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

Running head: **Methylated DNA in Blood for the Clinical HNSCC Management**

Andreas Schröck[1]*, Annette Leisse[1,2]*, Luka de Vos[1], Heidrun Gevensleben[2], Freya Dröge[3], Alina Franzen[1], Malin Wachendörfer[1,2], Friederike Schröck[1], Joerg Ellinger[4], Marcus Teschke[5], Timo Wilhelm-Buchstab[6], Jennifer Landsberg[7], Stefan Holdenrieder[8], Gunther Hartmann[9], John K. Field[10], Friedrich Bootz[1], Glen Kristiansen[2†], Dimo Dietrich[1†]

Affiliations:

[1] University Hospital Bonn, Department of Otolaryngology, Head and Neck Surgery, Bonn, Germany

[2] University Hospital Bonn, Institute of Pathology, Bonn, Germany

[3] University Hospital Essen, Ear, Nose and Throat Clinic, Essen, Germany

[4] University Hospital Bonn, Department of Urology, Bonn, Germany

[5] University Hospital Bonn, Department of Oral and Maxillofacial Surgery, Bonn, Germany

[6] University Hospital Bonn, Department of Radiology, Bonn, Germany

[7] University Hospital Bonn, Department of Dermatology, Bonn, Germany

[8] German Heart Centre Munich, Munich, Germany

[9] University Hospital Bonn, Institute of Clinical Chemistry and Clinical Pharmacology, Bonn, Germany

[10] University of Liverpool Cancer Research Center, Liverpool, UK

* Contributed equally

† These authors are joint senior authors on this work.

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List of abbreviations: HNSCC, head and neck squamous cell carcinoma; TKI, tyrosine-kinase inhibitors; mAb, monoclonal antibody; FDA, Food and Drug Administration, ccfDNA, circulating cell-free DNA, *SEPT9*, septin 9; *SHOX2*, short

stature homeobox 2; IQR, Interquartile Range; AUC, Area Under the Curve; ROC, Receiver Operating Characteristic; PPV, positive predictive value

List of human genes: *SHOX2*, short stature homeobox 2; *SEPT9*, septin 9; *ACTB*, actin beta

Abstract

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 (HNSCC) that represent a heterogeneous group of cancers with unsatisfactory treatment guidelines.

Methods: *SHOX2* and *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 prior to 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*: 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 post-therapeutic surveillance. The early detection of a recurrent/metastatic disease or a

second malignancy could lead to an earlier consecutive treatment, thereby improving patients' outcomes.

Head and neck squamous cell carcinomas (HNSCC) represent the 6th most common cancer worldwide with a five-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 loco-regional and/or distant recurrences [4] with a median survival of less than one year [5]. 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 patients' risk-stratification are urgently needed to achieve an effective individual treatment, while avoiding overtreatment. A pre-therapeutically 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 could lead to an 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 (TKI) or therapeutic monoclonal antibodies (mAbs). Although tyrosine-kinase inhibitors (e.g. erlotinib and gefitinib) may cause major side effects and lack satisfactory efficacy [6], mAbs show promising results [7]. Cetuximab has already received FDA (Food and Drug Administration) approval for locally advanced, recurrent, and metastatic HNSCC. Immunotherapeutic treatment with the mAb

pembrolizumab demonstrated an overall response rate of 18% in patients with advanced HNSCC [8] and has very recently received FDA approval for the treatment of recurrent and metastatic HNSCC. 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: [9]), thus requiring a sufficient remaining life expectancy to allow for the drug to be effective.

In addition to tumor recurrence, patients with 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 [11,12,13]. 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-tumorous 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*) gene locus is hypermethylated in colorectal precursor lesions and carcinomas [18], and its methylation in ccfDNA has been proven 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 HNSCC [20].

Methylation of the short stature homeobox 2 (*SHOX2*) gene locus is a validated lung cancer biomarker [21,22,23,24,25,26]. *SHOX2* methylation has previously been shown to be associated with the amplification of the 3q25 gene locus, which is a frequent event in HNSCC [27,28,29]. 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 and Study Design

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 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: 1) patients suffering from primary squamous cell carcinoma (SCC) of the larynx, pharynx, mouth; 2) patients with loco-regional tumor recurrence (lymph nodes or local tissue) of a previous HNSCC; and 3) 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 SCC than HNSCC for at least three years. All control patients were matched to HNSCC patients with respect to age, gender, and tobacco and alcohol

consumption. All control patients had a cancer-free history of at least three years and no prior history of SCC. Blood samples were taken prior to 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.

Clinical Endpoints

The following clinical endpoints / times-to-event were investigated: 1) death / overall survival; 2) development of a loco-regional recurrence (local lymph nodes or local tissue) / loco-regional recurrence-free survival; 3) development of distant metastases / distant metastases-free survival; 4) development of a loco-regional recurrence and/or distant metastases / progression-free survival. Second primary cancers were considered as a secondary clinical endpoint.

Sample Preparation and SHOX2 and SEPT9 Methylation Quantification

Plasma was prepared from EDTA-stabilized blood and the ccfDNA was bisulfite converted and purified as described in detail in **Supplemental Protocol 1** (all **Supplemental information** is available with the online version of this article). Methylation was determined using *SHOX2*/*SEPT9*/*ACTB* triplex qPCR as previously described in detail [30]. The qPCR target regions within the *SHOX2* and *SEPT9* genes are shown in **Supplemental Figure 1**. Water was used as a no template control to check for cross contamination and DNA carry over. Quantitative methylation levels from six (plasma, 10 µl template DNA each replicate) and three (tissue, 20 ng quantified via UV spectrophotometry) qPCR replicates were calculated

using the ΔΔCq method, and mean values were computed [24,30]. The analytical performance of the qPCR assay has previously been evaluated [30].

Statistical Analyses

ANOVA, Kendall's τ and Spearman's ρ rank correlations, *t*-tests, and Wilcoxon-Mann-Whitney tests were performed to compare *SHOX2* and *SEPT9* methylation levels among cancer patients and between controls and cancer patients. Median methylation levels were reported including Interquartile Ranges (IQR). The Area Under the Curve (AUC) of the Receiver Operating Characteristic (ROC) was computed as a measure of test diagnostic accuracy. Cox proportional hazards (univariate and multivariate with backward elimination) and Kaplan-Meier analyses were conducted to investigate the time-to-events. *P*-values refer to the log rank test and Wald test, respectively. Two-sided *P*-values <0.05 were considered statistically significant.

Results

SHOX2 and SEPT9 DNA methylation in tissues and matched plasma specimens

SHOX2 and *SEPT9* were found to be hypermethylated in HNSCC tissues compared to normal adjacent tissues (diagnostic accuracy: AUC_{SEPT9}=0.89, 95%CI [0.85–0.94], AUC_{SHOX2}=0.98, 95%CI [0.97–0.99]; **Supplemental Figure 2** in **Supplemental Results 1**). Furthermore, matched tumor samples and pre-therapeutic blood samples from 55 patients were analyzed. *SHOX2* methylation in plasma correlated significantly with tissue methylation levels (Spearman's ρ=0.36, *P*=0.007), and *SEPT9* showed a trend towards 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 clinico-pathological data reflected a representative Caucasian cohort of HNSCC patients (**Supplemental Table 1**, **Supplemental Table 2** and **Table 1**).

Blood plasma samples of HNSCC patients prior to first-line 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 (**Figure 1A**, AUC_{SEPT9}=0.79, 95%CI [0.74–0.85]; AUC_{SHOX2}=0.80, 95%CI [0.75-0.85]). The level of methylated ccfDNA was associated significantly with clinico-pathological tumor and nodal category, vascular and lymphatic invasion, and tumor grading (**Table 1** and **Supplemental Table 2**). A methylation cut-off value was introduced to dichotomize the quantitative methylation values (**Figure 1A**). The cut-off was chosen based on the methylation levels in control patients with 95% of controls showing methylation levels below the cut-off (specificity). Methylation levels below these cut-offs (*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 [21,22,23,24]. 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 [19,21,22,23,30,31]. Based on this cut-off, 50% (*SHOX2*) and 57% (*SEPT9*) of HNSCC patients showed a

positive test result (sensitivity), respectively. For diagnostic purposes, both biomarker levels were averaged (mean) in order to compute one value reflecting the overall concentration of tumorous ccfDNA better than a single biomarker. The mean averaged methylation level ($mean_{SHOX2/SEPT9}$=[*SHOX2*+*SEPT9*]/2) resulted in 59% sensitivity at 96% specificity based on the mean averaged cut-off of 0.16% (**Figure 1A**). A mean averaged methylation level led to an increased diagnostic accuracy ($AUC_{mean SEPT9/SHOX2}$=0.83, 95%CI [0.78–0.88], **Figure 1A**). 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 two biomarkers for monitoring purposes.

SHOX2 and SEPT9 DNA methylation in plasma for survival prediction

Eight out of 137 HNSCC patients with available plasma samples prior to 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 (*SEPT9*: HR=5.27, 95%CI [2.03–13.68], *P*=0.001; *SHOX2*: HR=2.32, 95%CI [1.12–4.83], *P*=0.024). This finding was confirmed in Kaplan-Meier analyses of overall survival as shown in **Figure 1B**.

