Free-Circulating Methylated DNA in Blood for Diagnosis, Staging, Prognosis, and Monitoring of Head and Neck Squamous Cell Carcinoma Patients: An Observational Prospective Cohort Study

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BACKGROUND: Circulating cell-free DNA methylation testing in blood has recently received regulatory approval for screening of colorectal cancer. Its application in other clinical settings, including staging, prognosis, prediction, and recurrence monitoring is highly promising, and of particular interest in head and neck squamous cell carcinomas (HNSCCs) that represent a heterogeneous group of cancers with unsatisfactory treatment guidelines.

METHODS: Short stature homeobox 2 (SHOX2) and septin 9 (SEPT9) DNA methylation in plasma from 649 prospectively enrolled patients (training study: 284 HNSCC/122 control patients; testing study: 141 HNSCC/102 control patients) was quantified before treatment and longitudinally during surveillance.

RESULTS: In the training study, 59% of HNSCC patients were methylation-positive at 96% specificity. Methylation levels correlated with tumor and nodal category (P < 0.001). Initially increased methylation levels were associated with a higher risk of death [SEPT9: hazard ratio (HR) = 5.27, P = 0.001; SHOX2: HR = 2.32, P = 0.024]. Disease recurrence/metastases were detected in 47% of patients up to 377 days earlier compared to current clinical practice. The onset of second cancers was detected up to 343 days earlier. In the testing study, sensitivity (52%), specificity (95%), prediction of overall survival (SEPT9: HR = 2.78, P = 0.022; SHOX2: HR = 2.50, P = 0.026), and correlation with tumor and nodal category (P < 0.001) were successfully validated.

CONCLUSIONS: Methylation testing in plasma is a powerful diagnostic tool for molecular disease staging, risk stratification, and disease monitoring. Patients with initially high biomarker levels might benefit from intensified treatment and posttherapeutic surveillance. The early detection of a recurrent/metastatic disease or a second malignancy could lead to an earlier consecutive treatment, thereby improving patients’ outcomes.

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Head and neck squamous cell carcinomas (HNSCCs) represent the sixth most common cancer worldwide with a 5-year survival rate of about 65% (1). Early-stage disease is managed currently with a single-modality treatment (surgery or radiotherapy), while advanced stages are treated using combined therapy (surgical resection and adjuvant radiotherapy, radio-chemotherapy, or definitive radio-chemotherapy) (2). A multimodal therapeutic approach is frequently accompanied by substantial side effects (3). Patients with early stage tumors have an increased probability of full recovery, whereas patients with more advanced stages often develop locoregional and/or distant recurrences (4) with a median survival of less than 1 year (5).

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11 Nonstandard abbreviations: HNSCC, head and neck squamous cell carcinoma; TKIs, tyrosine-kinase inhibitors; mAb, monoclonal antibody; FDA, Food and Drug Administration; cfDNA, circulating cell-free DNA; SCC, squamous cell carcinoma; qPCR, quantitative PCR; IDI, interquartile range; AUC, area under the curve; HR, hazard ratio; PPV, positive predictive value.
HNSCCs display a heterogeneous tumor biology regarding risk of recurrence, progression, and individual tendency to metastasize. Owing to the heterogeneous course of the disease, there are no exact guidelines defining the therapeutic approach for each patient. Additional diagnostic tools for patient risk stratification are urgently needed to achieve an effective individual treatment, while avoiding overtreatment. A pretherapeutically tested blood-based biomarker might identify patients at a higher risk of tumor recurrence. These patients might potentially benefit from a more aggressive treatment, whereas patients with a better prognosis might benefit from a treatment with reduced toxicity. A biomarker-based, intensified clinical disease monitoring method could lead to early detection of recurrent or metastatic disease, allowing for the timely initiation of a consecutive treatment, i.e., surgery, radio-chemotherapy, chemotherapy, and therapies with tyrosine kinase inhibitors (TKIs) or therapeutic monoclonal antibodies (mAbs). Although TKIs (e.g., erlotinib and gefitinib) may cause major side effects and lack satisfactory efficacy (6), mAbs show promising results (7). Cetuximab has already received US Food and Drug Administration approval for locally advanced, recurrent, and metastatic HNSCCs. Immunotherapeutic treatment with the mAb pembrolizumab demonstrated an overall response rate of 18% in patients with advanced HNSCCs (8) and has very recently received FDA approval for the treatment of recurrent and metastatic HNSCCs. The early detection of recurrent or metastatic disease is of particular importance in the context of immunotherapies, since late or delayed responses occurring after treatment initiation have been described for immune checkpoint inhibitors [for review see (9)], thus requiring a sufficient remaining life expectancy to allow for the drug to be effective.

