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PIK3CA Hotspot Mutations in Saliva as a Diagnostic Marker in Oral Squamous Cell Carcinoma Patients

KONSTANTIN KRÜGER[1*], AUDREY GRUST[1,2*], DAVID MUALLAH[1,2], ADRIAN PATENGE[1], HARRIET WIKMAN[3], MARIA MEDINA MESA[4], EIKE SÖREN KNUST[4], ALEXANDER SARTORI[4], RALF SMEETS[1,2], LAN KLUWE[1,5], REINHARD E. FRIEDRICH[1] and SIMON BURG[1]

[1]*Department of Oral and Maxillofacial Surgery, University Medical Center Hamburg-Eppendorf, Hamburg, Germany;*
[2]*Department of Oral and Maxillofacial Surgery, Division of Regenerative Orofacial Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany;*
[3]*Institute of Tumour Biology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany;*
[4]*Agena Bioscience GmbH, Hamburg, Germany;*
[5]*Laboratory for Tumour Genetics and Regenerative Medicine, Department of Neurology, University Hospital Hamburg-Eppendorf, Hamburg, Germany*

Abstract. *Background/Aim: This study aimed at the analogous detection of PIK3CA mutations, common in oral squamous cell carcinoma (OSCC), in matched tumor and saliva samples. Patients and Methods: Tissue and saliva samples were obtained from 29 patients diagnosed with primary OSCC. Saliva samples were obtained preoperatively; tissue specimens were acquired during tumor resection. Tumor DNA was extracted from both tissue and saliva samples. All samples were controlled for DNA quantity and quality and genetic matching of sample pairs was confirmed using the iPlex Pro Exome QC Panel. Variant detection was performed using the MassARRAY® System, a mass-spectrometry based detection system. Mutational analysis in tissue tumor DNA was made using the multiplexed ClearSEEK™ PIK3CA v1.0 Panel covering 20 hotspot mutations in PIK3CA. In saliva samples, variants were analyzed using both the ClearSEEK™ and the UltraSEEK® Lung v1.1 Panel, with a higher limit of detection but covering less PIK3CA variants. Results: Overall, a PIK3CA variant was found in seven of the 29 tumor tissue samples (24%) by ClearSEEK™ ; UltraSEEK® additionally confirmed the variant in four of these seven positive samples. Of the three variants not detected by UltraSEEK®, two were not included in the panel and one was included but not detected. Of the seven variants found in tissue, five could also be detected in the matching saliva samples (71%), either by utilizing ClearSEEK™ or UltraSEEK®. Conclusion: The detection of PIK3CA hotspot mutations in OSCC and their simultaneous occurrence in saliva underline the potential benefit of liquid biopsies for non-invasive cancer detection and follow-up care of OSCC patients.*

Around 450,000 carcinomas of the oral cavity are diagnosed worldwide annually (1). In this context, the oral squamous cell carcinoma (OSCC) represents the most frequent entity accounting for more than 90% of all oral cavity cancers (1-3). Besides the main risk factors for oropharynx cancer, which are extensive tobacco and alcohol consumption, a chronic infection with human papillomavirus (HPV) 16 and 18 further increases the risk for this life-threatening disease. The diagnosis of OSCC is often delayed due to the long symptom-free period as well as the misdiagnosis of mucosal lesions (*e.g.*, leukoplakia) in early cancer stages. Unfortunately, cervical lymph node metastasis is present in 40% of OSCC cases at the time of first diagnosis. Thus, the 5-year-survival rate remains poor with a percentage of only 70% (4). Another reason for the poor survival is the high risk of recurrence (3). Recurrent disease is difficult to detect because of the altered anatomy after ablative surgery and reconstruction, radiotherapy, oedema, and scar tissue formation. Consequently, therapy is often delayed, predisposing to a poorer outcome. Hence, it is of high clinical relevance to detect

*These Authors contributed equally to this study.

Correspondence to: Dr. med. Konstantin Krüger, Department of Oral and Maxillofacial Surgery, University Medical Center Hamburg-Eppendorf, Martinistrasse 52, 20251 Hamburg, Germany. Tel: +49 15229064363, e-mail: konkruger@gmail.com

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primary OSCC as well as its recurrence as early as possible. Whilst effective and early screening methods exist for other prominent carcinomas (*e.g.,* prostatic cancer), no such method is available in the case of initial or recurrent OSCC. Through extension into the oral cavity, carcinomas are constantly in contact with saliva thus already indicating a potential use for early detection and follow-up diagnostics of oral cancer (3, 5).

