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Droplet Digital PCR Development to Quantify the DNA Methylation Levels of *SEPT9* and *SHOX2* in Plasma from Patients with Head and Neck Squamous Cell Carcinoma

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Abstract: Head and neck squamous cell carcinomas (HNSCCs) develop from mucosa of the oral cavity, pharynx, and larynx. The methylation levels of Septin 9 (*SEPT9*) and short stature homeobox 2 (*SHOX2*) genes in ccfDNA are considered *pan*-cancer biomarkers and have shown prognostic value in preliminary reports in HNSCC. Liquid biopsy is a non-invasive procedure able to collect tumor-derived molecules including circulating cell-free DNA (ccfDNA). Here, we developed a ddPCR-based assay to detect the DNA methylation levels of plasma circulating *SEPT9* and *SHOX2* in patients with HNSCC. We first set up the assay on commercial methylated and unmethylated DNA. Then, the dynamic changes of the methylation levels of *SEPT9* and *SHOX2* were quantified in 20 patients during the follow-up. The results highlighted: i) the capability of the ddPCR-based assay in detecting very low copies of methylated molecules; ii) the significant decrease in methylation levels of *SEPT9* and *SHOX2* in plasma of HNSCC patients at the first time points of follow-up respect to T_0; iii) a different trend of longitudinally DNA methylation variations associable to the clinicopathological features of the patients. The absolute quantification of the methylation levels of *SEPT9* and *SHOX2* in HNSCC may be used for risk stratification and disease monitoring.

Keywords: liquid biopsy; ddPCR; DNA methylation; cell free DNA; HNSCC

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is a cancer of the squamous epithelium of the oral cavity, larynx, pharynx and nasal cavity. HNSCC is the seventh cause of human malignancy worldwide. The major risk factors for HNSCC are smoking, alcohol abuse, and infection by the human papillomavirus (HPV). The treatments of HNSCC include surgery, radiotherapy and chemotherapy. However, the prognosis of HNSCC is poor due to recurrent or metastatic HNSCC and in this case the curative options are very limited [1–3]. It is urgent to identify biomarkers that could help to improve patient outcome. The liquid biopsy is an important tool in molecular oncology because is an excellent source of biomolecules especially the circulating cell-free DNA (ccfDNA) released into the bloodstream by cell secretion or following apoptosis and necrosis [4,5]. Less than 1% of the ccfDNA is circulating tumor DNA (ctDNA) characterized by cancer hallmarks such as mutations or aberrant gene methylation and their detection can serve as molecular indicators for diagnosis, prognosis, and the identification of early recurrence [6]. Changes in DNA methylation profile are known to arise early during cancer development and the hypermethylation of the promoter region of tumor suppressor genes is involved in cancer onset and progression [7,8]. The DNA methylation is a stable covalent modification that can be detected in bio-fluids by PCR-based

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methods [9]. The DNA hypermethylation of *SEPT9* and *SHOX2* has been previously described in tissues as well as in ccfDNA from plasma of HNSCC patients by using qPCR assay [10]. Septin 9 (*SEPT9*), which belongs to the septin family and is involved in cytokinesis and cell cycle control, has been studied in many cancers including ovarian cancer, lung cancer, and colorectal carcinoma. As a tumor suppressor gene, the higher methylated level of *SEPT9* inhibits gene expression and promotes cancer progression [11]. The hypermethylation of the short stature homeobox 2 (*SHOX2*) gene has been found in various malignancies [12,13]. Circulating *SHOX2* and *SEPT9* hypermethylation was detected also in different human cancers and they are considered promising circulating tumor liquid biopsy biomarkers [14]. Two commercial kits based on the quantification of the DNA methylation levels on selected genes in plasma ccfDNA are used in clinics for the diagnosis of the colorectal cancer named "Epi proColon" (methylation of septin 9 gene, *SEPT*9) [15] and of lung cancer named "EpiproLung" (methylation of Prostaglandin E Receptor 4 and short stature homeobox 2 genes; *PTGER4* and *SHOX2)* [16]. More recently, a methylation-based plasma kit for the analysis of multiple genes has been validated for the diagnosis of the lung cancer (lung EpiCheck) [17]. Methods used to detect DNA methylation are usually based on qPCR. It is known that the commonly used Bio-Rad ddPCR technology provides greater sensitivity and absolute quantification of the template than conventional qPCR system. The target templates are partitioned into 20,000 water-in-oil droplets made by the "generator" each of them represents a nano-sized PCR environment. The PCR-positive and PCR-negative droplets are automatically counted by the "reader" to provide absolute quantification of target DNA in digital form [18,19]. To our knowledge, epigenetic studies in liquid biopsy from HNSCC patients using ddPCR are still very limited and the *SEPT9* and *SHOX2* methylation analysis by ddPCR is lacking [20]. Due to its importance for clinical implication, we developed here a ddPCR based-assay for the absolute quantification of *SEPT9* and *SHOX2* methylation levels in ccfDNA. In details, we analyzed *SEPT9* and *SHOX2* methylation in plasma from 20 HNSCC patients before the curative treatment (T_0), as a preliminary activity of a larger project for liquid biopsy in HNSCC ("Identify" project); and we also performed a quantitative longitudinal methylation analysis during different time-points of follow up of the same patients with intervals of 3 months (T_1, T_2, T_3) to verify whether methylated *SHOX2* and *SEPT9* may be reliable biomarkers to monitor the response to the treatment.