In addition to tumor recurrence, patients with an HNSCC are at high risk of developing a second primary malignancy. The cumulative incidence of a second cancer within 5 years after initial tumor diagnosis has been reported to be 17.9% (10). A blood-based pan-cancer test for the detection of other malignant processes besides HNSCC might allow for an early diagnosis when curative treatment is still an option.

Tumor-derived circulating cell-free DNA (ccfDNA) is a highly promising biomarker for the assessment of tumor progression and the evaluation of prognosis, diagnosis, and response to treatment (11–13). Tumor cells release ccfDNA into the bloodstream; however, the majority of ccfDNA is hematopoietic and usually not of cancerous origin (14). Tumor-specific DNA methylation allows for the discrimination of tumorous and non-tumor ccfDNA.

Septins are aberrantly expressed in several tumor entities (15). Septin 9 has been shown to play a critical role in mitotic cell division (16), inhibition of proliferation, tumor growth, and angiogenesis (15, 17). The septin 9 (SEPT9)12 gene locus is hypermethylated in colorectal precursor lesions and carcinomas (18), and its methylation in ccfDNA has been proven to be a powerful biomarker for colorectal cancer screening (19) that has received FDA approval. Furthermore, SEPT9 DNA hypermethylation has been suggested as a biomarker for the diagnosis of HNSCCs (20).

Methylation of the short stature homeobox 2 (SHOX2) gene locus is a validated lung cancer biomarker (21–26). SHOX2 methylation has been previously shown to be associated with the amplification of the 3q25 gene locus, which is a frequent event in HNSCCs (27–29). Furthermore, SHOX2 and SEPT9 methylation in combination have been shown to be clinically useful diagnostic and prognostic biomarkers in pleural effusions and ascites (30, 31). The present study explores the value of quantitative SEPT9 and SHOX2 methylation levels in ccfDNA for the clinical management of HNSCC patients.

Methods

PATIENTS

HNSCC patients (training cohort: n = 284, testing cohort: n = 141) and matched control patients with non-malignant diseases (training cohort: n = 122, testing cohort: n = 102) treated between July 2012 and November 2014 (training cohort) and between December 2014 and July 2016 (testing cohort) at the Departments of Head and Neck Surgery (training and testing cohorts) and Oral and Maxillofacial Surgery (testing cohort) at the University Hospital of Bonn were prospectively enrolled.

INCLUSION/EXCLUSION CRITERIA

The HNSCC cohort consisted of 3 groups: (a) patients suffering from primary squamous cell carcinoma (SCC) of the larynx, pharynx, mouth; (b) patients with locoregional tumor recurrence (lymph nodes or local tissue) of a previous HNSCC; and (c) HNSCC patients who were clinically followed up after treatment. Group 3 was included in the training cohort only. All HNSCC patients were free of other SCCs than HNSCCs for at least 3 years. All control patients were matched to HNSCC patients with respect to age, sex, and tobacco and alcohol consumption. All control patients had a cancer-free history of at least 3 years and no prior history of SCC. Blood samples were taken before treatment and longitudinally during clinical follow-up.

The study protocol was approved by the ethics committee of the University Hospital Bonn (vote no. 224/12). All patients provided written informed consent.

