Evaluation of DNA methylation levels of *SEPT9* and *SHOX2* in plasma of patients with head and neck squamous cell carcinoma using droplet digital PCR

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Abstract. Head and neck squamous cell carcinoma (HNSCC) is the seventh most commonly diagnosed cancer globally. HNSCC develops from the mucosa of the oral cavity, pharynx and larynx. Methylation levels of septin 9 (*SEPT9*) and short stature homeobox 2 (*SHOX2*) genes in circulating cell-free DNA (ccfDNA) are considered epigenetic biomarkers and have shown predictive value in preliminary reports in HNSCC. Liquid biopsy is a non-invasive procedure that collects tumor-derived molecules, including ccfDNA. In the present study, a droplet digital PCR (ddPCR)-based assay was developed to detect DNA methylation levels of circulating *SEPT9* and *SHOX2* in the plasma of patients with HNSCC. The assay was first set up using commercial methylated and unmethylated DNA. The dynamic changes in the methylation levels of *SEPT9* and *SHOX2* were then quantified in 20 patients with HNSCC during follow-up. The results highlighted: i) The ability of the ddPCR-based assay to detect very low copies of methylated molecules; ii) the significant decrease in *SEPT9* and *SHOX2* methylation levels in the plasma of patients with HNSCC at the first time points of follow-up with respect to T_0; iii) a different trend of longitudinally DNA methylation variations in small groups of stratified patients. The absolute and precise quantification of *SEPT9* and *SHOX2* methylation levels in HNSCC may be useful for studies with translational potential.

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Introduction

Head and neck squamous cell carcinoma (HNSCC) is a cancer of the squamous epithelium of the oral cavity, larynx, pharynx and nasal cavity (1). HNSCC is the seventh leading cause of human malignancy, accounting for 890,000 new diagnoses and 450,000 cancer related-deaths per year worldwide. According to GLOBOCAN 2020, the global incidence of HNSCC has been increasing in recent years, and this trend is partially attributed to the growing prevalence of human papillomavirus (HPV)-related oropharyngeal carcinoma (2,3). In Italy, HNSCC accounts for ~3% of all malignancies, most observed in the male population (3). The major risk factors for HNSCC are smoking, alcohol abuse, and HPV (1). Treatments of HNSCC include surgery, radiotherapy and chemotherapy (4). However, the prognosis of HNSCC is inauspicious due to recurrent or metastatic HNSCC; in this case, curative options are very limited (1,2,4). Liquid biopsy is an important tool in molecular oncology as it is an excellent source of biomolecules, particularly circulating cell-free DNA (ccfDNA) released into the bloodstream from cell secretion or as a result of apoptosis and necrosis (5,6). In total, <1% of the ccfDNA is circulating tumor DNA (ctDNA) characterized by cancer hallmarks, such as mutations or aberrant gene methylation; their detection can serve as molecular indicators for diagnosis, prognosis, and identification of early recurrence (7,8). Alterations in the DNA methylation profile are known to occur early during cancer development, and hypermethylation of the promoter region of tumor suppressor genes is involved in cancer onset and progression (9,10). DNA methylation is a stable covalent modification that mainly occurs at the 5C position of cytosine in CpG dinucleotides to form the 5-methylcytosine and can be detected in bio-fluids by PCR-based methods (11). DNA hypermethylation of septin 9 (*SEPT9*) and short stature homeobox 2 (*SHOX2)* has been previously described in tissues and in ccfDNA from plasma of patients with HNSCC using qPCR assay (12). *SEPT9* belongs to the septin family and