Coined by Pantel and Alix-Panabières in 2010, the term liquid biopsy (LB) refers to the yield of genetic information from tumors only through the analysis of patients' bodily fluids (6). This non-invasive diagnostic method avoids the risks of typical complications associated with a traditional tumor biopsy, such as bleeding, infection, seeding of tumor cells and sampling errors depending on tumor tissue heterogeneity. Exemplary target biomarkers for tumor diagnostics using LB are cell free DNA (cfDNA), circulating tumor cells or exosomes (6). A small fraction of cfDNA is tumor associated DNA (ctDNA) that originates from the tumor itself and is released during apoptosis, necrosis or by active secretion, thus carrying the same (epi-) genetic alterations as the tumor itself. The most frequent mutations of any given tumor entity are so-called hotspot mutations. Their detection in body fluids serves as a strong indicator for the presence of the respective tumor (5, 7).

Wang *et al*. performed a proof of principle study searching for hotspot mutations in the plasma and saliva of head and neck squamous cell carcinomas (HNSCC), emphasizing the superiority of detecting genetic markers over conventional biomarkers such as CD44 because of their specificity for neoplastic cells (5, 8). It was postulated that ctDNA detection of HNSCC has a higher accuracy in plasma, whereas the ctDNA detection of OSCC is notably more indicative in saliva because of the constant contact between tumor and fluid. This seems plausible given the mere anatomical proximity as well as Wang's statistical observation that saliva samples presented the highest measurable fraction of associated mutant DNA (5). Overall, saliva ctDNA proved more sensitive and specific for the detection of early OSCC stages than plasma ctDNA. In the case of HNSCC, detection of the associated hotspot mutations *TP53, PIK3CA, NOTCH1*, and *CDKN2A* was possible in 95% of cases. *PIK3CA* mutations are highly common to OSCC, as shown by Starzynska *et al.,* with their focus on *PTEN* loss and *PIK3CA* amplification (9).

The MassARRAY® system (Agena Bioscience, Hamburg, Germany), based on matrix-assisted laser desorption ionization-time of flight mass spectrometry, was used to detect genetic DNA targets such as somatic mutations based on their molecular mass. This method has recently been applied to LB of melanoma and lung cancer and showed additional information beyond currently detected biomarkers (10-14). However, whilst blood presented the principal focus of aforementioned studies, the MassARRAY® system equally allows for a quick and cost-effective analysis of saliva.

This study aimed to representatively detect *PIK3CA* mutation profiles of OSCC tissue, as well as in the corresponding preoperatively obtained saliva samples. The discovery of *PIK3CA* hotspot mutations in OSCC and their simultaneous occurrence in saliva could reveal the benefit of liquid biopsies as a useful tool for non-invasive early detection and follow-up care of OSCC patients. This would be in line with contemporary research, that already justifying PIK3CA mutations to be potent diagnostic markers of other carcinoma recurrences such in the case of rectal cancer residues (15).

Patients and Methods

Written informed consent was obtained prospectively from all participants. The Ethics Review Board of the University of Hamburg, Germany, approved the study (approval number: PV7012, Ärztekammer Hamburg).

Tissue as well as saliva samples were collected from 29 OSCC patients at the Department of Oral and Maxillofacial Surgery of the University Medical Center Hamburg-Eppendorf, Germany between October 2020 and August 2021 (Figure 1). The inclusion criteria were a minimal age of 18 years, a histologically confirmed OSCC that was clinically exposed to the oral cavity and sufficiently vast for biopsy without affecting routine pathological investigations. Sample pairs of tumor and saliva from each patient were pseudonymized.