12 Human genes: SHOX2, short stature homeobox 2; SEPT9, septin 9; ACTB, actin beta.
Results

SHOX2 AND SEPT9 DNA METHYLATION IN TISSUES AND MATCHED PLASMA SAMPLES

SHOX2 and SEPT9 were found to be hypermethylated in HNSCC tissues compared to normal adjacent tissues (diagnostic accuracy: AUCₜₑₚₑₚₑ = 0.89, 95% CI, 0.85–0.94; AUCₗₑₚₑ = 0.98, 95% CI, 0.97–0.99; see online Supplemental Fig. 2 in Supplemental Results 1). Furthermore, matched tumor samples and pretherapeutic blood samples from 55 patients were analyzed. SHOX2 methylation in plasma correlated significantly with tissue methylation levels (Spearman ρ = 0.36, P = 0.007), and SEPT9 showed a trend toward higher methylation in plasma from patients with SEPT9 hypermethylated tumors (Spearman’s ρ = 0.25, P = 0.067). Based on these results additional patients were recruited to build a training and a testing cohort.

SHOX2 AND SEPT9 DNA METHYLATION IN PLASMA FOR DIAGNOSIS AND MOLECULAR STAGING

A total of 284 HNSCC patients and 122 control patients with benign diseases were recruited for the training cohort. Baseline characteristics and distribution of clinicopathological data reflected a representative white cohort of HNSCC patients (see online Supplemental Tables 1 and 2, and Table 1).

Blood plasma samples of HNSCC patients before treatment were available in 48% (137/284) of patients. Matched control patients were tested to determine the basal level of sporadic methylation in plasma. Quantitative levels of tumorous ccfDNA indicated by hypermethylation of the SHOX2 and SEPT9 gene loci were significantly higher in HNSCC patients compared to control patients (Fig. 1A, AUCₚₑₚₑ = 0.79, 95% CI, 0.74–0.85; AUCₗₑₚₑ = 0.80, 95% CI, 0.75–0.85). The level of methylated ccfDNA was associated significantly with clinicopathological tumor and nodal category, vascular and lymphatic invasion, and tumor grading (Table 1; also see online Supplemental Table 2). A methylation cutoff value was introduced to dichotomize the quantitative methylation values (Fig. 1A). The cutoff was chosen based on the methylation levels in control patients, with 95% of controls showing methylation levels below the cutoff (specificity). Methylation levels below these cutoffs (SHOX2: 0.25%, SEPT9: 0.075%) were considered sporadic background methylation levels known to occur in blood from healthy individuals and patients with benign diseases (21–24). In agreement with previous studies and as expected from the analysis of tissues described above, sporadic background methylation was higher for the SHOX2 gene locus compared to the SEPT9 gene locus (19, 21–23, 30, 31). Based on this cutoff, 50% (SHOX2) and 57% (SEPT9) of HNSCC patients showed a positive test result (sensitivity), respectively. For diagnostic purposes, both biomarker levels were averaged (mean) to compute 1 value reflecting the overall concentration of tumorous ccfDNA better than a single biomarker. The mean methylation level \(\text{mean}_{\text{SHOX2+SEPT9}} = (\text{SHOX2} + \text{SEPT9})/2\) resulted in 59% sensitivity at 96% specificity based on the mean cutoff of 0.16% (Fig. 1A). A mean methylation level led to an increased diagnostic accuracy (AUCmeanₚₑₚₑ = 0.83, 95% CI, 0.78–
The advantage of combining both biomarkers is the increase of specificity from 95% to 96%. If reproducible in a larger cohort, a specificity increase from 95% to 96% would mean a 20% reduction of false-positive results from 5% to 4%. Furthermore, a single value facilitates the application of the 2 biomarkers for monitoring purposes.

**SHOX2 AND SEPT9 DNA METHYLATION IN PLASMA FOR SURVIVAL PREDICTION**

Eight out of 137 HNSCC patients with available plasma samples before primary treatment were treated in a palliative manner and thus were excluded from survival analyses. Due to the different biological relevance of SHOX2 and SEPT9, survival analyses were performed separately for each biomarker without the use of mean SHOX2/SEPT9.