is involved in cytokinesis and cell cycle control (13). DNA methylation of *SEPT9* has been found in different tumors and its increased methylation levels have been detected during the progression of cells to malignancy (14-17). In colon mucosa, *SEPT9* methylation levels were found gradually increasing in tissues from: Controls-not-advanced adenomas-advanced adenomas-invasive adenocarcinoma (14,15). Accordingly, a significant reduction of SEPT9 protein levels was identified in adenoma and tumor tissues (15). Similarly, *SEPT9* hypermethylation was observed in breast cancer (BC) tissues, but not in healthy breast tissues, and was inversely correlated with *SEPT9* mRNA expression in BC cell lines and tissues (16). In BC cells, DNA hypermethylation was revealed to inhibit the expression of *SEPT9,* which, in turn, altered the formation of structured filaments and increased the migratory potential of tumor cells by promoting cancer progression (13,18,19). Hypermethylation of *SHOX2* gene has been identified in several malignancies (20). The *SHOX2* gene is a member of the *SHOX* gene family and encodes for a protein containing a 60-amino acid DNA-binding domain, suggesting its role as a transcriptional regulator. The exact molecular mechanism of *SHOX2* or the role of *SHOX2* hypermethylation during carcinogenesis has not been determined (21). However, numerous studies have clearly evidenced the strong association between *SHOX2* hypermethylation and cancer progression (14,22,23). In colon mucosa, *SHOX2* methylation levels gradually increased during the progression from the non-cancerous stage to the adenoma and adenocarcinoma stages (14). Similarly, *SHOX2* methylation was found to be absent or low in non-malignant brain tissues and pilocytic astrocytomas, at intermediate values in lower-grade gliomas, and high in glioblastomas (23). In lung adenocarcinoma, *SHOX2* methylation levels gradually increased in accordance with disease stage (from stage 0-II) and cancer invasiveness (22). Hypermethylation of circulating *SHOX2* and *SEPT9* has been detected in several human cancers, and they are considered promising circulating tumor liquid biopsy biomarkers (24). Methods used to detect DNA methylation are usually based on qPCR. The commonly used droplet digital PCR (ddPCR) technology provides greater sensitivity and absolute quantification of the template than conventional qPCR systems (25-28). The target templates are partitioned into 20,000 water-in-oil droplets produced by a 'generator', each representing a nano-sized PCR environment (29). The PCR-positive and PCR-negative droplets are automatically counted by a 'reader' to provide absolute quantification of the target DNA in digital form (30,31). To the best of the authors' knowledge, epigenetic studies in liquid biopsies of patients with HNSCC using ddPCR are still very limited and the combined *SEPT9* and *SHOX2* methylation analysis by ddPCR is lacking (32). In the present study, a ddPCR-based-assay was developed for absolute quantification of *SEPT9* and *SHOX2* methylation levels in ccfDNA. The ability of ddPCR-based assays to detect very low copies of methylated *SEPT9* and *SHOX2* was demonstrated. Finally, the feasibility of measuring *SEPT9* and *SHOX2* DNA methylation levels in the plasma of 20 patients with HNSCC, before curative treatment (surgery, radiotherapy, chemotherapy) and during different follow-up time points of the same patients with intervals of 3 months, was revealed. The present study is preliminary research of a larger project of liquid biopsies in HNSCC ('Identify' project) to assess whether DNA methylation levels of *SHOX2* and *SEPT9* may vary during treatment.

Materials and methods

Plasma samples from patients with HNSCC. All patients enrolled in the present study (n=20) were recruited from the Unit of Otorhinolaryngology-Head and Neck Surgery, ASST Spedali Civili, Department of Medical and Surgical Specialties, Radiological Sciences and Public Health, University of Brescia (Brescia, Italy). Clinical and pathological characteristics are reported in Table I for each patient. All patients with HNSCC 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 American Joint Committee on Cancer (AJCC) staging system (33) iii) aged ≥18 years and written informed consent provided. Peripheral blood samples were collected in EDTA-coated tubes. All recruited patients were screened for HPV-related disease by determining the HPV status genotyping by PANA RealTyper HPV kit CE/IVD (cat. no. PNAM-5001; HLB PANAGENE). The p16 protein expression was assessed using the CINtec p16 histological test (cat. no. 06695256001; Roche Diagnostics) with strong and widespread nuclear and cytoplasmic staining in at least 70% of cells used as a reference for positivity (Fig. S1). All these clinical characteristics were determined from the medical records of the patients; therefore they were not investigated as part of the present study. The screenings were assessed routinely in HNSCC clinic management. Peripheral blood (10 ml/patient) from patients with HNSCC 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). Collecting liquid biopsies from patients that received the same type of treatment would have taken much longer. Plasma was obtained by centrifugation of peripheral blood at 200 x g for 10 min at 4˚C in an accuSpin Micro21 centrifuge (Thermo Fisher Scientific, Inc.). The plasma was transferred to a new tube and stored at -80˚C until DNA extraction. The study was approved by the Ethics Committee of Spedali Civili of Brescia (Protocol Identify, Ethics Committee approval no. NP 4551).