Preoperative collection of saliva samples was conducted as described by Goode *et al*. and Min *et al*. (16, 17). Patients spat into a tube with 5 ml buffer solution for stabilizing the DNA after rinsing the mouth with 5 ml isotonic sodium chloride solution. Tissue samples were obtained with a biopsy punch during surgical tumor resection by an experienced maxillofacial surgeon and stored in tubes with isotonic sodium chloride solution. All samples were blinded to the clinical data and stored at 4˚C before being processed in the laboratory within 24 h. The isolation of cfDNA from saliva samples was conducted as described by Goode *et al*. (16). Cell lysis was performed using the MasterPure DNA Purification Kit (Lucigen, Middleton, WI, USA), DNA was separated from proteins by a proteinase K (Macherey-Nagel, Düren, Germany), dried, and rehydrated. The tumor tissue was first lysed using the MasterPure DNA Purification Kit (Lucigen) and subsequently, the DNA was extracted using a Master Pure DNA-kit (Lucigen). The DNA was stored at -20˚C until further mutational analysis was performed.

Prior to mutational analysis, all DNA samples were controlled for quality, quantity, and genetic identity (sample QC) with the Exome QC Panel (Agena Bioscience, Hamburg, Germany). All expected sexes were confirmed, and sample pairs matched according to a 21-SNP profile (18). For somatic mutation detection in DNA from tumor tissue and saliva, genetic analyses were performed using the ClearSEEK™ *PIK3CA* v1.0 Panel (Agena Bioscience) with a limit of detection of >1%. The Panel covers 20 hotspot mutations in *PIK3CA* (Table I). First, a single multiplex PCR was performed using ~15 ng of tissue DNA and ~30 ng of saliva DNA according to the manufacturer's instructions (Agena Bioscience). Thermocycling was performed using a Labcycler Basic 96 (SensoQuest, Göttingen, Germany). Amplicons were treated with shrimp alkaline phosphatase (SAP). For variant assessment by single-base extension (SBE) with the terminator nucleotides being