In univariate Cox proportional hazards analysis of the remaining 129 cases, patients with positive SEPT9 and SHOX2 plasma levels were at a higher risk of death compared to negative patients \[\text{SEPT9}: \text{hazard ratio (HR)} = 5.27, 95\% \text{ CI}, 2.03–13.68, P = 0.001 \text{; SHOX2}: \text{HR} = 2.32, 95\% \text{ CI}, 1.12–4.83, P = 0.024\]. This finding was confirmed in Kaplan–Meier analyses of overall survival as shown in Fig. 1B.

To avoid overly optimistic results from the introduction of a methylation cutoff for result dichotomization, univariate and multivariate Cox proportional hazards analyses were conducted using SHOX2 and SEPT9 DNA methylation levels in plasma as continuous variables. In univariate Cox proportional hazards analyses, increased SEPT9 methylation levels were prognostic for an adverse overall survival, a higher risk of locoregional tumor recurrence, and the development of distant metastases (see online Supplemental Table 3). In multivariate
Fig. 1. Diagnostic and prognostic accuracy of SHOX2 and SEPT9 DNA methylation.
(A), Training cohort: SHOX2, SEPT9, and averaged \[\text{mean}_{SHOX2/SEPT9} = \frac{\text{SHOX2} + \text{SEPT9}}{2}\] DNA methylation levels in plasma from HNSCC (n = 137) and control patients (n = 122) and diagnostic accuracy (ROC analysis). (B), Training cohort: Kaplan–Meier survival analysis of 129 HNSCC patients treated with curative intent stratified according to plasma methylation levels. (C), Diagnostic accuracy in the testing cohort (n = 141 HNSCC and n = 102 control patients). (D), Validation cohort: Kaplan–Meier survival analysis of 137 HNSCC patients treated with curative intent.
Cox proportional hazards analyses including established prognostic factors, \textit{SEPT9} methylation proved to be an independent prognostic factor after backward elimination (see online Supplemental Table 3). \textit{SHOX2} plasma methylation levels, in contrast, were not prognostic in multivariate analysis for any of the analyzed clinical endpoints (death: $P = 0.78$, distant metastases: $P = 0.97$; locoregional recurrence: $P = 0.91$).

**SHOX2 and SEPT9 DNA Methylation in Plasma: Clinical Performance Validation**

Selected clinical performance characteristics were validated in the testing cohort, i.e., diagnostic accuracy (AUCs, sensitivity, and specificity), molecular staging (association with T category, N category, grade), and prognostic value. A total of 141 HNSCC patients and 102 control patients were recruited for the testing cohort. Baseline characteristics and distribution of clinicopathological data of the testing cohort were comparable to the training cohort (see online Supplemental Tables 4 and 5, and Table 2).

Four out of 141 HNSCC patients were treated in a palliative manner and thus were excluded from survival analyses.