To avoid overly optimistic results from the introduction of a methylation cut-off 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 loco-regional tumor recurrence, and the development of distant metastases (**Supplemental Table 3**). In multivariate Cox proportional hazards analyses including established prognostic factors, *SEPT9* methylation proved to be an independent prognostic factor after backward elimination (**Supplemental Table 3**). *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; loco-regional 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 clinico-pathological data of the testing cohort were comparable to the training cohort (**Supplemental Table 4**, **Supplemental Table 5**, and **Table 2**).

Seventy-four of 141 HNSCC patients (sensitivity: 52%) showed a mean$_{SHOX2/SEPT9}$ plasma level above the cut-off (0.16% as defined in the training cohort) and were true positive, while 97 of 102 control patients (specificity: 95%) were true negative. Mean$_{SHOX2/SEPT9}$ resulted in a diagnostic accuracy of AUC=0.80 (95%CI [0.75–0.86], **Figure 1C**). The strong correlation of *SHOX2* and *SEPT9* plasma hypermethylation with the tumor and nodal category was corroborated in the testing cohort (*P*<0.001 each, **Table 2**). In addition, *SHOX2* plasma hypermethylation was associated significantly with tumor grade (*P*=0.001).

Four out of 141 HNSCC patients were treated in a palliative manner and thus were excluded from survival 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, **Figure 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 post-therapeutic disease monitoring longitudinally. Plasma methylation levels during patients' post-therapeutic follow-up examinations were available in 90% (257/284) of patients. Twenty-two percent (56/257) of these patients reached one or more of the investigated clinical endpoints during surveillance. Twelve percent (31/257) of patients died, 8% (20/257) developed distant metastases, and 11% (27/257) relapsed loco-regionally. Distant and/or loco-regional progression of HNSCC was diagnosed in 17% (43/257) of patients. Furthermore, 5% (12/257) of patients developed a second primary tumor (four lung cancers, three HNSCC, two colorectal cancers, one gastric, one esophageal, and one pancreatic cancer). Seven of these twelve patients (58%) did not have a recurrence of the HNSCC. Thirteen percent (38/284) of patients had a HNSCC-specific tumor progression in 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 three of these 64 positive samples could be expected to be false positive results. Altogether, 16% (42/257) of patients showed one or more positive results. Of these patients, 62%

(26/42) had a single positive result, 31% (13/42) had two positive blood samples, and 7% (3/42) of patients had more than two positive results. **Supplemental Table 6** shows the results from the last negative and the first positive blood samples from all patients who reached one or more of the clinical endpoints during disease monitoring. All patients (n=16) with more than one positive test result reached one or more of the clinical endpoints. Of patients with only one positive test result, 92% (24/26) suffered from a disease progression or a second cancer and/or died within one 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, seven patients (**Supplemental Table 6**) showed high methylation levels in plasma prior to death without any diagnosed tumor recurrence or second primary cancer. Methylation levels in plasma above the cut-off were found in 47% (18/38) of patients with a loco-regional 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 clinico-pathological verification of the tumor progression. The tumor recurrence of the other four 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 loco-regional 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 (ductal pancreatic adenocarcinoma) and 343 days (oesophageal cancer) prior to the clinico-pathological diagnosis of the second cancer. Monitoring results from eight selected cases are described in detail in **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 HNSCC. 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 prior to therapy would benefit from a more aggressive first-line therapy and should receive more intensified post-therapeutic 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 prior to 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 which 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 expectation to be effective. Accordingly, the earlier detection of an incurable disease is likely to increase the quality-adjusted life span of these patients. Just as with the treatment of HNSCC, there are no validated guidelines defining the intervals and the exact

procedure of post-therapeutic follow-up examinations. To evaluate the impact of *SHOX2* and *SEPT9* in post-therapeutic 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 HNSCC. 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, seven patients showed high methylation levels in plasma prior to 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 post-therapeutic 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 one positive test result had a proven tumor recurrence. Accordingly, the retesting of a positive blood sample using a second blood specimen 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 pre-therapeutic blood test result. Patients whose tumor or pre-therapeutic blood sample is positive for only either *SEPT9* or *SHOX2* might benefit from a monitoring test that considers only the positive biomarker for the interpretation of the result.

SHOX2 and *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) [18,19,22,30,31,32] and could therefore be a promising biomarker candidate for the detection of occult second primary cancers during disease monitoring of HNSCC. Furthermore, these biomarkers might be a potential diagnostic tool for the management of malignant diseases other than HNSCC, as they appear to be non-specific 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 three major shortcomings: 1) 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; 2) 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; 3) The group of individuals with a high risk profile of developing HNSCC (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 *SEPT9* and *SHOX2* DNA methylation levels in ccfDNA from blood plasma are clinically valuable biomarkers for diagnosis, molecular staging, prognosis (risk-stratification), and post-therapeutic 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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Figure Legends

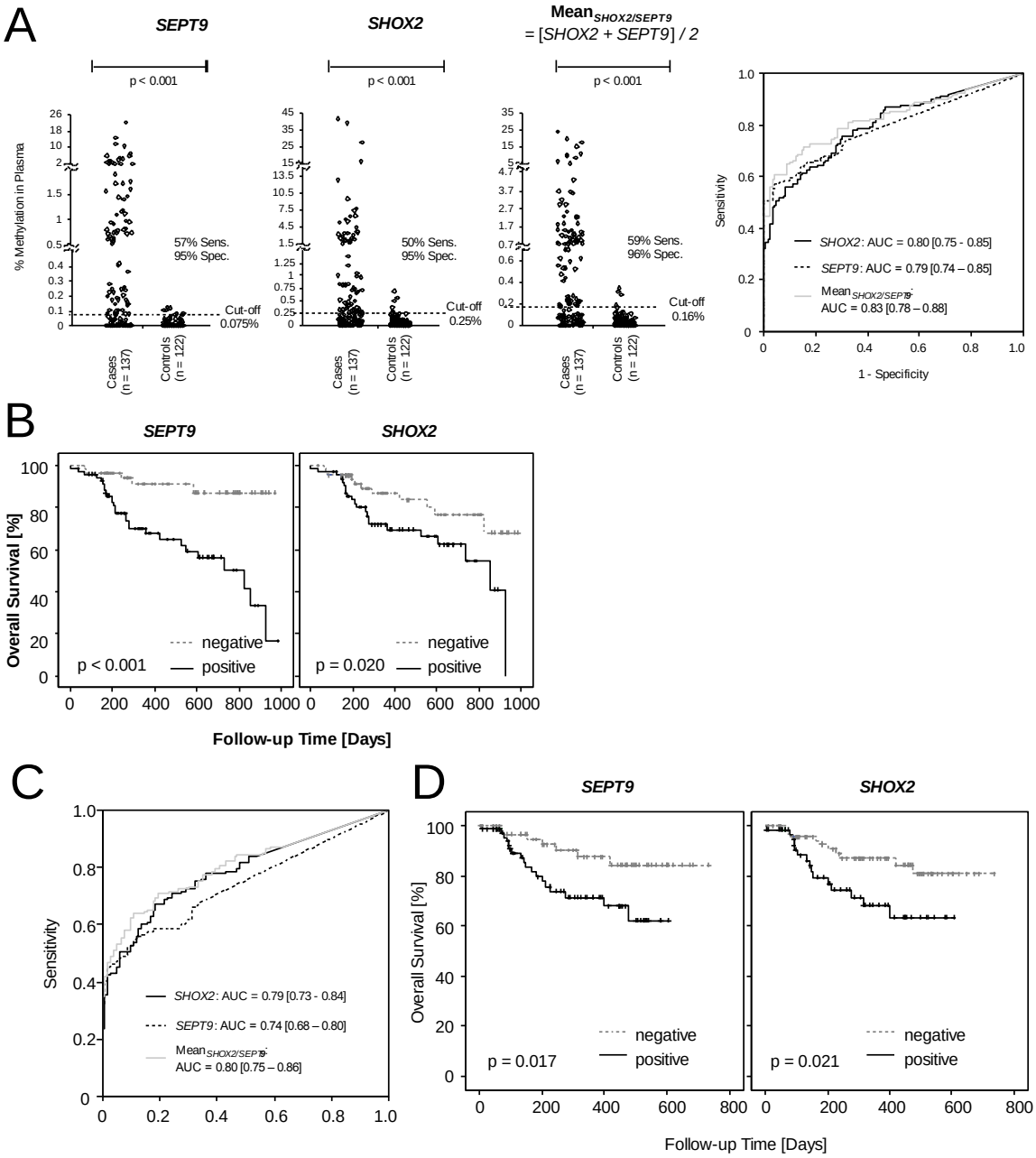


Figure 1: A: Training cohort: *SHOX2*, *SEPT9* and averaged (mean$_{SHOX2/SEPT9}$=[*SHOX2*+*SEPT9*]/2) DNA methylation levels in plasma from HNSCC (n=137) and control patients (n=122) and diagnostic accuracy (ROC analysis). B: Training cohort: Kaplan-Meier survival analysis of 129 HNSCC patients treated with curative intent stratified according to plasma methylation levels. C: Diagnostic accuracy in the validation cohort (n=141 HNSCC and n=102 control patients). D: Validation cohort: Kaplan-Meier survival analysis of 137 HNSCC patients treated with curative intent.