ccfDNA isolation from plasma and bisulfite conversion. According to the manufacturer's instructions, ccfDNA was isolated from 2 ml of plasma using MagMAX Cell-Free DNA isolation kit (cat. no. A29319; ThermoFisher Scientific, Inc.). 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 (cat. no. Q32854; Thermo Fisher Scientific, Inc.). Following the manufacturer's instructions, the remaining ccfDNA (29 μl) was used for bisulfite conversion using EZ DNA Methylation-Lightning kit (cat. no. D5030; Zymo Research Corp). A total of 500 ng of a methylated and non-methylated human DNA standard (Human Methylated & Non-methylated DNA Set; cat. no. D5014; Zymo Research Corp.) were converted with bisulfite as positive controls. Subsequently, 13 and 10 μl

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

ID of patient	Tumor site	Staging (VIII ed. AJCC)	[a]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 node 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	Oropharynx	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)
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)

[a]In HNSCC, HPV/p16 status is routinely assessed only in the oropharyngeal primary subsite. HNSCC, head and neck squamous cell carcinoma; HPV, human papilloma virus; SCC, squamous cell carcinoma; FU, follow-up; adj, adjuvant treatment; RT-CHT, radiotherapy-chemotherapy; NED, no evidence of disease.

of bisulfite-converted DNA were obtained from ccfDNA and methylated and non-methylated DNA, respectively, and stored at -80˚C until their use.

Methylation-specific ddPCR (MS-ddPCR) assays. The MS-ddPCR assays were optimized according to the principles of MS-PCR (34,35) to detect the methylation levels of *SEPT9* and *SHOX2*. MS-ddPCR experiments were performed using QX200™ ddPCR System (Bio-Rad Laboraties, Inc.) (36,37). The MS-ddPCR reaction mix consisted of the 2X ddPCR Supermix for Probes, and locus-specific primers and probes. For the SEPT9 assay, the primers and probe sequences were designed using Beacon Designer (Premier Biosoft International). Two sets of primers and probes were obtained and correspond to the bisulfite-modified methylated or unmethylated sequence: 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 by Beacon Designer to detect bisulfite-modified methylated *SHOX2* using a FAM-labelled probe set (named SHOX2) and to detect, after bisulfite conversion, a CpG-free region in the actin beta (*ACTB*) gene using a HEX-labelled probe set (named ACTB) (38). The complete list of all primer and probe sequences is provided in Table SI. The PCR mix was prepared in a 22-μl reaction volume containing 11 μl 2X ddPCR Supermix for Probes (no dUTP) (cat. no. 186-3024; Bio-Rad Laboratories, Inc.), 0.55 μl 20X PCR probe assay specific for the methylated loci (SEPT9-M or *SHOX2*) and 0.55 μl 20X PCR probe assay specific for the unmethylated *SEPT9* (SEPT9-U) or *ACTB*, and bisulfite-treated DNA, as a template. Each ddPCR assay mixture (20 μl) was loaded into a disposable droplet generator cartridge (cat. no. 1864008; Bio-Rad Laboratories, Inc.). Subsequently, 70 μl of droplet generation oil for probes (cat. no. 1863005; Bio-Rad Laboratories, Inc.) was loaded into each of the eight oil wells. The cartridge was then placed inside the QX200 droplet generator (Bio-Rad Laboratories, Inc.). After droplet generation was completed, the droplets were transferred to a 96-well PCR plate (cat. no. 12001925; Bio-Rad Laboratories, Inc.) using a multichannel pipette. The plate was heat-sealed with foil and placed in a T100 Thermal Cycler (Bio-Rad Laboratories, Inc.). Thermal cycling conditions were as follows: 95˚C for 10 min, 40 cycles at 94˚C for 30 sec and 52˚C (for the SEPT9 assay) or 57˚C (for the SHOX2 assay) for 1 min (ramp rate reduced to 2%), with a final step at 98˚C for 10 min and a 4˚C indefinite hold. QuantaSoft software version 1.7.4 (Bio-Rad Laboratories, Inc.) was used to verify the number of total droplets and positive droplets for methylated *SEPT9* or *SHOX2* in the FAM channel and for the unmethylated *SEPT9* or *ACTB* in the HEX channel. The *SEPT9* methylation level was calculated as a percentage: Concentration (copies/μl) for *SEPT9*-M/(concentration (copies/μl) for SEPT9-M + concentration (copies/μl) for SEPT9-U). In addition, due to the lack of primers/probe set for unmethylated *SHOX2*, the *SHOX2* methylation level was calculated as the ratio: Concentration (copies/μl) for *SHOX2*/concentration (copies/μl) *ACTB*.