**Table 2. Association of methylation levels with T and N categories and histological grade (testing cohort).**

<table>
<thead>
<tr>
<th>Clinicopathological parameters</th>
<th>Methylation in plasma prior to treatment</th>
<th>Median SEPT9, %; IQR</th>
<th>$P$ value$^b$</th>
<th>Median SHOX2, %; IQR</th>
<th>$P$ value$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>All HNSCC cases</td>
<td>141 (100%)</td>
<td>0.10; 0.80</td>
<td></td>
<td>0.20; 0.72</td>
<td></td>
</tr>
<tr>
<td>T category</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$T_0$</td>
<td>1 (1%)</td>
<td>0.00; NA</td>
<td></td>
<td>0.05; NA</td>
<td></td>
</tr>
<tr>
<td>$T_1$</td>
<td>31 (22%)</td>
<td>0.00; 0.08</td>
<td></td>
<td>0.05; 0.15</td>
<td></td>
</tr>
<tr>
<td>$T_2$</td>
<td>43 (30%)</td>
<td>0.17; 0.67</td>
<td></td>
<td>0.12; 1.19</td>
<td></td>
</tr>
<tr>
<td>$T_3$</td>
<td>29 (21%)</td>
<td>0.13; 0.54</td>
<td></td>
<td>0.23; 0.75</td>
<td></td>
</tr>
<tr>
<td>$T_4$</td>
<td>28 (20%)</td>
<td>0.93; 2.02</td>
<td>$&lt;0.001^c$</td>
<td>0.50; 0.66</td>
<td>$&lt;0.001^c$</td>
</tr>
<tr>
<td>NA$^d$</td>
<td>9 (6%)</td>
<td>0.00; 0.45</td>
<td></td>
<td>0.20; 5.63</td>
<td></td>
</tr>
<tr>
<td>N category</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$N_0$</td>
<td>38 (27%)</td>
<td>0.00; 0.22</td>
<td></td>
<td>0.07; 0.29</td>
<td></td>
</tr>
<tr>
<td>$N_1$</td>
<td>24 (17%)</td>
<td>0.09; 0.50</td>
<td></td>
<td>0.07; 0.30</td>
<td></td>
</tr>
<tr>
<td>$N_2$</td>
<td>54 (38%)</td>
<td>0.32; 1.33</td>
<td></td>
<td>0.37; 2.15</td>
<td></td>
</tr>
<tr>
<td>$N_3$</td>
<td>5 (4%)</td>
<td>2.22; 13.23</td>
<td>$&lt;0.001^c$</td>
<td>2.40; 3.94</td>
<td>$&lt;0.001^c$</td>
</tr>
<tr>
<td>$N_4$</td>
<td>20 (14%)</td>
<td>0.02; 0.56</td>
<td></td>
<td>0.21; 0.52</td>
<td></td>
</tr>
<tr>
<td>Histopathological grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$G_1$</td>
<td>10 (7%)</td>
<td>0.00; 0.67</td>
<td></td>
<td>0.03; 0.14</td>
<td></td>
</tr>
<tr>
<td>$G_2$</td>
<td>52 (37%)</td>
<td>0.12; 0.46</td>
<td></td>
<td>0.08; 0.35</td>
<td></td>
</tr>
<tr>
<td>$G_3$</td>
<td>33 (23%)</td>
<td>0.10; 1.33</td>
<td>0.13</td>
<td>0.45; 1.53</td>
<td>0.001$^c$</td>
</tr>
<tr>
<td>NA$^d$</td>
<td>46 (33%)</td>
<td>0.17; 0.82</td>
<td></td>
<td>0.29; 1.00</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ T (tumor) and N (node) categories and histological grade of 141 cancer cases included in the HNSCC patient testing cohort and their association with pretherapeutic \textit{SHOX2} and \textit{SEPT9} plasma DNA methylation levels. $T_0$, tumor in situ. A comprehensive list of methylation levels and their association with clinicopathological parameters (T, N, M (metastasis) category, grade, tumor localization, vascular invasion (V), lymphatic invasion (L), surgical margin (R), and tumor status (primary tumor vs recurrence)) can be found in online Supplemental Table 4.

$^b$ $P$ values refer to Kendall $\tau$ rank correlation.

$^c$ Significant feature.

$^d$ NA, not applicable or unknown because no surgical specimen analyzed (palliative treatment/supportive care, concurrent radio-chemotherapy) or data not available. TNM classification does not apply to cancer of unknown primary origin (CUP) and local lymph node recurrences. Grading does not apply to CUP and locoregional recurrences. IQR applies only to $\geq 4$ measurements.
analyses. In univariate Cox proportional hazards analysis of the remaining 137 cases, patients with positive SEPT9 and SHOX2 plasma levels were at higher risk of death compared to methylation-negative patients (SEPT9: HR = 2.78, 95% CI, 1.16–6.67, P = 0.022; SHOX2: HR = 2.50, 95% CI, 1.12–5.60, P = 0.026, Fig. 1D). Accordingly, the results obtained in the training study were successfully validated in the testing cohort.

