MJ, et al. Prognoses and improvement for head and neck cancers diagnosed in Europe in early 2000s: the EUROCARE-5 population-based study. *Eur J Cancer*. 2015;51(15):2130-2143.
- Vaamonde P, Martín C, Río M d, LaBella T. Second primary malignancies in patients with cancer of the head and neck. *Otolaryngol Head Neck Surg*. 2003;129(1):65-70.
- Braakhuis BJM, Leemans CR, Brakenhoff RH. Expanding fields of genetically altered cells in head and neck squamous carcinogenesis. *Semin Cancer Biol*. 2005;15(2):113-120.
- Schlichting JA, Pagedar NA, Chioreso C, Lynch CF, Charlton ME. Treatment trends in head and neck cancer: surveillance, epidemiology, and end results (SEER) patterns of care analysis. *Cancer Causes Control*. 2019;25(10 suppl):833-812.
- Eldeeb H, Macmillan C, Elwell C, Hammod A. The effect of the surgical margins on the outcome of patients with head and neck squamous cell carcinoma: single institution experience. *Cancer Biol Med*. 2012;9(1):29-33.
- Li M, Xie M, Zhou L, Wang S. The impact of surgical margin status on the outcomes of locally advanced hypopharyngeal squamous cell carcinoma treated by primary surgery. *Acta Otolaryngol*. 2018;138(12):1136-1145.
- Mitchell DA, Kanatas A, Murphy C, Chengot P, Smith AB, Ong TK. Margins and survival in oral cancer. *Br J Oral Maxillofac Surg*. 2018;56(9):820-829.
- Tabor MP, Brakenhoff RH, Ruijter-Schippers HJ, et al. Multiple head and neck tumors frequently originate from a single preneoplastic lesion. *Am J Pathol*. 2002;161(3):1051-1060.
- Braakhuis B, Tabor M, Kummer JA, Leemans CR, Brakenhoff RH. A genetic explanation of Slaughter's concept of field cancerization: evidence and clinical implications. *Cancer Res*. 2003;15(63):1727-1730.
- Slaughter DP, Southwick HW, Smejkal W. Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. *Cancer*. 1953;6(5):963-968.
- Du E, Ow TJ, Lo YT, et al. Refining the utility and role of frozen section in head and neck squamous cell carcinoma resection. *Laryngoscope*. 2016;126(8):1768-1775.
- Brennan JA, Mao L, Hruban RH, et al. Molecular assessment of histopathological staging in squamous-cell carcinoma of the head and neck. *N Engl J Med*. 1995;332(7):429-435.
- van Houten VMM, Leemans CR, Kummer JA, et al. Molecular diagnosis of surgical margins and local recurrence in head and neck cancer patients: a prospective study. *Clin Cancer Res*. 2004;10(11):3614-3620.
- Califano JA, van der Riet P, Westra WH, et al. Genetic progression model for head and neck cancer: implications for field cancerization. *Cancer Res*. 1996;56:2488-2492.
- Schirmer MA, Beck J, Leu M, et al. Cell-free plasma DNA for disease stratification and prognosis in head and neck cancer. *Clin Chem*. 2018;64(6):959-970.
- Wang Y, Springer S, Mulvey CL, Silliman N, Schaefer J, Sausen M. Detection of somatic mutations and HPV in the saliva and plasma of patients with head and neck squamous cell carcinomas. *Sci Transl Med*. 2015;7(293):1-8.
- Wu P, Xie C, Yang L, et al. The genomic architectures of tumour-adjacent tissues, plasma and saliva reveal evolutionary underpinnings of relapse in head and neck squamous cell carcinoma. *Br J Cancer*. 2021;125(6):854-864.
- Bettegowda C, Sausen M, Leary RJ, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med*. 2014;6(224):224ra24.
- Flach S, Howarth K, Hacking S, et al. Liquid biopsy for minimal residual disease detection in head and neck squamous cell carcinoma (LIONESS)—a personalised circulating tumour DNA analysis in head and neck squamous cell carcinoma. *Brit. J Cancer*. 2022;126(8):1186-1195.
- Mazurek AM, Rutkowski T, Fiszer-Kierzkowska A, Małusecka E, Skłodowski K. Assessment of the total cfDNA and HPV16/18 detection in plasma samples of head and neck squamous cell carcinoma patients. *Oral Oncol*. 2016;54:36-41.
- Egyud M, Sridhar P, Devaiah A, et al. Plasma circulating tumor DNA as a potential tool for disease monitoring in head and neck cancer. *Head Neck*. 2018;68(1):7-8.
- Dawson SJ, Tsui DWY, Murtaza M, et al. Analysis of circulating tumor DNA to monitor metastatic breast cancer. *N Engl J Med*. 2013;368(13):1199-1209.
- Murtaza M, Dawson SJ, Tsui DWY, et al. Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. *Nature*. 2013;497(7447):108-112.
- Weber P, Künstner A, Hess J, et al. Therapy-related transcriptional subtypes in matched primary and recurrent head and neck cancer. *Clin Cancer Res*. 2022;28(5):1038-1052.
- National Comprehensive Cancer Network guidelines. Accessed January 5, 2022. <https://www.nccn.org>
- Baumgarten L v, Kumbrink J, Jung A, et al. Therapeutic management of neuro-oncologic patients—potential relevance of CSF liquid biopsy. *Theranostics*. 2020;10(2):856-866.
- Yang H, Wang K. Genomic variant annotation and prioritization with ANNOVAR and wANNOVAR. *Nat Protoc*. 2015;10(10):1556-1566.
- Landrum MJ, Lee JM, Benson M, et al. ClinVar: improving access to variant interpretations and supporting evidence. *Nucleic Acids Res*. 2017;46(database issue):gkx1153.



29. Kopanos C, Tsiolkas V, Kouris A, et al. VarSome: the human genomic variant search engine. *Bioinformatics*. 2019;35(11):1978-1980.
30. Network TCGA. Comprehensive genomic characterization of head and neck squamous cell carcinomas. *Nature*. 2015;517(7536):576-582.

### SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Flach S, Kumbrink J, Walz C, et al. Analysis of genetic variants of frequently mutated genes in human papillomavirus-negative primary head and neck squamous cell carcinoma, resection margins, local recurrences and corresponding circulating cell-free DNA. *J Oral Pathol Med*. 2022;51(8):738-746. doi:[10.1111/jop.13338](https://doi.org/10.1111/jop.13338)