Tables

Table 1: Association of methylation levels with stage and histological grade (training cohort). T and N categories and histological grade of the 284 cancer cases included in the HNSCC patient cohort and their association with *SHOX2* and *SEPT9* plasma DNA methylation levels. Methylation levels in plasma prior to treatment were available for 137/284 patients. A comprehensive list of methylation levels and their association with clinico-pathologic parameters (T-, N-, M-category, grade, tumor localization, vascular invasion (V), lymphatic invasion (L), surgical margin (R), and tumor status (primary tumor versus recurrence)) can be found in Supplemental Table 2.

		Methylation in Plasma Prior to Treatment				
Clinico-pathologic Parameters	**Total Number (n)**	**n**	**Median *SEPT9* [%]; IQR**	***P*-Value‡**	**Median *SHOX2* [%]; IQR**	***P*-Value‡**
All HNSCC Cases	284 (100%)	137 (100%)	0.14; 0.87		0.25; 0.94	
T-Stage						
T_{is}	9 (3%)	1 (1%)	0.00; N/A		0.05; N/A	
T_1	65 (23%)	22 (16%)	0.00; 0.10		0.03; 0.10	
T_2	87 (31%)	42 (31%)	0.13; 0.65		0.27; 1.40	
T_3	56 (20%)	30 (22%)	0.07; 0.63		0.18; 0.78	
T_4	48 (17%)	32 (23%)	0.76; 1.90	*P*<0.001†	0.72; 2.21	*P*<0.001†
N/A¥	19 (7%)	10 (7%)	0.04; 2.31		0.37; 2.25	
Nodal Status						
N_0	110 (39%)	37 (27%)	0.07; 0.32		0.12; 0.35	
N_1	35 (12%)	19 (14%)	0.09; 1.40		0.31; 0.67	
N_2	89 (31%)	51 (37%)	0.56; 1.08		0.45; 2.66	
N_3	6 (2%)	2 (1%)	4.57; N/A	*P*<0.001†	14.72; N/A	*P*<0.001†
N_x	44 (16%)^	28 (20%)	0.00; 0.27		0.05; 0.56	
Histopathological Grade						
G_1	9 (3%)	2 (1%)	0.00; N/A		0.03; N/A	
G_2	127 (45%)	59 (43%)	0.09; 0.50		0.13; 0.42	
G_3	85 (30%)	51 (37%)	0.24; 1.20	*P*=0.027†	0.52; 3.15	*P*<0.001†
N/A¥	63 (22%)	25 (18%)	0.06; 1.50		0.25; 0.97	

¥ N/A: 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 CUP and local lymph node recurrences. Grading does not apply to CUP and loco-regional recurrences.
‡ *P*-values refer to Kendall's *τ* rank correlation.
† Significant feature

Table 2: Association of methylation levels with stage and histological grade (testing cohort). T and N categories and histological grade of 141 cancer cases included in the HNSCC patient testing cohort and their association with pre-therapeutic *SHOX2* and *SEPT9* plasma DNA methylation levels. A comprehensive list of methylation levels and their association with clinico-pathologic parameters (T-, N-, M-category, grade, tumor localization, vascular invasion (V), lymphatic invasion (L), surgical margin (R), and tumor status (primary tumor versus recurrence)) can be found in Supplemental Table 4.

	Methylation in Plasma Prior to First-line Treatment				
Clinico-pathologic Parameters	**n**	**Median *SEPT9* [%]; IQR**	***P*-Value[‡]**	**Median *SHOX2* [%]; IQR**	***P*-Value[‡]**
All HNSCC Cases	141 (100%)	0.10; 0.80		0.20; 0.72	
T-category					
T_{is}	1 (1%)	0.00; N/A		0.05; N/A	
T_1	31 (22%)	0.00; 0.08		0.05; 0.15	
T_2	43 (30%)	0.17; 0.67		0.12; 1.19	
T_3	29 (21%)	0.13; 0.54		0.23; 0.75	
T_4	28 (20%)	0.93; 2.02	*P*<0.001[†]	0.50; 0.66	*P*<0.001[†]
N/A[¥]	9 (6%)	0.00; 0.45		0.20; 5.63	
Nodal Status					
N_0	38 (27%)	0.00; 0.22		0.07; 0.29	
N_1	24 (17%)	0.09; 0.50		0.07; 0.30	
N_2	54 (38%)	0.32; 1.33		0.37; 2.15	
N_3	5 (4%)	2.22; 13.23	*P*<0.001[†]	2.40; 3.94	*P*<0.001[†]
N_x	20 (14%)	0.02; 0.56		0.21; 0.52	
Histopathological Grade					
G_1	10 (7%)	0.00; 0.67		0.03; 0.14	
G_2	52 (37%)	0.12; 0.46		0.08; 0.35	
G_3	33 (23%)	0.10; 1.33	*P*=0.13	0.45; 1.53	*P*=0.001[†]
N/A[¥]	46 (33%)	0.17; 0.82		0.29; 1.00	

[¥] N/A: 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 CUP and local lymph node recurrences. Grading does not apply to CUP and loco-regional recurrences.
[‡] *P*-values refer to Kendall's *τ* rank correlation.
[†] Significant feature

Supplemental Table 1: Patients' characteristics (training cohort). Characteristics of the HNSCC patient cohort (284 patients) and matched group of control patients (122 individuals). First-line treatment of HNSCC patients consisted of surgery in 32% (90/284), surgery and adjuvant radio-(chemo)therapy in 60% (171/284), or definitive radio-chemotherapy in 8% (23/284).

	Total	HNSCC Cohort	Matched Controls
Age	406 (100%)	284 (100%)	122 (100%)
≤ 50 Years	50 (13%)	30 (11%)	20 (16%)
51-60 Years	139 (34%)	97 (34%)	42 (35%)
> 60 Years	217 (53%)	157 (55%)	60 (49%)
Median Age [Years]	61	61	60
Mean Age [Years]	61.4	61.4	61.4
Age Range [Years]	32-89	32-89	32-87
Gender			
Female	80 (20%)	53 (19%)	27 (22%)
Male	326 (80%)	231 (81%)	95 (78%)
Smoking and Drinking Habits			
Non-smokers	40 (10%)	30 (10%)	10 (8%)
Smokers (Current and Former)	309 (76%)	215 (76%)	94 (77%)
Unknown Smoking Status	57 (14%)	39 (14%)	18 (15%)
Range Pack/Years	0-200	0-200	0-120
Median Pack/Years (Smokers only)	40	40	35
Mean Pack/Years (Smokers only)	42.0	44.1	37.1
Unknown Alcohol Consumption	76 (18%)	49 (18%)	27 (22%)
No Alcohol	32 (8%)	26 (9%)	6 (5%)
Occasional Alcohol	60 (15%)	40 (14%)	20 (16%)
Moderate Alcohol	157 (39%)	99 (35%)	58 (47%)
Strong Alcohol	32 (8%)	27 (9%)	5 (4%)
Alcoholic (Current and Former)	49 (12%)	43 (15%)	6 (5%)
Pre-existing Conditions			
None	56 (14%)	52 (18%)	4 (3%)
Diseases of Inner Ear, Equilibrium Organ and Middle Ear	40 (5%)	10 (4%)	30 (25%)
Diseases of Nose and Paranasal Sinuses	30 (10%)	0 (0%)	30 (25%)
Diseases of Oral Cavity, Larynx and Salivary Glands	1 (<1%)	0 (0%)	1 (1%)
Expanding Lesions Throat	19 (5%)	1 (<1%)	18 (15%)
Leucoplakia, Erythroplakia, Ulcers	5 (1%)	1 (<1%)	4 (3%)
Acute ENT-Infections und Abscesses	11 (3%)	2 (1%)	9 (7%)
Benign ENT-Tumors	8 (2%)	1 (<1%)	7 (6%)
Diseases of Gastrointestinal Tract	60 (15%)	48 (17%)	12 (10%)
Diseases of Cardiovascular System	200 (49%)	144 (51%)	56 (46%)
Diseases of Respiratory System	52 (13%)	40 (14%)	12 (10%)
Diseases of Metabolism or Endocrinological System	115 (28%)	83 (29%)	32 (26%)
Diseases of Kidney and Urinary Tract	17 (4%)	10 (4%)	7 (6%)
Diseases of Hepatic and Biliary System	20 (5%)	17 (6%)	3 (2%)
Infectious Diseases	3 (1%)	2 (1%)	1 (1%)
Ophthalmologic Diseases	4 (1%)	3 (1%)	1 (1%)
Skin Diseases	10 (2%)	5 (2%)	5 (4%)
Skeletal Diseases	35 (9%)	24 (8%)	11 (9%)
Neurological and Psychiatric Diseases	43 (11%)	32 (11%)	11 (9%)
Status after Other Malignant Tumors	21 (5%)*	18 (6%)	3 (2%)
Status after Other Benign Tumors (Excl. ENT Tumors)	3 (1%)	2 (1%)	1 (1%)

* Cases: breast cancer (n=1), uterine cancer (n=3), prostate cancer (n=1), colorectal cancer (n=1), esophageal cancer (n=2), lung cancer (n=3), CLL (n=1), multiple myeloma (n=1), basalioma (n=4); controls: prostate cancer (n=1), basalioma (n=2)

Supplemental Table 2: Clinico-pathologic parameters and methylation levels (training cohort). Clinico-pathologic parameters of the 284 cancer cases included in the HNSCC patient cohort and the association with *SHOX2* and *SEPT9* plasma DNA methylation levels. Methylation levels in plasma prior to treatment were available for 137/284 patients.