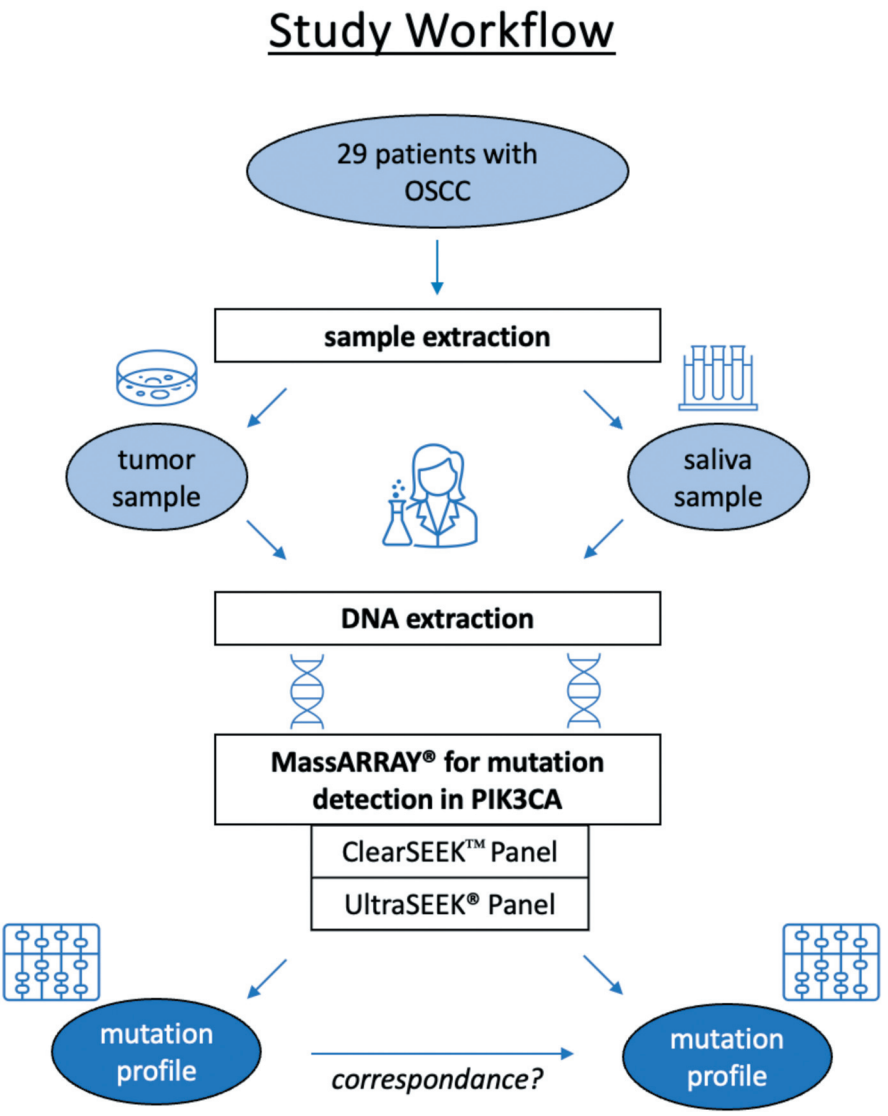

Figure 1. *Study workflow. Twenty-nine patients with oral squamous cell carcinoma (OSCC) were included, from whom saliva samples were obtained preoperatively and tumor samples intraoperatively via excision. DNA was extracted from the matching samples and investigated via MassARRAY® to detect PIK3CA mutations and their correspondence.*

specific to the mutant allele, PCR/SAP products were divided into three multiplex reactions spanning the 20 *PIK3CA* variants.

Transfer of the SBE products, liquid handling and target detection was performed in automated fashion using the MassARRAY® System with ChipPrep Module (Agena Bioscience). The turnaround-time of the workflow described was approximately 8 h excluding DNA extraction and sample QC.

Confirmational analysis of *PIK3CA* variants was performed using the UltraSEEK® Lung v1.1 Panel (Agena Bioscience) with a limit of detection of >0.1%. The panel detects 74 clinically relevant variants across *BRAF* [4], *EGFR* [46], *ERBB2* [4], *KRAS* [16], and *PIK3CA* [4], the latter being p.E542K, p.E545K, p.H1047L, and p.H1047R and thus a subset of the variants listed in Table I. Again, a single multiplex PCR was performed using ~15 ng of tissue DNA and ~30 ng of saliva DNA according to the manufacturer's instructions and amplicons were SAP treated. PCR/SAP products were divided into 12 aliquots for single-base extension, which was performed with biotinylated chain terminator nucleotides specific to the mutant allele. Streptavidin-coated magnetic beads were used to capture the single-base extended oligonucleotides. Beads with captured products were pelleted using a magnet, suspended with 13 μl of biotin competition solution, and finally incubated at 95˚C for 5 min. Eluted products were automatically transferred to the MassARRAY® System for variant detection as described above.

In both the ClearSEEK™ and the UltraSEEK® workflow, no wild type products are being generated. Therefore, amplicon

Table I. *Variant list of the ClearSEEK™ PIK3CA v1.0 Panel.*

Gene	Nucleotide change	Amino acid change	COSMIC ID	Genomic mutation ID
PIK3CA	c.1258T>C	p.C420R	757	COSV55874020
PIK3CA	**c.1624G>A**	**p.E542K**	**760**	**COSV55873227**
PIK3CA	c.1624G>C	p.E542Q	17442	COSV55894248
PIK3CA	**c.1633G>A**	**p.E545K**	**763**	**COSV55873239**
PIK3CA	c.1633G>C	p.E545Q	27133	COSV55878227
PIK3CA	c.1634A>C	p.E545A	12458	COSV55873209
PIK3CA	c.1634A>G	p.E545G	764	COSV55873220
PIK3CA	c.1634A>T	p.E545V	27155	COSV55892885
PIK3CA	c.1635G>C	p.E545D	27374	COSV55875881
PIK3CA	c.1635G>T	p.E545D	765	COSV55874040
PIK3CA	c.1636C>A	p.Q546K	766	COSV55873527
PIK3CA	c.1636C>G	p.Q546E	6147	COSV55882350
PIK3CA	c.1637A>C	p.Q546P	767	COSV55875400
PIK3CA	c.1637A>G	p.Q546R	12459	COSV55876869
PIK3CA	c.1637A>T	p.Q546L	25041	COSV55877455
PIK3CA	c.3139C>A	p.H1047N	5029128	COSV55912376
PIK3CA	c.3139C>T	p.H1047Y	774	COSV55876499
PIK3CA	c.3140A>C	p.H1047P	249874	COSV55888015
PIK3CA	**c.3140A>G**	**p.H1047R**	**775**	**COSV55873195**
PIK3CA	**c.3140A>T**	**p.H1047L**	**776**	**COSV55873401**

The four variants for PIK3CA that are also covered by the UltraSEEK® Lung v1.1 Panel are written in bold.

controls were added to the reactions to ensure, in the absence of a variant signal, that only the target region was amplified. Data analysis was performed using the automated Somatic Variant Report v1.0.5 (Agena Bioscience).