Establishing the efficiency of MS-ddPCR assays. Methylated and non-methylated human DNA standards (Zymo Research Corp.) converted with bisulfite were used to verify the efficiency of MS-ddPCR assays in detecting *SEPT9* and *SHOX2* methylation. By following the same experimental workflow used by Yu *et al* (39), two-fold serial dilutions of fully methylated DNA were prepared with water. A series of samples containing 20,000, 10,000, 5,000, 2,500, 1,250, 625, 312.5, 156.25, 78.125 and 0 pg of standard bisulfite-converted DNA was assessed for *SEPT9* and *SHOX2* by MS-ddPCR assays, as aforementioned. The range of the standard curve comprised the expected yield of DNA isolated from 1-2 ml of plasma. To verify the ability of MS-ddPCR to discriminate 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 (39). A negative template control (NTC) containing all components of the reaction except for the DNA template was included in each experiment.

MS-quantitative PCR (MS-qPCR). Commercial 100% methylated and non-methylated human DNA standards converted with bisulfite were used to verify the efficiency of MS-qPCR assays in detecting *SEPT9* and *SHOX2* methylation. Samples containing 20,000, 10,000, 5,000, 2,500, 1,250, 625, 312.5, 156.25, 78.125 and 0 pg of standard bisulfite-converted DNA were tested for *SEPT9* and *SHOX2* by MS-qPCR assays, as aforementioned. Furthermore, to verify the ability of MS-qPCR to discriminate 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. The qPCR reaction (20 μl/well) contained 10 μl of Taq-Man 2X Universal PCR Master Mix (Thermo Fisher Scientific, Inc.), 0.5 μl 20X PCR probe assay specific for the methylated loci (SEPT9-M or SHOX2), and 0.5 μl 20X PCR probe assay specific for unmethylated SEPT9 (SEPT9-U) or ACTB, and bisulfite-treated DNA, as a template. The PCR reactions were incubated at 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and 52˚C (for SEPT9 assay) or 57˚C (for SHOX2 assay) for 1 min. PCRs were performed in triplicate using the QuantStudio 3 Real-Time PCR system (Thermo Fisher Scientific, Inc.).

Detection of SEPT9 and SHOX2 methylation levels in ccfDNA of patients with HNSCC by MS-ddPCR. To assess the methylation levels of *SEPT9* and *SHOX2* in the plasma of patients with HNSCC, 6 μl of bisulfite-converted ccfDNA were used for both MS-ddPCR assays. Multiplex ddPCR assays and relative analysis were performed as aforementioned. Each experiment included the positive control wells for the methylated and unmethylated loci containing 4 μl (20 ng) of fully methylated DNA (Zymo Research Corp.) converted with bisulfite and 4 μl (20 ng) of completely unmethylated DNA (Zymo Research Corp.) converted with bisulfite. NTC wells were also included.

Statistical analysis. Statistical analysis was carried out using GraphPad Prism 7.0 software (Dotmatics). The linear regression between the calculated percentage of the DNA methylation levels of *SEPT9* and *SHOX2* and the percentage of input methylated DNA was performed to establish the efficiency of MS-ddPCR assays. The experiments were performed in triplicate. One-way ANOVA or two-way ANOVA, followed by Tukey's post hoc test, was used to compare the mean values