		Methylation in Plasma Prior to Treatment				
Clinico-pathologic Parameters	**Total Number (n)**	**n**	**Median *SEPT9* [%]; IQR**	***P*-Value‡**	**Median *SHOX2* [%]; IQR**	***P*-Value‡**
All HNSCC Cases	284 (100%)	137 (100%)	0.14; 0.87		0.25; 0.94	
Localisation						
Oral Cavity / Tongue / Lips	34 (12%)	15 (11%)	0.16; 0.74		0.30; 1.14	
Oropharynx and Tonsils	107 (38%)	53 (39%)	0.23; 1.04		0.27; 2.00	
Hypopharynx	26 (9%)	18 (13%)	0.09; 0.88		0.52; 4.64	
Larynx	92 (32%)	37 (27%)	0.05; 0.46		0.12; 0.45	
Others (Nasopharynx, Facial Skin)	18 (6%)	10 (7%)	0.86; 1.97		0.27; 0.92	
CUP	8 (3%)	4 (3%)	0.00; 1.20	*P*=0.46	0.36; 1.48	*P*=0.51
Tumor						
Primary Tumor	235 (83%)	107 (78%)	0.14; 0.81		0.28; 1.33	
Loco-regional Recurrence	50 (18%)	30 (22%)	0.12; 1.20	*P*=0.66	0.11; 0.64	*P*=0.075
T-Stage						
T_{is}	9 (3%)	1 (1%)	0.00; N/A		0.05; N/A	
T_1	65 (23%)	22 (16%)	0.00; 0.10		0.03; 0.10	
T_2	87 (31%)	42 (31%)	0.13; 0.65		0.27; 1.40	
T_3	56 (20%)	30 (22%)	0.07; 0.63		0.18; 0.78	
T_4	48 (17%)	32 (23%)	0.76; 1.90	*P*<0.001†	0.72; 2.21	*P*<0.001†
N/A¥	19 (7%)	10 (7%)	0.04; 2.31		0.37; 2.25	
Nodal Status						
N_0	110 (39%)	37 (27%)	0.07; 0.32		0.12; 0.35	
N_1	35 (12%)	19 (14%)	0.09; 1.40		0.31; 0.67	
N_2	89 (31%)	51 (37%)	0.56; 1.08		0.45; 2.66	
N_3	6 (2%)	2 (1%)	4.57; N/A	*P*<0.001†	14.72; N/A	*P*<0.001†
N_x	44 (16%)	28 (20%)	0.00; 0.27		0.05; 0.56	
Distant Metastases						
M_0	279 (98%)	132 (96%)	0.12; 0.80		0.23; 0.78	
M_1	5 (2%)	5 (4%)	1.56; 4.16	*P*=0.32	3.09; 26.62	*P*=0.022†
Histopathological Grade						
G_1	9 (3%)	2 (1%)	0.00; N/A		0.03; N/A	
G_2	127 (45%)	59 (43%)	0.09; 0.50		0.13; 0.42	
G_3	85 (30%)	51 (37%)	0.24; 1.20	*P*=0.027†	0.52; 3.15	*P*<0.001†
N/A¥	63 (22%)	25 (18%)	0.06; 1.50		0.25; 0.97	
Lymphatic Invasion						
L_0	132 (47%)	64 (46%)	0.15; 0.79		0.17; 0.67	
L_1	38 (13%)	28 (21%)	0.19; 0.74	*P*=0.83	0.43; 2.30	*P*=0.043†
N/A¥	114 (40%)	45 (33%)	0.06; 1.28		0.25; 1.89	
Vascular Invasion						
V_0	154 (54%)	79 (58%)	0.15; 0.72		0.21; 0.68	
V_1	17 (6%)	12 (9%)	0.87; 1.93	*P*=0.038†	0.83; 2.21	*P*=0.047†
N/A¥	113 (40%)	46 (34%)	0.05; 1.19		0.23; 1.81	
Surgical Margin						
R_0	182 (64%)	83 (61%)	0.15; 0.73		0.20; 0.65	
R_1	27 (9%)	19 (14%)	0.71; 1.41		0.83; 3.25	
R_2	2 (1%)	2 (1%)	1.00; N/A	*P*=0.090	0.78; N/A	*P*=0.014†
N/A¥	73 (26%)	33 (24%)	0.04; 0.89		0.21; 1.63	

¥ N/A: 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 CUP and local lymph node recurrences. Grading does not apply to CUP and loco-regional recurrences.

‡ *P*-values refer to the following tests: Wilcoxon-Mann-Whitney test (R_0 vs. $R_{1,2}$; L_0 vs. L_1; V_0 vs. V_1; M_0 vs. M_1; primary tumor vs. loco-regional Recurrence), Kendall's τ rank correlation (T stage, N stage, grade), ANOVA (tumor localization).

† Significant feature

Supplemental Table 3: Survival analyses (training cohort). Univariate and multivariate Cox proportional hazards regression of overall, distant metastases-free, loco-regional recurrence-free, and progression-free survival for *SEPT9* DNA methylation levels (as continuous variable), tumor category (T), nodal category (N), vascular invasion (V), lymphatic invasion (L), grade (G), tumor status (primary tumor or loco-regional recurrence of an earlier HNSCC). Patients receiving only palliative treatment or supportive care were excluded from survival analyses.

					Univariate		Multivariate	
Survival / Time-to-Event	**Clinico-pathologic Factor**	**n**	**Events**	**Censored**	***P*-value**	**Hazard Ratio [95% CI]**	***P*-value**	**Hazard Ratio [95% CI]**
Overall Survival	T_4, T_3, T_2, T_1, T_{is}	123	33	90	0.001†	1.84 [1.30 – 2.60]	0.050	1.82 [1.00 – 3.29]
	N_3, N_2, N_1, N_0	103	26	77	0.16	1.35 [0.89 – 2.04]		
	G_3, G_2, G_1	110	27	83	0.24	1.52 [0.75 – 3.05]		
	L_1 vs. L_0	91	25	66	0.25	1.60 [0.72 – 3.56]		
	V_1 vs. V_0	90	25	65	0.004†	3.83 [1.56 – 9.43]	0.011†	4.27 [1.40 – 13.1]
	$R_{2,1}$ vs. R_0	102	25	77	0.001†	3.69 [1.67 – 8.16]		
	Loco-regional Recurrence vs. Primary Tumor	129	33	96	0.025†	1.34 [1.04 – 1.72]		
	SEPT9 Methylation	129	33	96	<0.001†	1.14 [1.07 – 1.22]	0.005†	1.23 [1.07 – 1.41]
Distant Metastases-free Survival	T_4, T_3, T_2, T_1, T_{is}	123	16	107	0.021	1.78 [1.09 – 2.91]		
	N_3, N_2, N_1, N_0	103	14	89	0.22	1.42 [0.81 – 2.50]		
	G_3, G_2, G_1	110	15	95	0.70	1.20 [0.48 – 3.00]		
	L_1 vs. L_0	91	13	78	0.21	2.02 [0.68 – 6.01]		
	V_1 vs. V_0	90	13	77	0.91	0.89 [0.12 – 6.88]		
	$R_{2,1}$ vs. R_0	102	13	89	0.45	1.65 [0.45 – 6.00]		
	Loco-regional Recurrence vs. Primary Tumor	129	17	112	0.19	1.29 [0.88 – 1.88]		
	SEPT9 Methylation	129	17	112	0.002†	1.28 [1.09 – 1.50]	0.012†	1.25 [1.05 – 1.49]
Loco-regional Recurrence-free Survival	T_4, T_3, T_2, T_1, T_{is}	123	19	104	0.60	1.12 [0.73 – 1.72]		
	N_3, N_2, N_1, N_0	103	16	87	0.49	0.83 [0.49 – 1.41]		
	G_3, G_2, G_1	110	15	95	0.27	0.60 [0.24 – 1.49]		
	L_1 vs. L_0	91	15	76	0.38	0.57 [0.16 – 2.01]		
	V_1 vs. V_0	90	15	75	0.64	0.62 [0.08 – 4.71]		
	$R_{2,1}$ vs. R_0	102	14	88	0.60	1.41 [0.39 – 5.08]		
	Loco-regional Recurrence vs. Primary Tumor	129	21	108	0.001†	1.64 [1.22 – 2.20]	0.024†	1.72 [1.07 – 2.76]
	SEPT9 Methylation	129	21	108	<0.001†	1.21 [1.10 – 1.33]	0.005†	1.32 [1.09 – 1.59]
Progression-free Survival	T_4, T_3, T_2, T_1, T_{is}	123	32	91	0.16	1.27 [0.91 – 1.78]		
	N_3, N_2, N_1, N_0	103	27	76	1.00	1.00 [0.67 – 1.48]		
	G_3, G_2, G_1	110	27	83	0.43	0.61 [0.43 – 1.64]		
	L_1 vs. L_0	91	26	65	0.95	1.03 [0.45 – 2.37]		
	V_1 vs. V_0	90	26	64	0.71	0.76 [0.18 – 3.21]		
	$R_{2,1}$ vs. R_0	102	25	77	0.99	1.00 [0.34 – 2.92]		
	Loco-regional Recurrence vs. Primary Tumor	129	35	94	<0.001†	1.57 [1.24 – 2.00]	0.008†	1.68 [1.14 – 2.48]
	SEPT9 Methylation	129	35	94	<0.001†	1.21 [1.11 – 1.33]	0.002†	1.19 [1.10 – 1.56]