2. Results

2.1. Establishing the efficiency of MS-ddPCR assays for the detection of SEPT9 DNA methylation

In the present study, we developed two multiplex assays for measuring the methylation levels of SEPT9 and SHOX2 using ddPCR technology, defined as methylation-specific ddPCR (MS-ddPCR). MS-ddPCR for SEPT9 consisted of: i) a TaqMan probe-based assay designed with FAM reporter to detect the methylated bisulfite-converted DNA (SEPT9-M); ii) a TaqMan probe-based assay with HEX reporter to detect the unmethylated bisulfite-converted DNA (SEPT9-U) (Figure 1A). The sensitivity and specificity of the assays were tested using commercial methylated DNA and unmethylated DNA after bisulfite conversion. The 2-dimensional (2D) amplitude plot showed that SEPT9-M set detected only the methylated template (Figure 1B, positive droplets in blue, left) and on the contrary SEPT9-U set detected only the unmethylated template (Figure 1B, positive droplets in green, right) in multiplex ddPCR experiments. Next, we evaluated the performance of the MS-ddPCR assay by considering its ability to detect the SEPT9 DNA methylation levels in samples with low amounts of DNA input as well as in the presence of unmethylated DNA background. The MS-ddPCR for SEPT9 displayed dose-dependent trend and the methylation level was detectable by using a starting input of commercial bisulfite-treated DNA as low as 78.125 pg (Figure 1C). To assess the ability of the assay to detect methylated SEPT9 molecules in unmethylated DNA background, we diluted the methylated DNA with unmethylated DNA at different percentages (99%, 90%, 70%, 50%, 30%, 10%, 1%) and multiplex MS-ddPCR was performed on 20 ng of the bisulfite-treated DNA mixtures. The concentration of methylated target (copies/µl, in blue) and the concentration of unmethylated target (copies/µl, in green) decreased and increased respectively according to the

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percentage of methylated DNA. SEPT9-M and SEPT9-U assays were able to detect up to 1% of methylated SEPT9 and unmethylated SEPT9 resulting in a concentration of 0.14 copies/µl and 1.5 copies/µl, respectively (Figure 1D). The level of methylated SEPT9 (%) was calculated as described in the materials and methods section. The standard curve demonstrated the good linearity between the level of methylated SEPT9 (expressed as percentage, %) and the percentage of commercial bisulfite-treated methylated DNA loaded in each reaction (R^2=0.92; Figure 1E).

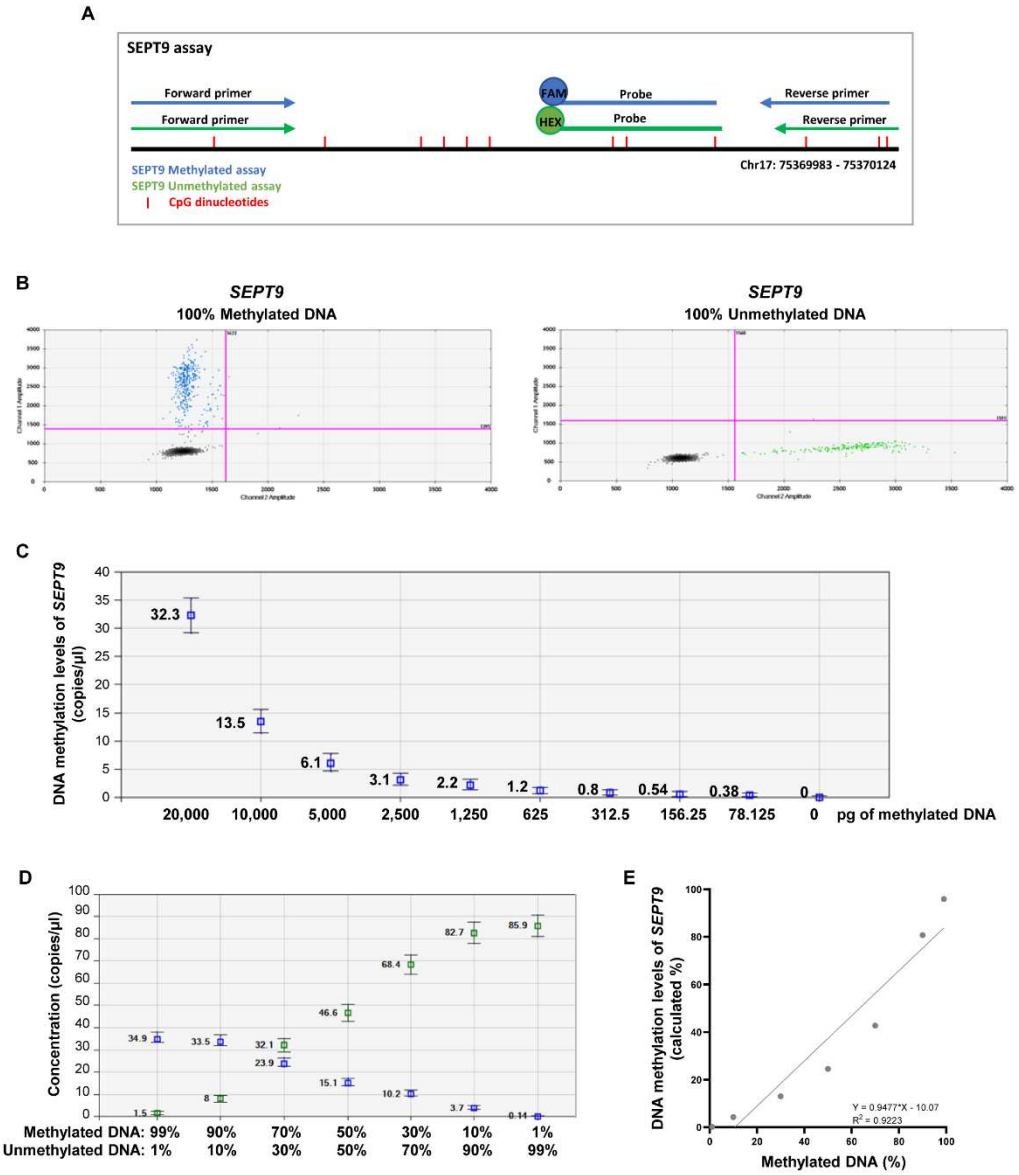

Figure 1. Efficiency of MS-ddPCR assays for the detection of SEPT9 DNA methylation. A) Schematic representation of the Methylation Specific-ddPCR (MS-ddPCR) assay used to detect the methylation levels of SEPT9. Multiplex ddPCR for the analysis of SEPT9 methylation was performed on bisulfite-converted DNA using the set specific for methylated DNA (in blue) and the set specific for unmethylated DNA (in green). Methylation-specific probe was designed with the fluorescence dye FAM and unmethylation-specific probe was designed with the fluorescence dye HEX. B) Example of 2D amplitude plot of the multiplex assay for SEPT9 using commercial methylated DNA (left) and unmethylated DNA (right) converted with bisulfite. For FAM and HEX dyes, a threshold was manually set to select positive droplets. Droplets positive for methylated SEPT9 were blue (Channel 1, FAM); droplets positive for unmethylated SEPT9 were green (Channel 2, HEX), negative droplets

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were dark grey. C) Two-fold dilution series of commercial 100% methylated DNA converted with bisulfite were prepared. ddPCR was able to detect the methylated SEPT9 as low as 78 pg of methylated DNA input. D) We prepared samples containing commercial methylated DNA and unmethylated DNA in different percentages (20 ng of total input DNA for each well) to verify the ability of MS-ddPCR assay to detect methylated SEPT9 molecules in an unmethylated DNA background. Concentrations (copies/µl) were reported for the assay specific for methylated SEPT9 (in blue) and the assay specific for unmethylated SEPT9 (in green). E) Standard curve of quantification was obtained using the SEPT9 methylation level detected in function of the percentage values of fully methylated DNA loaded in each reaction. SEPT9 methylation level was calculated as percentage: Conc. (copies/µl) for FAM / (Conc. (copies/µl) for FAM + Conc. (copies/µl) for HEX).