Results

In total, 29 OSCC patients from the Department of Oral and Maxillofacial Surgery of the University Medical Center Hamburg-Eppendorf in Germany were investigated between October 2020 and August 2021. The cohort comprised 11 males and 19 females, out of which 14 were smokers and 16 non-smokers (Table II). The lowest UICC stage was I (3 patients), the highest IVb (4 patients). No UICC stage IVc was included. The lowest T classification was pT1 (4 patients) and the highest pT4a (13 patients). No pT4b tumor was included. The lowest N classification was pN0 (17 patients) and the highest pN3b (4 patients). As the presence of metastasis would have been a contraindication for operative therapy, no patients with metastatic disease were included in the study.

The mean age was 63.9 years (±13.8), mean tumor depth 14.6 mm (±11.8), tumor diameter 39.3 mm (±18.0), and the mean number of positive lymph nodes 4.4 (±16.2) within a mean number of resected lymph nodes of 48.1 (±29.8) (Table III).

As previously described, tissue and saliva were initially analyzed using the ClearSEEK™ *PIK3CA* Panel. The UltraSEEK® Lung Panel was used for variant confirmation. Overall, in seven out of 29 patients (24%) a *PIK3CA* variant

Table II. *Demographic data: oncological classification.*

Parameter	Number of cases	Percentage (%)
Sex		
Male	11	27
Female	19	63
Smoker		
Yes	14	47
No	16	53
UICC stadium		
0	0	0
I	3	10
II	7	23
III	3	10
IVa	11	37
IVb	4	13
IVc	0	0
T classification		
pT1	4	13
pT2	5	17
pT3	7	23
pT4a	13	43
pT4b	0	0
N classification		
pN0	17	57
pN1	2	7
pN2a	1	3
pN2b	2	7
pN2c	2	7
pN3a	1	3
pN3b	4	13

Table III. *Demographic data: mean values of patient and tumor characteristics.*

	Age (years)	Tumor depth (mm)	Largest tumor diameter (mm)	Positive lymph nodes	Resected lymph nodes
Mean (standard deviation)	63.9 (13.8)	14.6 (11.8)	39.3 (18.0)	4.4 (16.2)	48.1 (29.8)

Table IV. *Mutation detection in corresponding tumor and saliva samples.*

Tissue sample	ClearSEEK™ Panel	UltraSEEK® Panel	Saliva sample	ClearSEEK™ Panel	UltraSEEK® Panel
T0019	WT	WT	S0019	WT	WT
T0020	WT	WT	S0020	WT	WT
T0021	WT	WT	S0021	WT	WT
T0023	WT	WT	S0023	WT	WT
T0026	WT	WT	S0026	WT	WT
T0028	WT	WT	S0028	WT	WT
T0029	WT	WT	S0029	WT	WT
T0030	WT	WT	S0030	WT	WT
T0031	**E545Q***	**-NA-**	**S0031**	**E545Q***	**-NA-**
T0032	WT	WT	S0032	WT	WT
T0038	WT	WT	S0038	WT	WT
T0040	**H1047R**	**H1047R**	**S0040**	**H1047R**	**H1047R**
T0037	WT	WT	S0037	WT	WT
T0054	**H1047R**	**H1047R**	**S0054**	**WT**	**H1047R**
T0055	WT	WT	S0055	WT	WT
T0057	**H1047R**	**H1047R**	**S0057**	**H1047R**	**H1047R**
T0178	WT	WT	S0178	WT	WT
T0179	WT	WT	S0179	WT	WT
T0182	WT	WT	S0182	WT	WT
T0185	**E542K**	**WT**	**S0185**	**WT**	**E542K**
T0192	WT	WT	S0192	WT	WT
T0200	WT	WT	S0200	WT	WT
T0218	WT	WT	S0218	WT	WT
T0219	WT	WT	S0219	WT	WT
T0221	*E545A**	*-NA-*	*S0221*	*WT*	*-NA-*
T0226	*H1047R*	*H1047R*	*S0226*	*WT*	*WT*
T0248	WT	WT	S0248	WT	WT
T0249	WT	WT	S0249	WT	WT
T0266	WT	WT	S0266	WT	WT

Bold: Variant detected in tissue and saliva. Italics: Variant detected in tissue only. WT: Wild type; NA: not available. *Variant not covered by UltraSEEK® Lung Panel.

was detected in the respective tumor tissue. In five of these cases (71%) the same variant could also be detected in the matching saliva sample (Table IV). In two out of these five cases the variants (namely E542K and H1047R,) were only detectable by the UltraSEEK® Lung Panel with its significantly higher analytical sensitivity.