† Significant feature

Supplemental Table 4: Patients' characteristics (testing cohort). Characteristics of the HNSCC patient testing cohort (141 patients) and matched group of control patients (102 individuals). First-line treatment of HNSCC patients consisted of surgery in 31% (44/141), surgery and adjuvant radio-(chemo)therapy in 53% (75/141), or definitive radio-chemotherapy in 16% (22/141).

	Total	HNSCC Cohort	Matched Controls
Age	243 (100%)	141 (100%)	102 (100%)
≤ 50 Years	24 (10%)	11 (8%)	13 (13%)
51-60 Years	67 (28%)	37 (26%)	30 (29%)
> 60 Years	152 (63%)	93 (66%)	59 (58%)
Median Age [Years]	63	63	62
Mean Age [Years]	63.6	64.5	62.6
Age Range [Years]	36-83	37-93	36-86
Gender			
Female	57 (23%)	26 (18%)	31 (30%)
Male	186 (77%)	115 (82%)	71 (70%)
Smoking and Drinking Habits			
Non-smokers	50 (21%)	28 (20%)	22 (22%)
Smokers (Current and Former)	175 (72%)	103 (73%)	72 (71%)
Unknown Smoking Status	18 (7%)	10 (7%)	8 (8%)
Range Pack/Years	0-130	0-130	0-100
Median Pack/Years (Smokers only)	30	30	30
Mean Pack/Years (Smokers only)	31.6	31.2	31.9
Unknown Alcohol Consumption	26 (11%)	13 (9%)	13 (13%)
No Alcohol	43 (18%)	28 (20%)	15 (15%)
Occasional Alcohol	43 (18%)	19 (13%)	24 (24%)
Moderate Alcohol	73 (30%)	36 (26%)	37 (36%)
Strong Alcohol	11 (4%)	5 (4%)	6 (6%)
Alcoholic (Current and Former)	47 (19%)	40 (28%)	7 (7%)
Pre-existing Conditions			
None	30 (12%)	25 (18%)	5 (5%)
Diseases of Inner Ear, Equilibrium Organ and Middle Ear	38 (16%)	5 (4%)	33 (32%)
Diseases of Nose and Paranasal Sinuses	26 (11%)	0 (0%)	26 (25%)
Diseases of Oral Cavity, Larynx and Salivary Glands	0 (0%)	0 (0%)	0 (0%)
Expanding Lesions Throat	21 (9%)	1 (1%)	20 (20%)
Leucoplakia, Erythroplakia, Ulcers	18 (7%)	7 (5%)	11 (11%)
Acute ENT-Infections und Abscesses	15 (6%)	0 (0%)	15 (15%)
Benign ENT-Tumors	15 (6%)	1 (1%)	14 (14%)
Diseases of Gastrointestinal Tract	33 (14%)	25 (18%)	8 (8%)
Diseases of Cardiovascular System	183 (75%)	114 (81%)	69 (68%)
Diseases of Respiratory System	48 (20%)	26 (18%)	22 (22%)
Diseases of Metabolism or Endocrinological System	50 (21%)	26 (18%)	24 (24%)
Diseases of Kidney and Urinary Tract	19 (8%)	15 (11%)	4 (4%)
Diseases of Hepatic and Biliary System	13 (5%)	7 (5%)	6 (6%)
Infectious Diseases	2 (1%)	2 (1%)	0 (0%)
Ophthalmologic Diseases	5 (2%)	4 (3%)	1 (<1%)
Skin Diseases	14 (6%)	5 (4%)	9 (9%)
Skeletal Diseases	24 (10%)	7 (5%)	17 (17%)
Neurological and Psychiatric Diseases	34 (14%)	20 (14%)	14 (14%)
Status after Other Malignant Tumors	25 (10%)*	12 (9%)	13 (13%)
Status after other Benign Tumors (Excl. ENT Tumors)	4 (2%)	3 (2%)	1 (1%)

* Cases: breast cancer (n=3), uterine cancer (n=4), prostate cancer (n=3), colorectal cancer (n=1), CLL (n=1); controls: breast cancer (n=2), uterine cancer (n=3), prostate cancer (n=1), thyroid carcinoma (n=2), colorectal cancer (n=1), CLL (n=2), basalioma (n=2)

Supplemental Table 5: Clinico-pathologic parameters and methylation levels (testing cohort). Clinico-pathologic parameters of 141 cancer cases included in the HNSCC patient testing cohort and association with pre-therapeutic *SHOX2* and *SEPT9* plasma DNA methylation levels.

		Methylation in Plasma Prior to First-line Treatment			
Clinico-pathologic Parameters	**n**	**Median *SEPT9* [%]; IQR**	***P*-Value[‡]**	**Median *SHOX2* [%]; IQR**	***P*-Value[‡]**
All HNSCC Cases	141 (100%)	0.10; 0.80		0.20; 0.72	
Localisation					
Oral Cavity / Tongue / Lips	38 (27%)	0.08; 0.79		0.14; 0.48	
Oropharynx and Tonsils	41 (29%)	0.23; 1.53		0.23; 2.37	
Hypopharynx	17 (12%)	0.08; 1.13		0.48; 0.95	
Larynx	33 (23%)	0.05; 0.39		0.07; 0.32	
Others (Nasopharynx, Facial Skin)	9 (6%)	0.03; 1.48		0.31; 1.13	
CUP	3 (2%)	0.47; N/A	*P*=0.097	0.23; N/A	*P*=0.20
Tumor					
Primary Tumor	112 (79%)	0.16; 0.83		0.22; 0.81	
Loco-regional Recurrence	29 (21%)	0.05; 0.45	*P*=0.41	0.09; 0.45	*P*=0.30
T-category					
T_{is}	1 (1%)	0.00; N/A		0.05; N/A	
T_1	31 (22%)	0.00; 0.08		0.05; 0.15	
T_2	43 (30%)	0.17; 0.67		0.12; 1.19	
T_3	29 (21%)	0.13; 0.54		0.23; 0.75	
T_4	28 (20%)	0.93; 2.02	*P*<0.001[†]	0.50; 0.66	*P*<0.001[†]
N/A[¥]	9 (6%)	0.00; 0.45		0.20; 5.63	
Nodal Status					
N_0	38 (27%)	0.00; 0.22		0.07; 0.29	
N_1	24 (17%)	0.09; 0.50		0.07; 0.30	
N_2	54 (38%)	0.32; 1.33		0.37; 2.15	
N_3	5 (4%)	2.22; 13.23	*P*<0.001[†]	2.40; 3.94	*P*<0.001[†]
N_x	20 (14%)	0.02; 0.56		0.21; 0.52	
Distant Metastases					
M_0	138 (98%)	0.10; 0.82		0.20; 0.72	
M_1	3 (2%)	0.00; N/A	*P*=0.53	0.06; N/A	*P*=1.0[†]
Histopathological Grade					
G_1	10 (7%)	0.00; 0.67		0.03; 0.14	
G_2	52 (37%)	0.12; 0.46		0.08; 0.35	
G_3	33 (23%)	0.10; 1.33	*P*=0.13	0.45; 1.53	*P*=0.001[†]
N/A[¥]	46 (33%)	0.17; 0.82		0.29; 1.00	
Lymphatic Invasion					
L_0	69 (49%)	0.09; 0.90		0.10; 0.51	
L_1	29 (21%)	0.14; 0.53	*P*=0.45	0.12; 1.67	*P*=0.46
N/A[¥]	43 (30%)	0.26; 0.82		0.27; 0.93	
Vascular Invasion					
V_0	86 (61%)	0.09; 0.53		0.10; 0.55	
V_1	9 (6%)	0.23; 1.34	*P*=0.47	0.09; 0.94	*P*=0.93
N/A[¥]	46 (33%)	0.17; 0.84		0.30; 1.01	
Surgical Margin					
R_0	90 (64%)	0.09; 0.70		0.15; 0.71	
R_1	11 (8%)	0.01; 0.18		0.06; 0.12	
R_2	2 (1%)	5.65; N/A	*P*=0.71	24.44; N/A	*P*=0.35
N/A[¥]	38 (27%)	0.27; 0.91		0.29; 1.28	

[¥] N/A: 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 CUP and local lymph node recurrences. Grading does not apply to CUP and loco-regional recurrences.