2.2 Establishing the efficiency of MS-ddPCR assays for the detection of SHOX2 DNA methylation

MS-ddPCR for SHOX2 consisted of: i) a TaqMan probe-based assay labelled with FAM for methylated SHOX2; ii) a TaqMan probe-based assay labelled with HEX for a region without CpGs of the ACTB gene (Figure 2A). The specificity of SHOX2 assays was tested by following the procedures described for SEPT9. Briefly, only methylated DNA treated with bisulfite resulted amplified by using the SHOX2 assay (Figure 2B, positive droplets in blue). As expected, the ACTB assay amplified methylated as well as unmethylated DNA (Figure 2B, positive droplets in green). The MS-ddPCR assay for SHOX2 displayed dose-dependent trend and it was able to detect methylated SHOX2 as low as 78.125 pg of commercial bisulfite-treated DNA (Figure 2C). The concentration of ACTB (copies/µl, in green) remained quite stable, meanwhile the concentration of methylated SHOX2 (copies/µl, in blue) increased accordingly to the percentage of the methylated DNA input with good linearity (R^2=0.98; Figure 2D-E).

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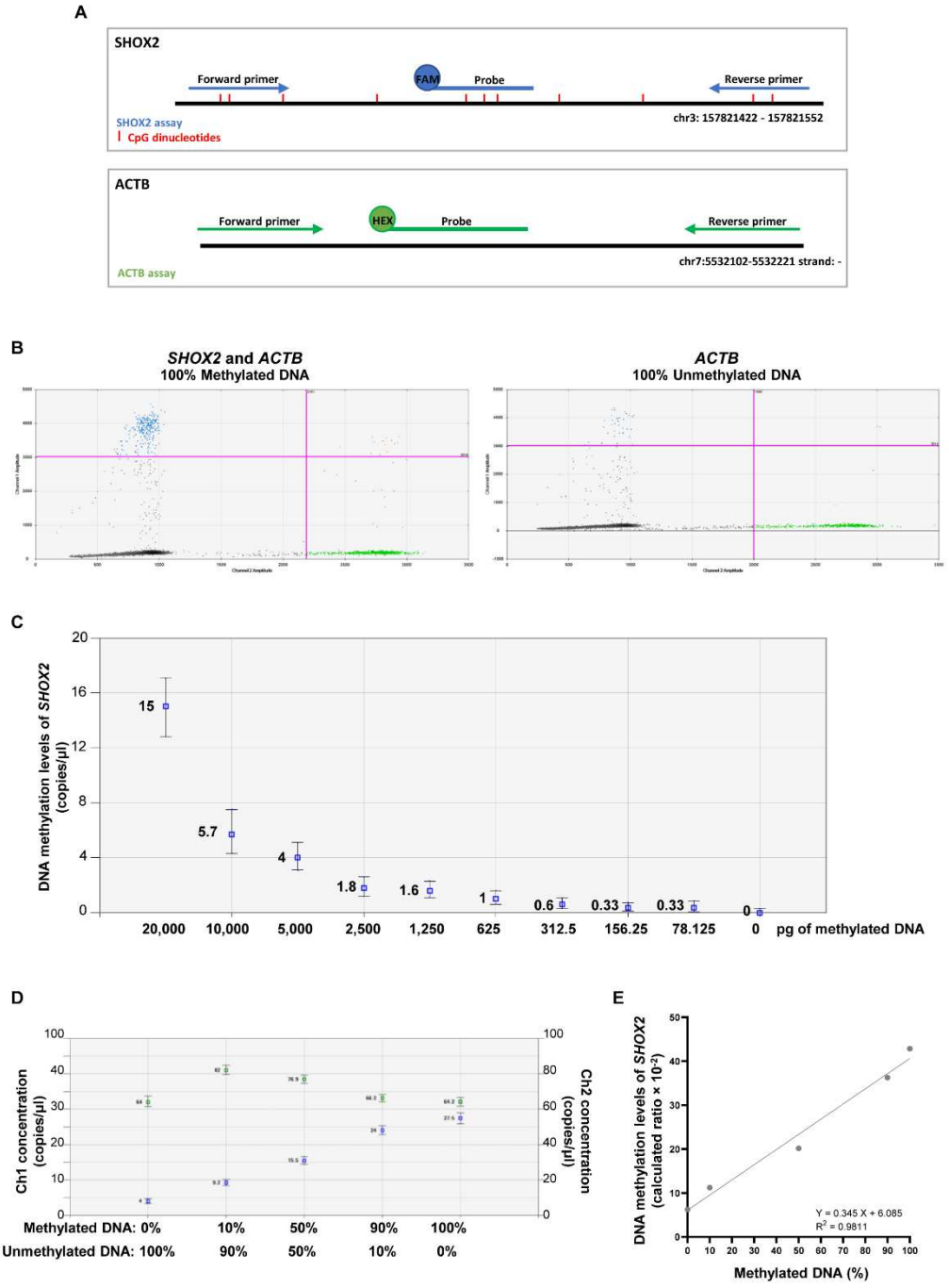