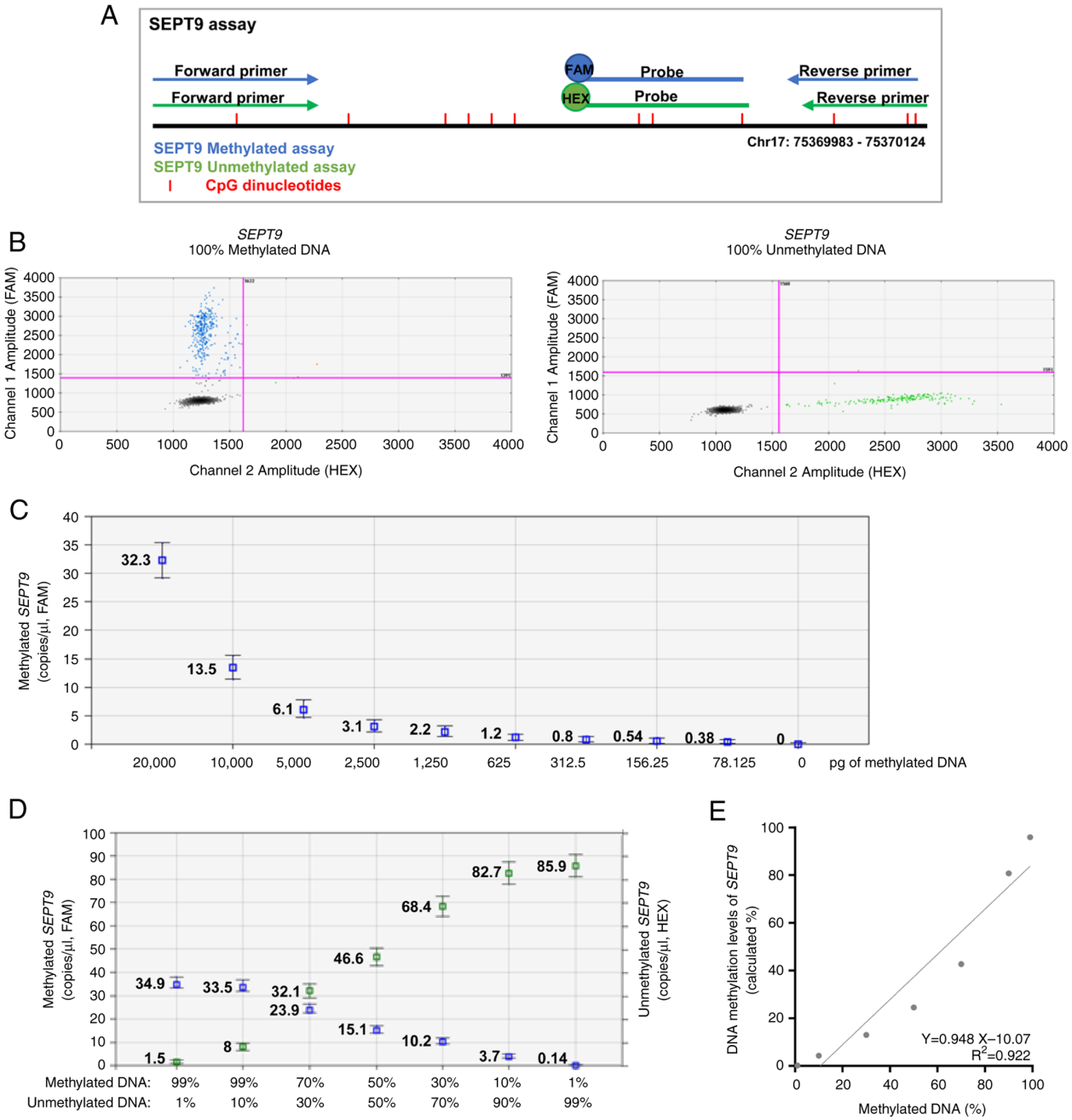

Figure 1. Efficiency of MS-ddPCR assays for the detection of *SEPT9* DNA methylation. (A) Schematic representation of the MS-ddPCR assay used to detect the methylation levels of *SEPT9*. Multiplex ddPCR for the analysis of *SEPT9* methylation was performed on bisulfite-converted DNA using the set specific for methylated DNA (in blue) and the set specific for unmethylated DNA (in green). A methylation-specific probe was designed with the FAM fluorescence dye, and an unmethylation-specific probe was designed with the HEX fluorescence dye. Vertical red lines represent the CpG dinucleotides; blue arrows and lines are the primers and probe, respectively, used for the detection of methylated *SEPT9*; green arrows and lines are the primers and probe, respectively, used for the detection of unmethylated *SEPT9*; the type of fluorescence dye is indicated as FAM or HEX. (B) An example of a 2D amplitude plot of the multiplex assay for *SEPT9* using commercial methylated DNA (left) and unmethylated DNA (right) converted with bisulfite. A threshold was manually set for FAM and HEX dyes to select positive droplets. Positive droplets for methylated *SEPT9* were blue (Channel 1, FAM); positive droplets for unmethylated *SEPT9* were green (Channel 2, HEX); negative droplets were dark grey. (C) Two-fold serial dilutions of commercial 100% methylated DNA converted with bisulfite were prepared. ddPCR detected the methylated *SEPT9* as low as 78 pg of input methylated DNA. (D) Samples containing commercial methylated DNA and unmethylated DNA in different percentages (20 ng of total input DNA for each well) were prepared to verify the ability of an MS-ddPCR assay to detect methylated *SEPT9* molecules in an unmethylated DNA background. Concentrations (copies/μl) were reported for the specific assay for methylated *SEPT9* (in blue) and the specific assay for unmethylated *SEPT9* (in green). (E) A standard quantification curve was obtained using the *SEPT9* methylation level detected in the function of the percentage values of fully methylated DNA loaded in each reaction. The *SEPT9* methylation level was calculated as a percentage: Concentration (copies/μl) for FAM/[concentration (copies/μl) for FAM + concentration (copies/μl) for HEX]. *SEPT9*, septin 9; MS-ddPCR, methylation-specific droplet digital PCR; ddPCR, droplet digital PCR.

of methylation levels for *SEPT9* and *SHOX2* in ccfDNA among the different follow-up time points. The histograms are presented as the mean values ± standard error of the mean (SEM). The mean values of *SEPT9* and *SHOX2* methylation levels at each time point (T_0, T_1, T_2, T_3) were used to determine the trend, shown as the red line, for longitudinal analysis of