[‡] *P*-values refer to the following tests: Wilcoxon-Mann-Whitney test (R_0 vs. $R_{1,2}$; L_0 vs. L_1; V_0 vs. V_1; M_0 vs. M_1; primary tumor vs. loco-regional Recurrence), Kendall's τ rank correlation (T stage, N stage, grade), ANOVA (tumor localization).

[†] Significant feature

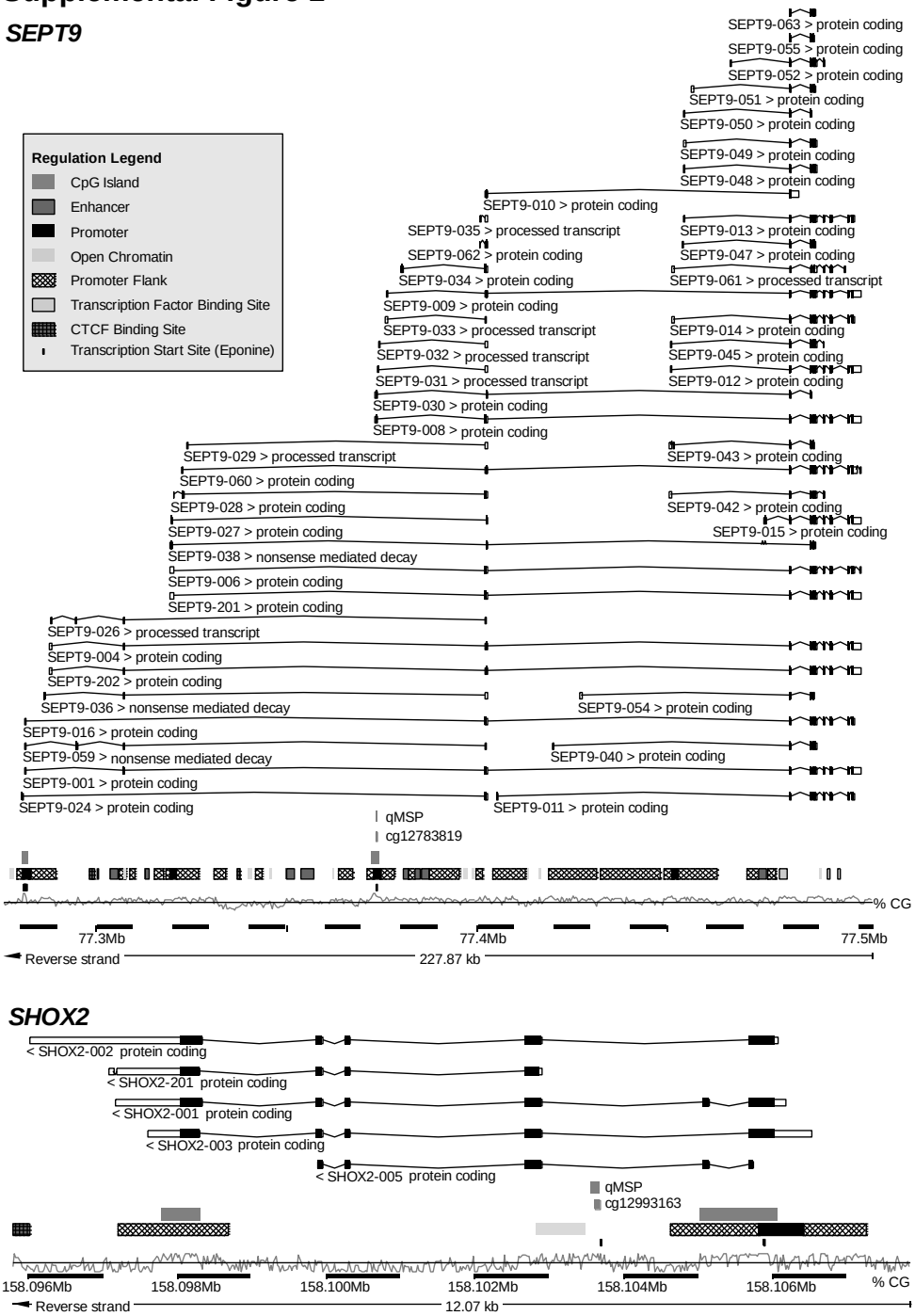
Supplemental Table 6: Disease monitoring. Results of the last negative and first positive blood tests are shown where applicable. The difference between the day of the clinical endpoints (or the diagnosis of a second cancer) and the day of the first positive blood test were calculated. A positive time difference indicates an earlier diagnosis via DNA methylation biomarkers compared to standard diagnostic work-up. Results are shown from all study patients who reached one or more clinical endpoints. Only patients with at least one blood sample available during follow-up are listed.

	Test Result [Day* of Testing / Test Result]		Day* of Reaching Clinical Endpoint					Difference [Day* of Reaching Clinical Endpoint – Day* of First Positive Test Result]				
Patient	Last Negative Test Result* [Day / Methylation Value]	First Positive Test Result* [Day / Methylation Value]	Death	Distant Metastases	Loco-regional Recurrence	Progression (Distant Metastases or Loco-regional Recurrence)	Development of a 2nd Cancer	Death	Distant Metastases	Loco-regional Recurrence	Progression (Distant Metastases or Loco-regional Recurrence)	Development of a 2nd Cancer
1	30 / 0.05	x	144		77	77		x		x	x	
2	37 / 0.01	x	215		78	78		x		x	x	
3	44 / 0.03	50 / 0.71	195					145				
4	13 / 0.04	61 / 10.89	166		83	83		105		22	22	
5	6 / 0.01	62 / 1.22	228					166				
6	28 / 0.15	x	165					x				
7	x	36 / 11.04	275	241	29	29		239	205	-7	-7	
8	4 / 0.04	x	361		113	113		x		x	x	
9	8 / 0.03	83 / 0.85	209					126				
10	19 / 0.01	x	76					x				
11	384 / 0.01	475 / 1.19	734	126		126		259	-349		-349	
12	13 / 0.04	x	293		110	110		x		x	x	
13	14 / 0.13	204 / 0.30	525					321				
14	13 / 0.01	x	70					x				
15	7 / 0.05	61 / 2.84	159		147	147		98		86	86	
16	x	7 / 0.30	171					164				
17	194 / 0.01	285 / 2.02	550	458		458	432 i	265	173		173	147
18	142 / 0.06	211 / 0.48	427	220		220		216	9		9	
19	337 / 0.02	x	421	205		205		x	x		x	
20	4 / 0.02	17 / 0.30					129 ii					112
21	4 / 0.02	x			134	134				x	x	
22	51 / 0.06	x		198		198			x		x	
23	336 / 0.04	x			370	370				x	x	
24	356 / 0.01	x			391	391				x	x	
25	55 / 0.02	328 / 0.67			336	336				8	8	
26	x	9 / 0.20			252	252				243	243	
27	184 / 0.08	275 / 0.19		401		401			126		126	
28	5 / 0.05	x					6 ii					x
29	680 / 0.01	x		667		667			x		x	
30	553 / 0.01	x		582		582			x		x	
31	721 / 0.01	x		272		272			x		x	
32	11 / 0.159	363 / 3.54	851	420		420		488	57		57	
33	169 / 0.14	218 / 0.33	586		491	491	8 iii	368		273	273	-210
34	11 / 0.04	x	822		118	118		x		x	x	
35	47 / 0.01	845 / 0.24					845 iv					0
36	833 / 0.05	x					370 iii					x
37	740 / 0.01	894 / 1.24		991		991			97		97	
38	1640 / 0.02	x					1633 iii					x
39	x	3273 / 0.48	3477		3428	3428		204		155	155	
40	1164 / 0.04	x		1164		1164			x		x	
41	114 / 0.05	247 / 7.82		290		290			43		43	
42	467 / 0.01	551 / 0.92			234	234	234 iv			-317	-317	-317
43	4111 / 0.01	x			3943	3943	3941 iv			x	x	x
44	125 / 0.01	x			215	215				x	x	
45	792 / 0.01	x		1011		1001			x		x	
46	178 / 0.04	x			178	178				x	x	
47	x	547 / 0.35	925		542	542		378		-5	-5	
48	7/0.09	34 / 1.37			34	34				0	0	
49	230 / 0.03	321 / 0.21		334	334	334			13	13	13	
50	63 / 0.13	121 / 9.53	267	202	202	202		146	81	81	81	
51	8 / 0.05	x	66					x				
52	x	345 / 0.17		722		722			377		377	
53	x	8 / 0.37	38					30				
54	71 / 0.01	x	125					x				
55	15 / 0.04	x	276					x				
56	7 / 0.07	x					613 iii					x
57	x	17 / 3.32	133					116				
58	140 / 0.01	x	263	127		127		x	x		x	
59	180 / 0.01	271 / 1.23		587	281	281	317 v		316		10	46
60	1485 / 0.01	x			1538	1538				x	x	
61	288 / 0.05	x			309	309				x	x	
62	9 / 0.02	100 / 0.23			259	259				159	159	
63	793 / 0.01	912 / 0.26					1255 vi					343