Figure 2. Efficiency of MS-ddPCR assays for the detection of SHOX2 DNA methylation. A) Schematic representation of MS-ddPCR assay used to detect the methylation levels of SHOX2. The assay was designed to detect methylated SHOX2 using methylation-specific primers and probe (in blue) and a CpG free region of the actin beta (ACTB) on bisulfite-converted DNA. The location of the primers (arrows) as well as probes and CpG dinucleotides (red vertical lines) is indicated in grey boxes. Methylation-specific probe was designed with the fluorescence dye FAM and ACTB-specific probe was designed with the fluorescence dye HEX. B) Example of 2D amplitude plot of the multiplex assay for SHOX2 using commercial methylated DNA (left) and unmethylated DNA (right) converted with bisulfite. For FAM and HEX dyes, a threshold was manually set to select positive droplets. Droplets positive for methylated SHOX2 were blue (Channel 1, FAM), droplets positive for ACTB (sequence without CpG) were green (Channel 2, HEX), and negative droplets are dark grey. C) Two-fold dilution series of commercial 100% methylated DNA converted with bisulfite were prepared. ddPCR was able to detect the methylated SHOX2 as low as 78 pg of methylated DNA input. D) We prepared

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samples containing commercial methylated DNA and unmethylated DNA in different percentages (20 ng of total input DNA for each well) to verify the ability of SHOX2 assay to detect methylated SHOX2 molecules in an unmethylated DNA background. Concentrations (copies/µl) were reported for the assay specific for methylated SHOX2 (in blue) and the assay specific for ACTB (in green). E) Standard curve of quantification was obtained using the SHOX2 methylation level detected in function of the percentage values of fully methylated DNA loaded in each reaction. SHOX2 methylation level was calculated as ratio: Conc. (copies/µl) for FAM / Conc. (copies/µl) for HEX.

2.3 Methylation levels of SEPT9 and SHOX2 in ccfDNA from plasma of HNSCC patients

By using the MS-ddPCR technology, we measured the methylation levels of SEPT9 and SHOX2 in plasma from 20 patients with HNSCC. In order to verify whether the treatment may influence the methylation levels of these genes in ccfDNA, we analyzed the SEPT9 and SHOX2 methylation levels in plasma from each patient before the treatment (T_0) and at 3-months intervals during the follow-up (T_1=3 months after treatment; T_2=6 months after treatment). The results are reported for the patients with 3 time points of follow-up (n=18; BS008 and BS014 were excluded). As reported in Figure 3A, the methylation of SEPT9 was detectable in 13 (72%) patients before the treatment (T_0). Interestingly, the mean methylation level of SEPT9 decreased during the follow-up showing a strong reduction at T_1 (fold change of 0.4 versus T_0) and a significant drop at 6 months after the treatment (T_2) ($p < 0.05$, fold change of 0.2 versus T_0). Eight (44%) patients displayed SHOX2 methylation in ccfDNA at T_0 and we obtained a significant decrease of the mean methylation levels of SHOX2 at T_1 and T_2 time-points of the follow-up (Figure 3B; $p < 0.05$, fold change of 0.09 and 0.07 respectively). Of these 8 patients, 5 showed a concomitant SEPT9 methylation in ccfDNA at T_0.

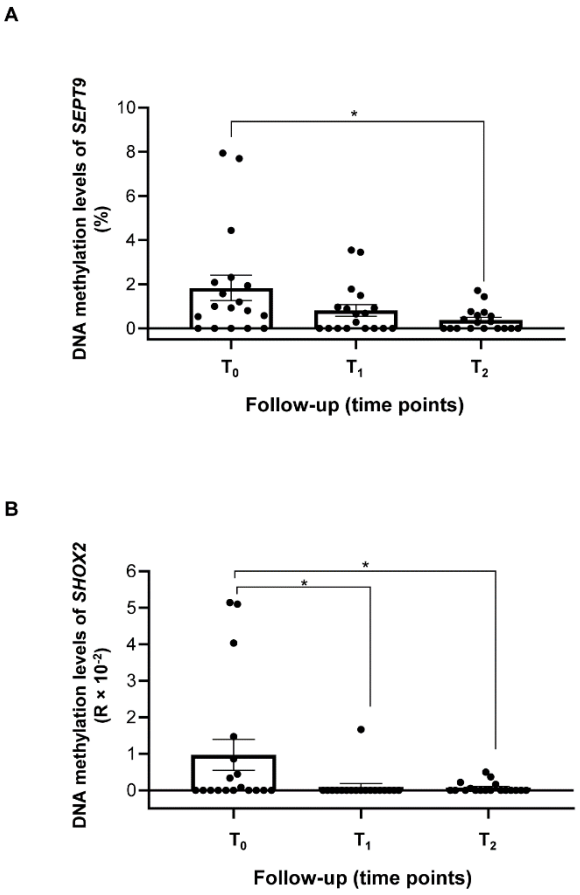

Figure 3. Methylation levels of SEPT9 and SHOX2 in ccfDNA from plasma of HNSCC patients before and after treatment. Mean methylation levels of SEPT9 (A) and SHOX2 (B) in plasma collected at the pre-treatment time-point (T_0) and at 3-months intervals (T_1=3 months and T_2=6 months after

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treatment) during the follow-up of HNSCC patients (n=18). The histograms indicate the means and bars are the SEM; one-way ANOVA, followed by Tukey's test, was used to compare the different groups, * $p < 0.05$.

2.4 Longitudinal variations of methylated SEPT9 and SHOX2 in ccfDNA from plasma of HNSCC patients

Among the 18 patients followed-up longitudinally, 10 reached the time point of 12 months after the treatment (T_3) at the time of writing the manuscript. For the SEPT9 analysis, 2 out of 18 patients were not included because of the undetectable methylation levels in all the time points. By monitoring the longitudinal methylation levels of SEPT9, we depicted four different trends of variation. As reported in Figure 4 (A-B), 6 patients showed a drop to 0% of methylated SEPT9 in plasma at the first time-point post-treatment (T_1). Among these, for 2 patients the methylation levels remained unchanged at the following available time points (Figure 4A), but in the remaining patients the methylation levels increased at T_2 (BS002, BS011, BS018) or T_3 (BS006) (Figure 4B). Five patients exhibited a decrease of SEPT9 methylation levels at T_2 (Figure 4C). On the contrary, we obtained a significant rising trend of SEPT9 methylation levels in 5 patients at the first time-point post-treatment, then at T_2, the SEPT9 methylation levels decreased ($p<0.05$; Figure 4D). In order to disclose a potential relation between the methylation levels of plasmatic SEPT9 and the treatment response, we divided all patients on the basis of the status of disease: not evidence of disease (NED), n=13 for each time points of follow-up; and patients with progressive disease (PD), n=6 patients at T_0 and n=4 at T_1 and T_2. By considering the average methylation level of SEPT9, a significant decrease was found at T_2 in the group of disease-free patients versus T_0; conversely, no variations were observed in the PD group (Figure S1 A).