SEPT9 and *SHOX2* methylation levels during the follow-up of patients with HNSCC. P<0.05 was considered to indicate a statistically significant difference.

Results

Establishing the efficiency of MS-ddPCR assays for the detection of SEPT9 DNA methylation. In the present study, two multiplex assays were used for measuring the methylation levels of *SEPT9* and *SHOX2* using ddPCR technology, defined as 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) and ii) a TaqMan probe-based assay with HEX reporter to detect the unmethylated bisulfite-converted DNA (SEPT9-U) (Fig. 1A). The sensitivity and specificity of the assays were assessed using commercial methylated DNA and unmethylated DNA after bisulfite conversion. The two-dimensional (2D) amplitude plot showed that the SEPT9-M set detected only the methylated template (Fig. 1B, positive droplets in blue, left) and, by contrast, the SEPT9-U set detected only the unmethylated template (Fig. 1B, positive droplets in green, right) in multiplex ddPCR experiments. Next, the performance of the MS-ddPCR assay was evaluated by considering its ability to detect the *SEPT9* DNA methylation levels in samples with low amounts of DNA input, and in the presence of an unmethylated DNA background. MS-ddPCR for *SEPT9* displayed a dose-dependent trend, and the methylation level was detectable using a starting input of commercial bisulfite-treated DNA as low as 78.125 pg (Fig. 1C). To assess the ability of the assay to detect methylated *SEPT9* molecules in an unmethylated DNA background, the methylated DNA with unmethylated DNA was diluted at different percentages (99, 90, 70, 50, 30, 10 and 1%) and multiplex MS-ddPCR was performed on 20 ng of the bisulfite-treated DNA mixtures. The concentration of the methylated target (copies/μl, in blue) and that of the unmethylated target (copies/μl, in green) decreased and increased, respectively, according to the percentage of methylated DNA. The SEPT9-M and SEPT9-U assays detected up to 1% methylated *SEPT9* and unmethylated *SEPT9*, with a concentration of 0.14 and 1.5 copies/μl, respectively (Fig. 1D). The level of methylated *SEPT9* (expressed as percent, %) was calculated as described in the Materials and methods section. The standard curve demonstrated good linearity between the level of methylated *SEPT9* (expressed as percent, %) and the percentage of commercial bisulfite-treated methylated DNA loaded in each reaction (R^2=0.92; Fig. 1E).