* after treatment start

[i] pancreatic cancer, [ii] colorectal cancer, [iii] lung cancer, [iv] HNSCC, [v] gastric cancer, [vi] esophageal cancer

Supplemental Figure 1



Supplemental Figure 1: Genomic organization (introns, exons, transcripts, regulatory elements, CG content) of the *SHOX2* and *SEPT9* genes and regions targeted for methylation analyses. *SEPT9* is located on chromosome 17:77,280,569-77,500,596 (forward strand) and has 47 transcripts. The qMSP and the Infinium bead cg12783819 used for methylation analyses target a region within a CpG island of a putative alternative promoter found in close proximity to the transcription start sites (Eponine, [1]) of transcript variants 008, 009, 030-035, and 062. *SHOX2* is encoded on chromosome 3:158,095,954-158,106,503 (reverse strand) and has 5 protein coding transcripts. The qMSP and the Infinium bead cg12993163 assess the methylation level at the putative transcription start site (Eponine) of transcript 201. All information was taken from Ensembl genome assembly GRCh38.p7 (www.ensembl.org).

Reference

1. Down TA, Hubbard TJP. Computational detection and location of transcription start sites in mammalian genomic DNA. Genome Res 2002;12:458-61.

Supplemental Results 1

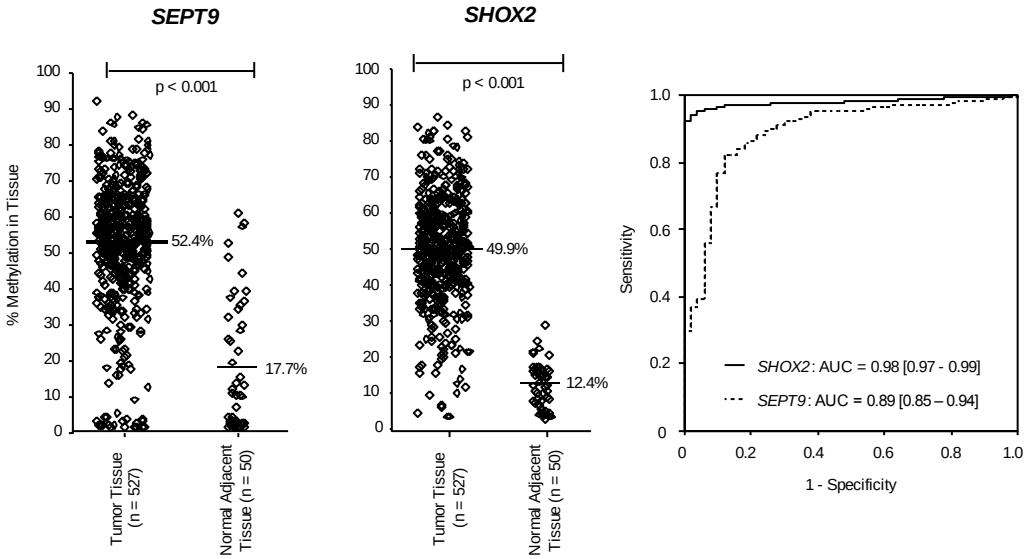
Supplemental Results 1

SHOX2 and SEPT9 DNA methylation in tissues and matched plasma specimens

Methylation levels of 527 HNSCC and 50 normal adjacent tissues from TCGA (http://cancergenome.nih.gov/.) were analyzed. The methylation data generated by the TCGA Research Network were created by means of the Infinium HumanMethylation450 BeadChip (Illumina, Inc., San Diego, CA, USA). HumanMethylation450 data of level 2 including background-corrected methylated (Intensity_M) and unmethylated (Intensity_U) summary intensities as extracted by the R package 'methylumi' were downloaded. The two beads cg12783819 and cg12993163, that hybridize to CpG-sites within the target region of the *SEPT9* and *SHOX2* qPCR assays (Supplemental Figure 1), were evaluated. Methylation values for each bead were calculated by the formula 100% x Intensity_M / (Intensity_M + Intensity_U). *SHOX2* and *SEPT9* were found to be hypermethylated in tumor tissues compared to normal adjacent tissues (diagnostic accuracy: AUC_{SEPT9}=0.89, 95%CI [0.85–0.94], AUC_{SHOX2}=0.98, 95%CI [0.97-0.99], P<0.001; Supplemental Figure 2). While mean methylation levels of both genes in HNSCC tissue were similar (*SEPT9*: 52.4% vs. *SHOX2*: 49.9%), twenty-one out of 527 (4%) tumors showed only low level *SEPT9* methylation <5%. However, these 21 tumor tissues had a mean average *SHOX2* methylation of 51.7%. In contrast, only 3/527 (0.6%) of HNSCC tissues had a *SHOX2* methylation <5%. All these three tumors were *SEPT9* hypermethylated. Thus, both biomarkers analyzed as a biomarker panel added independent information. Interestingly, mean *SEPT9* methylation in normal adjacent tissues (NAT) was higher (17.7%) compared to mean *SHOX2* methylation (12.4%). However, Supplemental Figure 2 illustrates that the *SHOX2* methylation in NAT was homogeneously distributed around the mean, while *SEPT9* methylation was characterized by a higher methylation variance within NAT. Four out of 50 (8%) NATs showed a *SEPT9* methylation higher than the mean *SEPT9* methylation in HNSCC. On the other hand, 22/50 (44%) NATs showed only low level methylation <5%, while 41/50 (82%) NATs exhibited *SHOX2* methylation >5%. Hence, *SHOX2* background methylation in plasma can be expected in a higher number of individuals without HNSCC compared to *SEPT9*. Interestingly, *SHOX2* and *SEPT9* methylation in HNSCC from smokers correlated significantly with the number of pack years (*SHOX2*: Spearman's $\rho = 0.15$, $P = 0.012$; *SEPT9*: $\rho = 0.17$, $P = 0.003$).

The feasibility to detect *SEPT9* and *SHOX2* hypermethylated ccfDNA in blood plasma from HNSCC patients was tested in a small patient group. Matched tumor samples and pre-therapeutic blood

samples from 55 patients were analyzed. For DNA methylation analyses of formalin-fixed and paraffin-embedded tumor tissues, bisulfite DNA was prepared using the innuCONVERT All-In-One Bisulfite Kit (Analytik Jena, Jena, Germany) according to the manufacturer's protocol. Mean methylation in these 55 HNSCC tissues was 51.1% (*SEPT9*) and 71.88% (*SHOX2*), respectively. Mean methylation in the 55 corresponding plasma samples was 2.08% (*SEPT9*) and 3.87% (*SHOX2*), respectively. *SHOX2* methylation in plasma correlated significantly with tissue methylation levels (ρ = 0.36, *P* = 0.007), while *SEPT9* only showed a trend towards higher methylation in plasma from patients with *SEPT9* hypermethylated tumors (ρ = 0.25, *P* = 0.067). As already found in the TCGA HNSCC cohort, a higher number of tumors (18/55, 33%) showed only low level (< 5%) *SEPT9* methylation in tissue, while only 1/55 (2%) tumors exhibited *SHOX2* methylation < 5%. Mean *SHOX2* methylation in the 18 HNSCC tissues with *SEPT9* methylation <5% was 94.6% and mean *SHOX2* plasma methylation was 6.2% with 12/18 (67%) plasma samples showing *SHOX2* methylation >1%, thus confirming the additive value of both biomarkers.



Supplemental Figure 2: *SHOX2* and *SEPT9* DNA methylation levels in HNSCC tumor (n=527) and normal adjacent tissues (n=50). Each rhombus reflects one sample measurement, and mean values (black bars) are given. *P*-values refer to Wilcoxon-Mann-Whitney test. ROC analysis and the resulting AUCs are shown. The results shown here are based upon data generated by the TCGA Research Network: http://cancergenome.nih.gov/.

Supplemental Results 2

SHOX2 and SEPT9 DNA methylation in plasma for disease monitoring: selected cases

Monitoring results of eight exemplary patients are described in Supplemental Figure 3. A 61-year old man (patient 11) diagnosed with a recurrent carcinoma of the larynx (T4, N2, G2, V1, L1, R0, M0) was treated with surgery and adjuvant radio-chemotherapy. Before treatment, 0.41% methylation were detected in plasma. On day eleven after surgery, blood methylation was below the cut-off (0.08%) and remained negative when measured on day 120 and 384 after surgery. Strongly positive results were first recorded on day 475 (1.19%) and 566 (4.36%), respectively. However, pulmonary metastases were already diagnosed on day 126 via computer tomography (CT). In this case, the diagnosis of systemic progression via CT imaging was superior to methylation testing in blood.