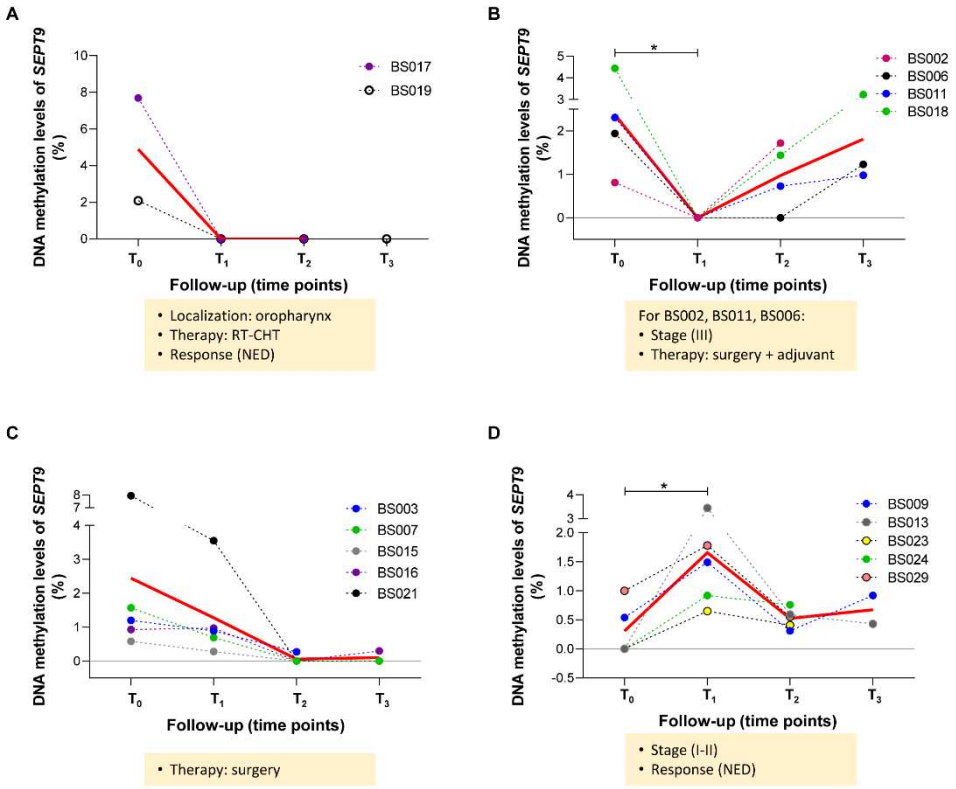

Figure 4. Longitudinal variation of SEPT9 DNA methylation levels in plasma from HNSCC patients. The graphs show the amount of methylated SEPT9 (%) detected in the plasma of HNSCC patients before (T_0) and after the treatment (T_n). Each line represents a single patient, and the dots indicate the methylation levels of SEPT9 at each blood withdrawal time point (T_1=3 months, T_2=6 months and T_3=12 months after treatment). The continuous red line represents the average trend of SEPT9 methylation for each group; the clinical characteristics shared by grouped patients are reported in the

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yellow box for each trend. Four trends were depicted. (A-B) The SEPT9 methylation drastically dropped at the first time-point post-treatment (T_1) or (C) slightly decreased and remained at low levels till the last time points of follow-up; (D) the SEPT9 methylation levels increased at T_1 and diminished at the following time-points. One-way ANOVA followed by Tukey's test was used to compare SEPT9 methylation levels among the different follow-up time points, *p<0.05.

For SHOX2 analysis, we excluded 6 patients out of 18 because of undetectable methylation levels in all the time points. In the remaining patients, we observed three different longitudinal trends during the follow-up (Figure 5). In details, 5 patients displayed a high methylation level of SHOX2 before the treatment followed by a drop at T_1 (or T_2 for BS013); the methylation levels remained undetectable at the following time points (BS015, BS017, BS018, BS024) ($p<0.05$ T_0 versus T_2; Figure 5A). Three patients showed different methylation levels of SHOX2 at T_0 followed by a drop to undetectable level at T_1 and a slightly increase at T_2 (BS011 and BS023) or T_3 (BS016) (Figure 5B). Finally, we found that the methylation levels of SHOX2 were absent at T_0 and T_1 in 4 patients, but it rose at T_2 (BS003, BS019, BS029) and at T_3 (BS006) (Figure 5C). In addition, it dropped to undetectable level in BS019 patient at 12 months of follow-up (T_3) (Figure 5C). Regarding the treatment response of HNSCC patients, no significant variations were found in SHOX2 methylation level among the different time points of follow-up in NED and PD groups (Figure S1 B).