SHOX2 and SEPT9 DNA Methylation in Plasma for Disease Monitoring

Owing to the increased sensitivity and specificity noted with tandem use of both biomarkers (mean SEPT9/SHOX2), their combination was tested for posttherapeutic disease monitoring longitudinally. Plasma methylation levels during patients’ posttherapeutic follow-up examinations were available in 90% (257/284) of patients. Twenty-two percent (56/257) of these patients reached 1 or more of the investigated clinical end points during surveillance. Twelve percent (31/257) of patients died, 8% (20/257) developed distant metastases, and 11% (27/257) relapsed locoregionally. Distant and/or locoregional progression of HNSCCs was diagnosed in 17% (43/257) of patients. Furthermore, 5% (12/257) of patients developed a second primary tumor (4 lung cancers, 3 HNSCCs, 2 colorectal cancers, 1 gastric, 1 esophageal, and 1 pancreatic cancer). Seven of these 12 patients (58%) did not have a recurrence of the HNSCC. Thirteen percent (38/284) of patients had a HNSCC-specific tumor progression in the absence of a second primary tumor.

Plasma methylation levels of 671 blood samples from all 257 monitored patients were analyzed. Sixty-four (10%) blood samples were above the selected cut-point and therefore positive. According to 96% specificity, approximately 3 of these 64 positive samples could be expected to be false-positive results. Altogether, 16% (42/257) of patients showed 1 or more positive results. Of these patients, 62% (26/42) had a single positive result, 31% (13/42) had 2 positive blood samples, and 7% (3/42) of patients had more than 2 positive results. Online Supplemental Table 6 shows the results from the last negative and the first positive blood samples from all patients who reached 1 or more of the clinical end points during disease monitoring. All patients (n = 16) with more than 1 positive test result reached 1 or more of the clinical end points. Of patients with only 1 positive test result, 92% (24/26) suffered from a disease progression or a second cancer and/or died within 1 year after the first positive blood test, which is in line with the specificity of the test. Deceased patients had a positive biomarker testing in 18/31 (58%) cases. The test positivity occurred a mean of 213 (range: 30–488) days before death. However, 7 patients (see online Supplemental Table 6) showed high methylation levels in plasma before death without any diagnosed tumor recurrence or second primary cancer. Methylation levels in plasma above the cutoff were found in 47% (18/38) of patients with a locoregional or a distant tumor recurrence in the absence of a second primary tumor. Of these patients, 78% (14/18) had a positive test result a mean of 105 (range 8–377) days before the clinicopathological verification of the tumor progression. The tumor recurrence of the other 4 patients (4/18) was diagnosed on the day of treatment, as patients were not available for testing beforehand. In detail, 43% (6/14) developed a locoregional recurrence, 43% (6/14) a distant recurrence, and 14% (2/14) both. Two patients who developed a second primary cancer in the absence of a HNSCC-specific tumor recurrence (n = 7) showed a suspicious increase in biomarker levels 112 days (duodenal pancreatic adenocarcinoma) and 343 days (esophageal cancer) before the clinicopathological diagnosis of the second cancer. Monitoring results from 8 selected cases are described in detail in online Supplemental Results 2.

Discussion

Previously, the therapeutic approach for HNSCC patients has mainly relied on clinical staging parameters. However, there are no validated guidelines defining the exact treatment of HNSCCs. Powerful biomarkers are therefore urgently needed to enable precise and reliable diagnosis, treatment, and prognosis, particularly in the context of novel therapeutic options, e.g., immunotherapies.

The aggressiveness of adjuvant treatment has been controversial and a topic of frequent discussions. Novel prognostic factors besides clinical tumor staging might improve risk stratification and lead to more personalized therapy. SHOX2 and SEPT9 proved to be powerful prognostic and molecular staging biomarkers for identifying patients at higher risk of tumor recurrence. Consequently, patients with high methylation levels before therapy would benefit from a more aggressive first-line therapy and should receive more intensified posttherapeutic disease monitoring. Of note, in contrast to strong prognostic parameters, i.e., pathologic tumor and nodal category, SEPT9 and SHOX2 methylation in blood are accessible before a surgical resection of the tumor, thus providing a chance to adapt first-line treatment in accordance with the prognosis of the patient. Currently, neoadjuvant immunotherapies are under investigation and might represent an option for patients who are unlikely to benefit from a surgery with curative intent.