In two tumor samples, p.E545A and p.E545Q *PIK3CA* variants were detected with the ClearSEEK™ Panel. These two variants are not covered by the UltraSEEK® Lung Panel and could therefore not be confirmed. However, the *PIK3CA* p.E545Q variant was also detected in the matching saliva sample.

Discussion

Early detection of primary tumors and recurrences of OSCC remains a significant challenge. In addition to clinical examination of patients and radiological imaging, another modality of diagnostics is required to allow for significantly earlier detection. This improvement is essential to improve currently unsatisfactory treatment prospects. Detection of mutation-associated ctDNA in saliva could mean simple, painless, and accurate tumor detection, especially for repeated follow-up analyses aimed at the mitigation and prevention of tumor recurrence. This study aimed at the detection of *PIK3CA*

mutations, being particularly expressed in OSCC, both in the tumor and saliva obtained preoperatively. It was clearly demonstrated that this detection is possible and therefore, prevalent hot-spot mutations such as those in *PIK3CA* can serve as pertinent diagnostic markers for the onset of OSCC. Whilst certainly never considered in the context of OSCC, other studies have already shown *PIK3CA* mutations to be relevant as diagnostic markers in other cancer recurrences such as rectal carcinomas (15).

We investigated ctDNA in saliva due to its prime suitability for LB, given its relative anatomical proximity to the tumor tissue and comparably high levels of associated mutant DNA. Detection of ctDNA has been significantly easier in saliva, as shown by Nonaka and Wong (19). Indeed, Wang *et al*. convincingly synopsized how sensitivity for OSCC-associated ctDNA detection peaks with the focus lying on the oral cavity (20). Equally, saliva ctDNA does not undergo organic decomposition, unlike blood, which is subject to degradation by the kidney, liver, spleen, nucleases, and phagocytes (21-23). A dual approach with both saliva and plasma as biomarkers might therefore be most suitable for a comprehensive assessment of therapeutic benefits (19).

Saliva presents a potent, multicomponent bodily fluid that is easily accessible whilst offering a wide-ranging potential for targeted analysis. The chosen method of saliva collection is crucial for ctDNA detection as well as differentiation from otherwise present cfDNA. Unnecessary contamination with further fragment cfDNA through cell lysis occurring after sampling must be avoided. This would allow for already known disadvantages associated with saliva as a diagnostic tool to be minimized and mitigated. Disadvantages include a generally low concentration of biomarkers, challenges regarding measurement sensitivity, as well as standardization of initial sample collection (24).

As ctDNA concentration is very low, especially in early cancer stages, ultrasensitive ctDNA assays have been developed (23). Instead of approaching the OSCC ctDNA in an untargeted manner, *i.e.,* by searching for any copy number aberrations, we searched for *PIK3CA* mutations specifically, as those are known to be particularly prevalent in OSCC tumors (9). Overall, a *PIK3CA* variant was found in 7 out of 29 patients (24%), for whom the same variant could be detected in five saliva samples (71%). The *PIK3CA* mutation detection rate in tumor tissue is in line with previous reports (9, 25). *PIK3CA* mutation detection thus presents a suitable target for saliva LB approaches for a subset of patients. LB could thus be implemented in follow-up diagnostics besides clinical investigation and radiological imaging to detect recurrent disease as early as possible. However, more studies with larger cohorts are needed to corroborate these results.

Outlook. After surgical resection, possibly combined with radiotherapy or chemoradiotherapy, about one fifth of patients experience a local tumor recurrence – usually within the first six months (26). These relapses do not always lead to symptoms and can only be detected above a certain tumor size using radiological imaging techniques, such as CT and MRI, which are already routinely used in tumor follow-up. CtDNA quantity and volume of the tumor in radiological imaging both relate to tumor burden. Early detection of rebound ctDNA could thus trigger even more targeted diagnostics, for example in the form of PET/CT, untargeted follow-ups at random intervals or even only when recurrence symptoms manifest (27). Considering breast cancer, studies have successfully detected minimal residual disease by the increase in ctDNA concentration several months after primary tumor removal. It therefore seems to be a suitable instrument for recurrence monitoring (23, 28, 29).