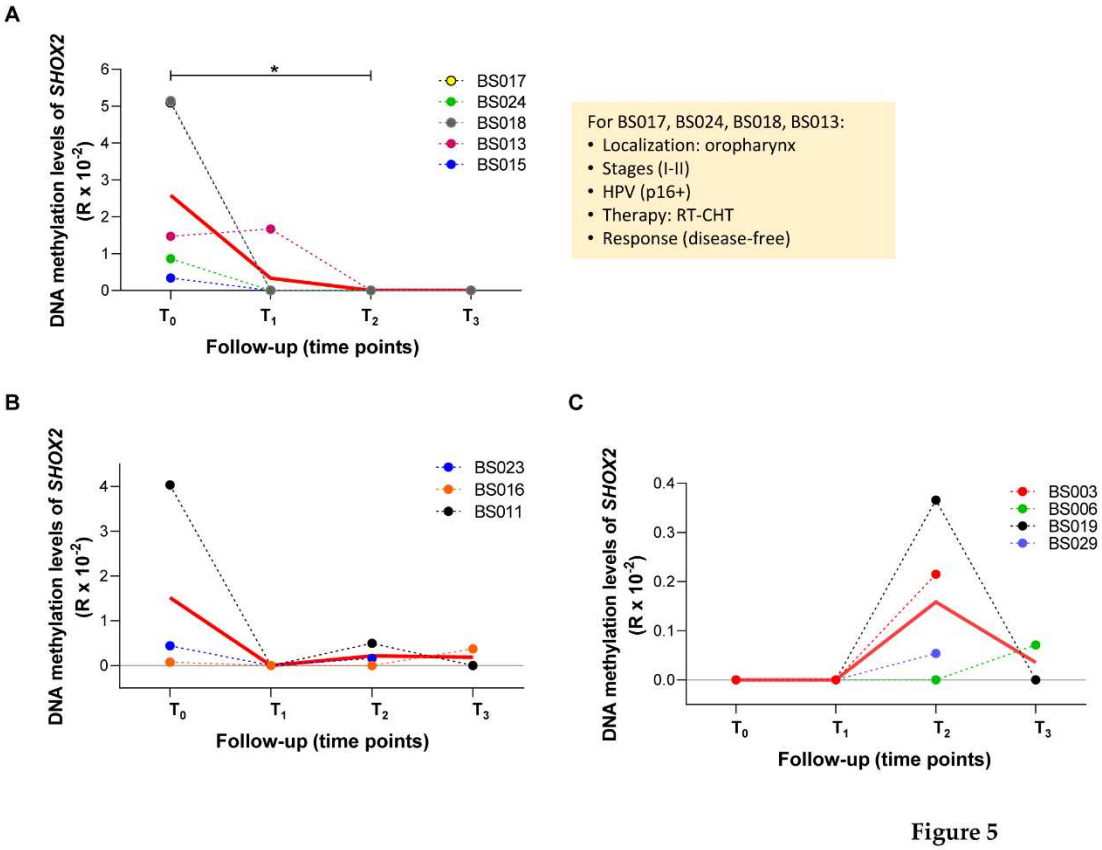

Figure 5

Figure 5. Longitudinal variation of SHOX2 DNA methylation levels in plasma from HNSCC patients. The graphs show the amount of methylated SHOX2 detected in the plasma of HNSCC patients before (T_0) and after the treatment (T_n). Each line represents a single patient, and the dots indicate the methylation levels of SHOX2 at each blood withdrawal time point (T_1=3 months, T_2=6 months and T_3=12 months after treatment). The continuous red line represents the average trend of SHOX2 methylation for each group; the clinical characteristics shared by grouped patients are reported in the yellow box for each trend. Three trends were depicted. (A) The SHOX2 methylation drastically dropped at the first or second time-points post-treatment (T_1 and T_2). (B) The SHOX2 methylation

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levels drastically dropped at T_1 and slightly increased at the following time points (T_2 or T_3). (C) The SHOX2 methylation levels were absent at T_0 and T_1, but increased at the last time points of follow-up (T_2 or T_3). One-way ANOVA followed by Tukey's test was used to compare SEPT9 methylation levels among the different follow-up time points, *$p<0.05$.

3. Discussion

The liquid biopsy, as a minimally invasive procedure, has been widely used in molecular oncology to collect circulating tumor cells (CTCs), extracellular vesicles (EVs) and circulating tumor DNA (ctDNA) released when the cells die via apoptosis, necrosis, ferroptosis, senescence or active secretion [23]. The methylation levels of *SEPT9* and *SHOX2* in circulating cell-free DNA (ccfDNA) are considered biomarkers of diagnosis, staging and prognosis for head and neck squamous cell carcinoma (HNSCC) as well as for other malignancies [10,24,25]. It has been demonstrated that the circulating levels of methylated *SEPT9* and *SHOX2* correlated with some clinico-pathological features of HNSCC patients such as tumor and nodal category, and high methylation levels were associated with a higher risk of death [10]. ccfDNA concentration varies ranging from 1-15 ng/ml plasma in healthy individuals to 100 ng/ml plasma in cancer patients. The total amount of ctDNA can also be less than 1% of total ccfDNA [26] and these low concentrations make the detection challenging. The accurate and precise quantification of the genomic alterations with prognostic and predictive values may be of great importance for clinical management. For this reason, we considered useful and innovative the development of a ddPCR based-assay with the aim to improve the detection of the circulating levels of the methylated *SEPT9* and *SHOX2* in plasma from patients with HNSCC. In this study, we combined the methylation-specific assay to the ddPCR technology (MS-ddPCR or MethyLight ddPCR) that has a high detection sensitivity and allows the absolute quantification of the target template in a very low concentration [27,28]. In general, for liquid biopsy, there are still few data on the levels of DNA methylated molecules of cancer-associated genes using ddPCR. Here, we report for the first time the use of ddPCR to quantify the plasma amount of methylated *SEPT9* and *SHOX2* in HNSCC.

By using a set of commercial fully methylated and non-methylated DNA, we were able to detect up to 78 pg of methylated DNA and we quantified up to 1% of methylated DNA in a background of non-methylated DNA. As mentioned above, this amount and the relative percentages may reflect those detected in circulation.

We then tested the ddPCR assay on a discovery cohort of 18 HNSCC patients to determine the methylation levels of *SEPT9* and *SHOX2* in plasma before the start of the therapies and during the monitoring of the treatment response at 3 different time points of follow-up. At the time of the writing this manuscript, we collected plasma samples up to 1 year (T_3) from the end of the treatment (surgical resection of the tumor, chemotherapy, radiotherapy) with intervals of 3 months. Most patients are still under surveillance, and we will proceed with methylation analysis at the available follow-up times.

We found a significant reduction of the mean methylation plasma levels of *SEPT9* and *SHOX2* in patients at T_2 (*SEPT9*) and T_1-T_2 (*SHOX2*) monitoring times, that is 3 (T_1) and 6 (T_2) months after the end of the treatment. In this context, Bergheim *et* al. demonstrated that post-therapeutic *SHOX2* and *SEPT9* methylation ccfDNA showed a trend towards decreased levels in colorectal cancer patients with localized disease and no decrease in patients with distant metastases [25]. According to Krausewitz *et* al., the high levels of *SEPT9* and *SHOX2* methylation characterized patients with metastatic disease in prostate cancer [29]. In HNSCC, the baseline positivity of *SEPT9* and *SHOX2* methylation in plasma was found in 15 patients (15/20, 75%) and the methylation levels were used to monitor the responses to treatment in 6 patients finding a decrease under systemic therapy [24]. The significant decrease of the mean methylation levels of *SEPT9* and *SHOX2* that we found in the discovery cohort may be due to the positive effects of the treatments, being all the patients except 1 (BS002) (n=17) disease-free at the monitoring time T_2. This encourages extending the analysis to a larger number of patients to confirm these findings.