Blood from a 72-year old man (patient 5) was negative 6 days after surgical resection of a recurrent carcinoma of the oropharynx (T4, N0, G2, L1, V0, R0, M0). A blood sample prior to surgery was not available. Methylation testing was negative (0.01%) on day 6 after surgery. The first strongly positive test result (1.22%) was obtained on day 62. Adjuvant radio-chemotherapy started on day 110. The patient died 238 days after surgery without diagnosis of a local or systemic progression. However, all five blood test between day 6 and 181 were strongly positive, indicating that the patient in fact died from an occult tumor progression.

Patient 17, a 60-year old man, was diagnosed with a T3, Nx, G2, V0, L0, R0, M0 squamous cell carcinoma on the tip of his nose and was surgically treated. The blood testing was slightly positive before surgery (0.18%). Adjuvant radiotherapy started on day 59. A second blood sample was analyzed on day 194 with negative results (0.01%). The next blood samples on days 285 and 313 were strongly positive. Eventually, a second primary cancer (pancreatic ductal adenocarcinoma) was diagnosed on day 432, one hundred and forty-seven days after the first positive blood sample. However, the pancreatic cancer was diagnosed at an advanced (unresectable) stage, and the patient died on day 550. While an earlier diagnosis of the second cancer based on methylation testing would have been possible, it is not clear whether the patient would have benefitted due to the adverse prognosis of the second cancer and the lack of therapeutic options.

A 57-year old man (patient 50) had a methylation positive (0.93%) blood test prior to a definitive radio-chemotherapy of a T3, N2, G2, M0 carcinoma of the hypopharynx. In the course of radio-chemotherapy, methylation levels in the blood decreased to 0.13% on day 63. A dramatic recurrence

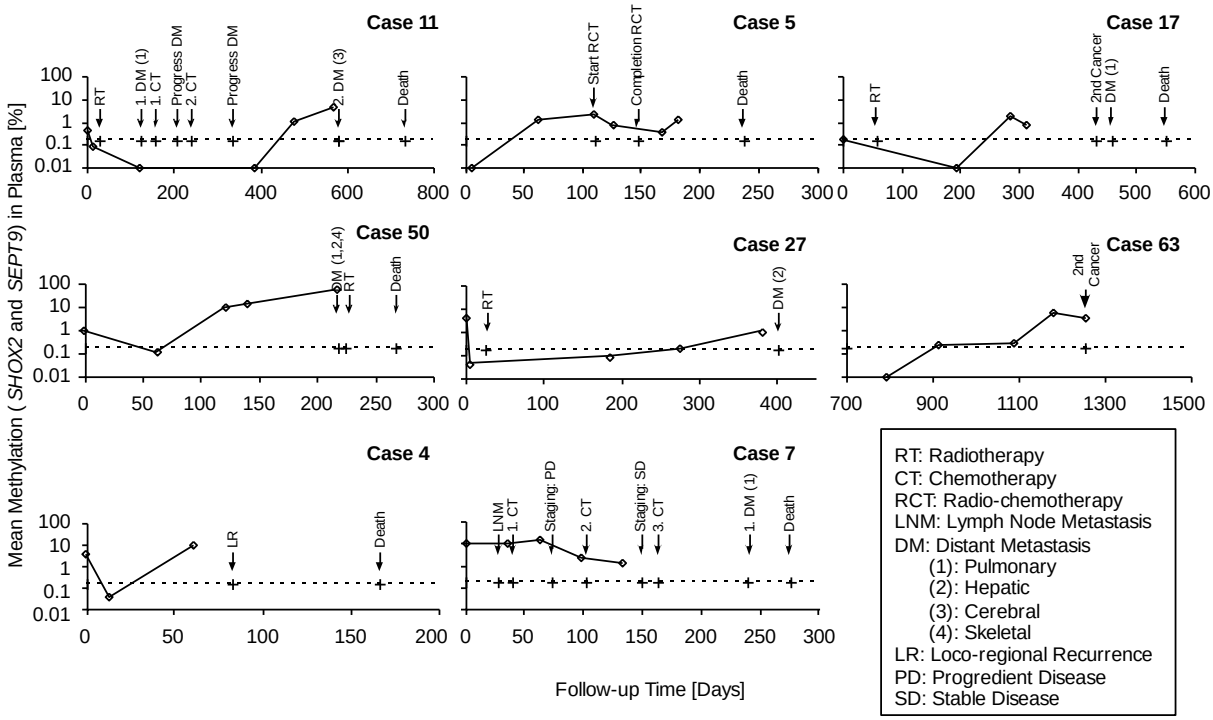
of methylated tumor ccfDNA was detected on day 121 (9.53%), 140 (14.65%), and 217 (54.93%). The patient died 267 days after the initiation of the therapy. Multiple distant metastases (pulmonary, skeletal, hepatic) were diagnosed on day 219, ninety-eight days after the first strongly positive blood result.

Methylation levels in the blood from patient 27 (male, 69 years old), who suffered from a surgically resected T2, N2, G2, L0, V0, R0, M0 carcinoma of the larynx, was strongly positive (0.85%) before treatment started. Negative blood test results were obtained on day 5 and 184 after surgery. On day 275, a first moderately positive (0.19%) test result was observed. Distant (hepatic) metastases were diagnosed on day 401, one hundred and twenty-six days after the first positive test result.

Patient 4 was a 69-year old male with a surgically resected T1, N2, G3, L0, V0, R0, M0 SCC of the oral cavity. Strongly positive methylation levels (3.55%) were found before treatment but decreased below the cut-off on day 13 after surgery. A fulminant recurrent methylation level (10.89%) was detected on day 61 after surgery. Twenty-two days later, a loco-regional relapse was diagnosed.

The 61 years old male patient 63 was a heavy smoker (75 pack years) and former alcoholic, who received surgery and adjuvant radio-chemotherapy because of a T3, N1, G3, L1, V1, R0, M0 laryngeal SCC. No methylated DNA was detected in the blood on day 793 after surgery. Slightly elevated methylation levels above the cut-off were measured on days 912 and 1087 before strong positive test results were obtained on day 1178 and 1255. A second cancer (N1, M0, oesophageal squamous cell carcinoma) was diagnosed on day 1255, a total of 343 days after the first positive blood test result.

Patient 7 (70 years old, male) showed strongly positive methylation levels prior to and after surgical resection of a T4, N2, G3, L1, V0, R1, M0 carcinoma of the hypopharynx. A lymph node metastasis was diagnosed on day 29, and the patient received a palliative cisplatin/docetaxel-based chemotherapy starting on day 41. However, blood methylation levels remained strongly positive on day 64, 99, and 134. Accordingly, progressive disease was diagnosed on day 74.



Supplemental Figure 3: Eight selected cases are depicted in detail. Methylation levels (*SHOX2* and *SEPT9* methylation mean averaged) below 0.01% were set to 0.01% in order to allow for a logarithmic illustration.

Supplemental Protocol 1

Plasma Preparation

Blood was collected using S-Monovette® (9 ml) K3 EDTA collection tubes (Sarstedt AG & Co., Nümbrecht, Germany). The blood was centrifuged for 6 min at 1.350 g, and the plasma was transferred to a new tube. A second centrifugation step was performed for 6 min at 3,000 g, and 3 ml plasma were transferred to a new 15 ml centrifugation tube. Plasma was prepared within 2 hours after sampling and immediately stored at -20°C for up to 6 months.

Bisulfite Conversion

3 ml Silane lysis/binding buffer (viral NA, Thermo Fisher Scientific, Waltham, MA, USA) were added to 3 ml plasma and incubated for 10 min at room temperature. 65 µl Dynabeads® SILANE (Thermo Fisher Scientific) and 2.2 ml ethanol (absolute, molecular biology grade) were added, and the mixture was incubated 45 min at 20 rpm in a rotator. The reaction tube was transferred to a DynaMag™-15 magnet (Thermo Fisher Scientific), and the supernatant was discarded. The beads were washed with wash buffer I (50% [v/v] Silane lysis/binding buffer (viral NA), 50% [v/v] ethanol) and eluted with 100 µl water. A total of 150 µl ammonium bisulfite (65%, pH 5.3, TIB Chemicals AG, Mannheim, Germany) and 25 µl denaturation buffer (70 mg/ml trolox [(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid] in THFA [tetrahydrofurfuryl alcohol]) were added to the eluted DNA, and the reaction mixture was incubated for 45 min at 85°C. After conversion reaction, 15 µl Dynabeads® SILANE and 1 ml wash buffer I were added and incubated for 45 min at 1,000 rpm and 23°C in a thermomixer. The tube was transferred to a DynaMag™-2 magnet (Thermo Fisher Scientific), and the bound DNA was washed once with wash buffer I and three times with wash buffer II (15% [v/v] water and 85% [v/v] ethanol, absolute). Finally, the bisulfite converted DNA was eluted with 65 µl elution buffer (10 mM Tris-HCl, pH 8.0).