Besides the early detection of recurrent tumors by measuring the ctDNA concentration, LB could further decode potentially altered molecular profiles. Therefore, it would be possible to investigate both the heterogeneity of oral cavity carcinomas and the clonal development of the tumor over time in a non-invasive way. Tumor heterogeneity and therefore the existence of a whole array of dissimilar populations of cancerous cells within a single tumor may facilitate therapy resistance. Whilst combined therapy approaches already mitigate associated risks to some extent, the use of a combination of diagnostic means, *i.e.*, ctDNA and CTCs, could further enhance therapeutic outcomes overall. Epigenetic alterations, such as methylation patterns and histone modification, could also be included in this context. Patients can thus be offered an early, individualized, repeatable, and more effective non-invasive diagnostic method, which would allow for a more targeted and potent therapy, therefore improving quality of life as well as therapy adherence.

Further standardization of sample collection, ctDNA investigation and bioinformatic or statistical analysis is currently needed (23, 30). Cut-off values must be developed and other components of LB, such as CTCs or extracellular vesicles, should be further investigated to strengthen clinical application and improve predictive accuracy (31, 32). Salivary cytokines, such as TNF-alpha, may also be elevated in tumor patient samples. Thus, considering their predictive and diagnostic potential alongside clinical investigations is equally important (33). Whilst standardized tubes and fixatives would be desirable for all biomarkers, their heterogeneity might render this impossible.

Study limitations. This study evaluated the combined detection of *PIK3CA* mutations in OSCC tissue and their corresponding ctDNA in saliva samples *via* mass spectrometry. A clear congruity could be verified. Yet, pertinent limitations must also be considered such as the small cohort size of 29 patients and the restriction towards the detection of *PIK3CA* variants. The resulting sensitivity of 24% falls within the expected range for *PIK3CA* alone. However, expanding the targets to include more mutant genes associated with OSCC may

counter this trend, thereby increasing detection rates further and improving the actual diagnostic utility.

It should also be noted that no metastatic status was included. In this regard, the reason was that tumor recurrence in metastatic OSCC is normally not treated *via* operation but by (radio-) chemotherapy, which in turn prevents the resection of tissue samples.

In addition to its primary goal of avoiding conventional tumor biopsies, LB aims to mitigate sampling errors caused by intratumoral heterogeneity. Furthermore, this study only investigated a small portion of the resected tumor and not the tumor in its entirety. Lastly, it is worth noting that OSCCs primarily come into contact with saliva due to their superficial growth. However, for tumors growing more deeply, ctDNA concentration in saliva may be lower or even undetectable.

Conclusion

The detection of *PIK3CA* hotspot mutations in OSCC, alongside their simultaneous occurrence in saliva, underlines the potential benefit of LB for non-invasive early cancer detection and follow-up care in OSCC patients.

Conflicts of Interest

Agena Bioscience GmbH, Hamburg, Germany, performed the genetic investigation of the tumor and saliva samples using the MassARRAY® System, a mass-spectrometry based detection system, for free. The Authors have no further competing interests to declare.

Authors' Contributions

Konstantin Krüger: Conceptualization, Investigation, Methodology, Project administration, Visualization, Roles/Writing – original draft. Audrey Grust: Conceptualization, Roles/Writing – original draft, Methodology, Validation. David Muallah: Data curation, Formal analysis, Writing – review & editing, Validation. Adrian Patenge: Conceptualization, Investigation, Methodology, Writing – review & editing. Harriet Wikman: Validation, Methodology, Supervision, Project administration Writing – review & editing. Maria Medina Mesa: Data curation, Software, Formal analysis. Eike Sören Knust: Data curation, Software, Formal analysis. Alexander Sartori: Supervision, Project administration, Data curation, Validation, Software, Formal analysis, Methodology, Writing – review & editing. Ralf Smeets: Supervision, Resources, Writing – review & editing. Lan Kluwe: Supervision, Data curation, Formal analysis, Validation, Writing – review & editing. Martin Gosau: Supervision, Resources. Reinhard E. Friedrich: Writing – review & editing. Simon Burg: Supervision, Resources, Methodology, Project administration, Writing – review & editing.

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