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Interestingly, we observed 4 different trends of *SEPT9* methylation levels by representing the data according to the longitudinal quantification for each patient during surveillance. Among them, we detected a significant decrease of *SEPT9* methylation levels at the monitoring time T_1 in a group of 4 patients (3 out of 4 with tumor stage T_3 and all 3 underwent to surgical resection and adjuvant therapy). A significant increase of *SEPT9* methylation levels at the monitoring time T_1 compared to T_0 followed by a drastic decrease at T_2 was found in 5 patients with tumor stages T_1-T_2 and without evidence of tumor progression at least until the monitoring time T_3. For *SHOX2*, a group of 5 patients displayed a significant decrease of the methylation levels at the surveillance time T_2 compared to T_0, and 4 patients out of 5 had the same clinico-pathological features in terms of tumor localization (oropharynx), tumor stage (T1-T2), HPV p16 infection, type of treatment (chemotherapy and radiotherapy) and response (NED).

We think that the preliminary results obtained from the longitudinally absolute quantification of the methylated *SEPT9* and *SHOX2* are promising and constitute important premises to continue the surveillance and to measure the circulating methylation levels during the follow-up. At the moment, at least to our knowledge, only 3 studies evaluated the impact of both *SEPT9* and *SHOX2* in post-therapeutic surveillance and as biomarkers of prognosis and early diagnosis of tumor recurrence in HNSCC. Schrock *et* al. correlated the *SEPT9* and *SHOX2* methylation levels determined by qPCR to diagnosis, prognosis, staging and monitoring of HNSCC patients [10]. de Vos *et* al. evaluated the impact of *SEPT9* and *SHOX2* DNA methylation in the diagnosis of HNSCC and in the treatment response assessing relative and quantitative determinations by qPCR [14, 24].

We believe that the use of ddPCR to detect small amounts of circulating methylated *SEPT9* and *SHOX2* as well as the monitoring of their dynamic changes at pre-established multiple times during the clinic surveillance are novel advancements in HNSCC with translational potential in clinical routine.

In conclusion, the extensive validation of *SEPT9* and *SHOX2* as circulating methylated biomarkers able to stratify groups of HNSCC patients on the basis of homogenous clinico-pathological characteristics is very important to improve the clinical management of the patients. For this purpose, we are continuing the multicenter study to collect liquid biopsies from 8 different hospitals to investigate the methylation levels of *SEPT9* and *SHOX2* as risk and monitoring biomarkers in a large cohort of Italian patients.

4. Materials and Methods

4.1. Plasma samples from HNSCC patients.

All patients enrolled in this study (n=20) were recruited from Spedali Civili of Brescia (Italy). The clinical and pathological characteristics are reported in Table 1 for each patient. All HNSCC patients met the following criteria: i) histologically confirmed squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx or larynx; ii) clinical stage I-IV according to the VIII edition of the AJCC (American Joint Committee on Cancer) staging system; iii) age ≥ 18 years old and written informed consent. Peripheral blood samples were collected in EDTA-coated collection tubes. All recruited patients were screened for HPV virus-related disease and background pathology. The peripheral blood (10 mL/patient) of HNSCC patients was collected before the start of the first treatment (T_0) and at intervals after the first treatment, including surgery, radiotherapy and chemotherapy (T_1 = 3 months, T_2 = 6 months, T_3 = 12 months after treatment). Plasma was obtained by centrifugation of peripheral blood at 1600 rpm for 10 min at 4°C. The plasma was transferred to a new tube and stored at -80°C until DNA extraction. The study was approved by the ethical committee of Spedali Civili of Brescia (Protocol Identify, Ethical Committee approval n. NP 4551).

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Table 1. Clinical characteristics of HNSCC patients enrolled in the study.

ID patient	Tumor Site	Staging (VIII ed. AJCC)	HPV (SCC Oropharynx)	Treatment Type	Status of Disease (at last FU)	Time point of FU with blood sample collected
BS002	Larynx	III		Surgery + adj	Right neck lymph nodes recurrence + pulmonary metastasis at 6 months of FU	6 months (T_2)
BS003	Oral cavity	II		Surgery + adj	Local recurrence at 8 months of FU	6 months (T_2)
BS006	Oral cavity	III		Surgery + adj	NED (FU 18 months)	12 months (T_3)
BS007	Oral cavity	II		Surgery	NED (FU 18 months)	12 months (T_3)
BS008	Oral cavity	II	p16+, HPV DNA+	RT-CHT	Tumor persistence at T_1	Pre-treatment (T_0)
BS009	Oral cavity	II		Surgery + adj	NED (FU 18 months)	12 months (T_3)
BS010	Larynx	II		CHT neo + RT	NED (FU 12 months)	6 months (T_2)
BS011	Oral cavity	III		Surgery + adj	Second primary tumor (SCC of the right tonsil) at 15 months of FU	12 months (T_3)
BS013	Oropharynx	II	p16+	RT-CHT	NED (FU 18 months)	12 months (T_3)
BS014	Larynx	III		Surgery + adj	Progressive disease with pulmonary metastasis at 4 months of FU	Pre-treatment (T_0)
BS015	Hypopharynx	III		Surgery + adj	Pulmonary metastasis at 9 months of FU	6 months (T_2)
BS016	Larynx	II		Surgery + adj	NED (FU 18 months)	12 months (T_3)
BS017	Oropharynx	I	p16+	RT-CHT	NED (FU 15 months)	12 months (T_3)

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BS018	Oropharynx	II	p16+	RT-CHT	NED (FU 15 months)	12 months (T_3)
BS019	Oropharynx	IV	NEG	RT-CHT	NED (FU 15 months)	12 months (T_3)
BS020	Oropharynx	II	p16+	RT-CHT	NED until T_2, then lost at FU	6 months (T_2)
BS021	Oral cavity	III		Surgery + adj	NED (FU 18 months)	12 months (T_3)
BS029	Larynx	II		Surgery	NED (FU 15 months)	6 months (T_2)
BS023	Oropharynx	I	p16+, HPV DNA+	RT-CHT	NED (FU 12 months)	6 months (T_2)
BS024	Oropharynx	I	p16+	RT-CHT	NED (FU 12 months)	6 months (T_2)

HPV, Human Papilloma Virus; RT-CHT, radiotherapy-chemotherapy; FU, follow-up; NED, no evidence of disease; adj, adjuvant treatment .