To evaluate the efficiency of qPCR in detecting the DNA methylation levels of *SEPT9*, the sensitivity and specificity in the same conditions were assessed. The SEPT9-M set reached the detection limit of qPCR (cycle threshold, Ct >35) with 625 pg of input methylated DNA, and the SEPT9-U set with 1,250 pg of input unmethylated DNA (Fig. 2A). As revealed in Fig. 2B, ddPCR was able to detect positive droplets up to 78.125 pg of input DNA for both sets. Analysis of the ability of the assay to detect methylated *SEPT9* molecules in an unmethylated DNA background, revealed that qPCR detected up to 30% of methylated *SEPT9* but the threshold cycles for 10 and 1% of methylated *SEPT9* were above the cutoff (Ct >35) (Fig. 2C). These results indicated the higher sensitivity and specificity of ddPCR-based assays than qPCR.

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 labeled with FAM reporter for methylated *SHOX2* and ii) a TaqMan probe-based assay labeled with HEX reporter for a region CpG-free in the *ACTB* gene (Fig. 3A). The specificity of SHOX2 assays was tested by following the procedures described for *SEPT9*. Only methylated DNA treated with bisulfite was amplified using the SHOX2 assay (Fig. 3B, positive droplets in blue). As expected, the ACTB assay amplified methylated and unmethylated DNA (Fig. 3B, positive droplets in green). The MS-ddPCR assay for *SHOX2* displayed a dose-dependent trend and could detect methylated *SHOX2* as low as 78.125 pg of commercial bisulfite-treated DNA (Fig. 3C). The concentration of *ACTB* (copies/μl, in green) remained stable with values between 69.1 and 78.2 copies/μl; meanwhile, the concentration of methylated *SHOX2* (copies/μl, in blue) increased accordingly to the percentage of input methylated DNA with good linearity (R^2=0.98; Fig. 3D and E).

To evaluate the efficiency of qPCR in detecting DNA methylation levels of *SHOX2*, the sensitivity and specificity in the same conditions were assessed. The SHOX2 set reached the detection limit of qPCR (Ct >35) with 156.25 pg and the ACTB set with 1,250 pg of input methylated DNA (Fig. 4A). As shown in Fig. 4B, ddPCR was able to detect positive droplets up to 78.125 pg of input DNA for both sets. Analysis of the ability of the assay to detect methylated *SHOX2* molecules in unmethylated DNA background showed that qPCR detected up to 50% of methylated *SHOX2* with threshold cycles below the cut-off (Ct=35) (Fig. 4C). These results indicated the higher sensitivity and specificity of ddPCR-based assays compared with qPCR.

Methylation levels of SEPT9 and SHOX2 in ccfDNA from the plasma of patients with HNSCC. Using the MS-ddPCR technology, the methylation levels of *SEPT9* and *SHOX2* in the plasma of 20 patients with HNSCC were assessed (Table I). The *SEPT9* and *SHOX2* methylation levels in the plasma of each patient before the treatment (T_0) and at 3-month intervals during follow-up (T_1=3 months and T_2=6 months after treatment) were analyzed. Considering all the patients with 2 time points of follow-up (n=18; BS008 and BS014 developed distant metastasis or tumor persistence and thus they were excluded from the subsequent analysis), methylation of *SEPT9* was detectable in 13 (72%) patients at T_0 (Fig. 5A). The mean methylation level of *SEPT9* (m*SEPT9*) decreased during follow-up, showing a reduction at T_1 (mean m*SEPT9* T_0=1.84±2.44, mean m*SEPT9* T_1=0.81±1.12; fold change of 0.4) and a significant drop at T_2 (P<0.05; mean m*SEPT9* T_2=0.377±0.519; fold change of 0.2 vs. T_0). A total of 8 (44%) patients displayed *SHOX2* methylation (m*SHOX2*) in ccfDNA at T_0 (mean m*SHOX2* T_0=0.97±1.798), and a significant decrease in the mean methylation levels of *SHOX2* at T_1 and T_2 follow-up time points (Fig. 5B; P<0.05; mean m*SHOX2* T_1=0.093±0.39, mean m*SHOX2* T_2=0.072±0.146; fold change of 0.09 and 0.07, respectively) was obtained. Of these 8 patients, 5 exhibited a concomitant *SEPT9* methylation in ccfDNA at T_0.