Following treatment, the timely detection of disease recurrence or metastases is of particular importance. The rapid development of novel immunotherapies, in particular, implies treatment options even for patients with an advanced or metastatic disease (8). However, immunotherapies require a minimal remaining life expectancy to be effective. Accordingly, the earlier detection of an incurable disease is likely to increase the quality-adjusted lifespan of these patients. Just as with the treatment of HNSCCs, there are no validated guidelines defining the
intervals and the exact procedure of posttherapeutic follow-up examinations. To evaluate the impact of \textit{SHOX2} and \textit{SEPT9} in posttherapeutic monitoring, biomarker levels in blood plasma were investigated longitudinally during follow-up examinations. The sensitivity of the biomarkers for the detection of tumor recurrences was only slightly lower compared to the sensitivity of the detection of the primary HNSCCs. In test-positive patients with tumor recurrences, suspicious biomarker levels were found significantly earlier compared to the common clinical practice. However, shorter time intervals between biomarker testing during follow-up care could lead to an even higher efficiency of such a test. In this study cohort, 7 patients showed high methylation levels in plasma before death without any diagnosed tumor recurrence or development of a second primary cancer. Owing to the high specificity of the biomarkers (95%–96%), it can be speculated that an occult tumor progression was present in these cases. Unfortunately, body-imaging examinations that could have clarified the status of these patients were not feasible. Several patients with a posttherapeutic positive blood sample were retested over time. Those with positive results in consecutively taken blood samples proved to suffer from disease progression in all cases, whereas only 92% of patients with only 1 positive test result had a proven tumor recurrence. Accordingly, the retesting of a positive blood sample using a second blood sample is likely to increase the specificity of the monitoring test. The performance of the monitoring test might further be improved by individualizing the algorithm for the results interpretation based on the analysis of the tumor tissue or based on the inclusion of the pretherapeutic blood test result. Patients whose tumor or pretherapeutic blood sample is positive for only either \textit{SEPT9} or \textit{SHOX2} might benefit from a monitoring test that considers only the positive biomarker for the interpretation of the result.

\textit{SHOX2} and \textit{SEPT9} DNA hypermethylation additionally has been linked to other cancers associated with alcohol and tobacco consumption (i.e., tumors of the lung, colon, stomach, liver, biliary tract, and pancreas) (18, 19, 22, 30–32) and could therefore be a promising biomarker candidate for the detection of occult second primary cancers during disease monitoring of HNSCCs. Furthermore, these biomarkers might be a potential diagnostic tool for the management of malignant diseases other than HNSCCs, as they appear to be nonspecific regarding tumor-site and organ.

The use of ccfDNA for cancer screening purposes is controversial. This study indicates that neither of these biomarkers is well suited for HNSCC screening owing to 3 major shortcomings. (a) Even a relatively high specificity of 95%–96% results in a positive predictive value (PPV) that is too low leading to additional effort and costs for validation of patients with positive screening results. (b) The sensitivity of the investigated biomarkers is higher for advanced tumor stages compared with early stage cancers. Accordingly, small and clinically occult tumors that would have the highest chance of cure are likely to be missed. (c) The group of individuals with a high-risk profile of developing HNSCCs (main risk factors: alcohol consumption and smoking) usually do not exhibit health-conscious behavior and therefore would probably not participate in a screening program. The latter limitation, however, is a general problem regarding HNSCC patients and is not related to the clinical performance of a biomarker.

In conclusion, this study demonstrates that quantitative \textit{SEPT9} and \textit{SHOX2} DNA methylation levels in ccfDNA from blood plasma are clinically valuable biomarkers for diagnosis, molecular staging, prognosis (risk stratification), and posttherapeutic monitoring of HNSCC patients. However, overdiagnosis is a common problem in clinical oncology and needs to be avoided (33). Accordingly, a multicenter, randomized, prospective, and interventional study is being currently planned that will investigate whether the application of such biomarkers leads to an improved survival with a high quality of life or to a reduction of costs.

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