4.2. ccfDNA isolation from plasma and bisulfite conversion.

Circulating cell-free DNA (ccfDNA) was isolated from 2 mL of plasma using MagMAX Cell-Free DNA isolation kit (ThermoFisher Scientific; Waltham, MA, USA) according to manufacturer's instruction. Purified ccfDNA was eluted in a 30 µL volume and 1 µL ccfDNA was used for ccfDNA quantification using Qubit Fluorometer and Qubit dsDNA HS (High Sensitivity) Assay Kit (ThermoFisher Scientific). The remaining ccfDNA (29 µl) was used for the bisulfite conversion using EZ DNA Methylation-Lightning kit (Zymo Research; Irvine, CA, USA) following the manufacturer's instruction. Five hundred nanograms of a methylated and non-methylated human DNA standard (Human Methylated & Non-methylated DNA Set, Zymo Research) were converted with bisulfite to use then as positive controls. 13 and 10 µL of bisulfite-converted DNA were obtained from ccfDNA and methylated and non-methylated DNA respectively and stored at −80°C until their use.

4.3. Methylation specific-droplet digital PCR (MS-ddPCR) assays.

The methylation specific-droplet digital PCR (MS-ddPCR) assays were optimized for the detection of the methylation levels of *SEPT9* and *SHOX2*. The MS-ddPCR experiments were performed using QX200™ ddPCR System (Bio-Rad, Hercules, CA, USA) [21,22]. The MS-ddPCR reaction mix consisted of the 2X ddPCR Supermix for Probes, and locus specific primers and probes. For *SEPT9* assay, we designed the primers and probe sequences using Beacon Designer (PREMIER Biosoft; San Francisco, CA, USA). Two sets of primers and probes were obtained: the set with primers and probe with the fluorescent FAM reporter for methylated *SEPT9* (named SEPT9-M) and the set with primers and probe with HEX reporter for unmethylated *SEPT9* (named SEPT9-U). For *SHOX2*, the assay was designed to detect the methylated *SHOX2* using a set with FAM-labelled probe (named SHOX2) and to detect a region without CpG in actin beta gene using a set with HEX-labelled probe (named ACTB). The complete list of all primer and probe sequences is provided in the Table S1. The PCR mix was prepared in a 22 µL reaction volume containing 11 µL 2 × ddPCR Supermix for Probes without dUTP (Bio-Rad), 0.55 µL 20 × PCR probe assay specific for the methylated loci (SEPT9-M or SHOX2) and 0.55 µL 20 × PCR probe assay specific for the unmethylated *SEPT9* (SEPT9-U) or *ACTB*, and bisulfite-treated DNA, as template. Each ddPCR assay mixture (20 µL) was loaded into a disposable droplet generator cartridge (Bio-Rad). Then, 70 µL of droplet generation oil for probes (Bio-Rad) were loaded into each of the eight wells for oil. The cartridge was then placed inside the

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QX200 droplet generator (Bio-Rad). When droplet generation was completed, the droplets were transferred to a 96-well PCR plate (Bio-Rad) using a multichannel pipette. The plate was heat-sealed with foil and placed in a conventional thermal cycler. Thermal cycling conditions were: 95 °C for 10 min, 40 cycles of 94 °C for 30 s and 52 °C (for *SEPT9* assay) or 57 °C (for *SHOX2* assay) for 1 min (ramping rate reduced to 2%), with a final step at 98 °C for 10 min and a 4 °C indefinite hold. QuantaSoft software (Bio-Rad) was used to check the number of total droplets as well as the droplets positive for the methylated *SEPT9* or *SHOX2* in FAM channel and for the unmethylated *SEPT9* or *ACTB* in HEX channel. *SEPT9* methylation level was calculated as percentage: Concentration (copies/µl) for SEPT9-M / (Concentration (copies/µl) for SEPT9-M + Concentration (copies/µl) for SEPT9-U). Meanwhile, *SHOX2* methylation level was calculated as ratio: Concentration (copies/µl) for *SHOX2* / Concentration (copies/µl) *ACTB*.

4.4. Establishing the efficiency of MS-ddPCR assays.

The methylated and non-methylated human DNA standards (Zymo Research) converted with bisulfite were used to verify the efficiency of MS-ddPCR assays in the detection of *SEPT9* and *SHOX2* methylation. Two-fold serial dilutions of the fully methylated DNA were prepared with water. A set of samples containing 20,000 pg, 10,000 pg, 5000 pg, 2500 pg, 1250 pg, 625 pg, 312.5 pg, 156.25 pg, 78.125 pg, and 0 pg of standard bisulfite-converted DNA were tested for *SEPT9* and *SHOX2* by MS-ddPCR assays as described above. To verify the ability of MS-ddPCR in discriminating the methylated DNA from the DNA background, 20 ng of total DNA containing the following percentages of fully methylated DNA -99%, 90%, 70%, 50%, 30%, 10%, and 1%- were tested. A negative template control (NTC) containing all the components of the reaction except for the DNA template was included in each experiment.

4.5. Detection of SEPT9 and SHOX2 methylation levels in ccfDNA of HNSCC patients by MS-ddPCR.

To evaluate the methylation levels of *SEPT9* and *SHOX2* in plasma of HNSCC patients, 6 µL of bisulfite-converted ccfDNA were used for both MS-ddPCR assays. The multiplex ddPCR assays and the relative analysis were performed as described above. Each experiment included the positive control wells for the methylated and unmethylated loci containing 4 µl (20 ng) of fully methylated DNA (Zymo Research) converted with bisulfite and 4 µl (20 ng) of completely unmethylated DNA (Zymo Research) converted with bisulfite. Negative template control (NTC) wells were also included.

4.6. Statistical analysis.

Statistical analysis was carried out using GraphPad Prism 7.0 software (San Diego, CA, USA). One-way ANOVA or two-way ANOVA followed by Tukey's test were used to compare the mean values of methylation levels for *SEPT9* and *SHOX2* in ccfDNA among the different time-points of the follow-up.

Supplementary Materials: The following supporting information can be downloaded at: Preprints.org, Table S1. Sequence of primers and probes used for MS-ddPCRs assays to detect the DNA methylation levels of *SEPT9* and *SHOX2*. Figure S1. DNA Methylation levels of *SEPT9* and *SHOX2* in plasma from HNSCC patients with different treatment response at the time points of follow-up.

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