Longitudinal variations of methylated SEPT9 and SHOX2 in ccfDNA from the plasma of patients with HNSCC.

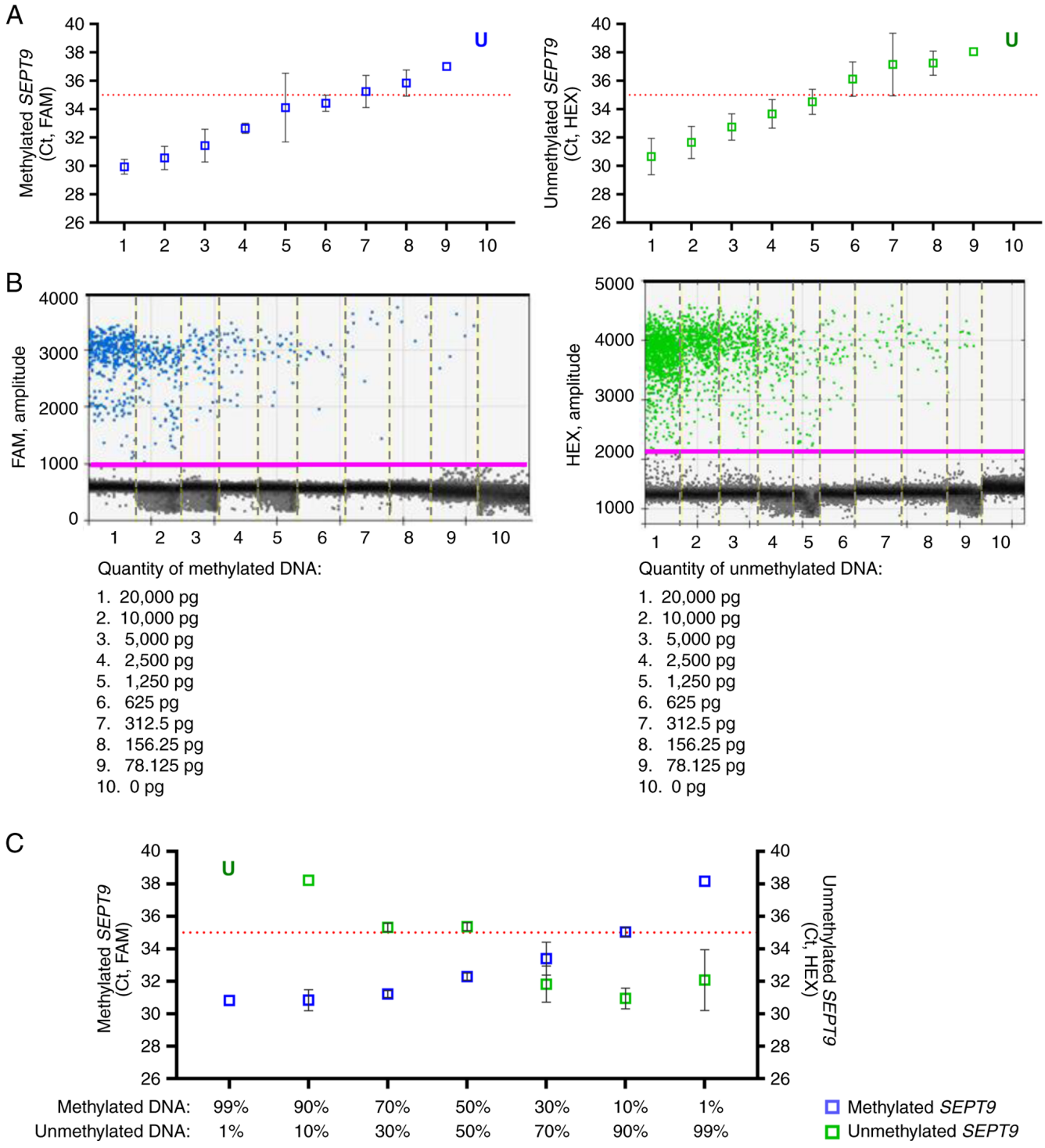

Figure 2. MS-qPCR assay for the detection of *SEPT9* DNA methylation. (A) Two-fold serial dilutions of commercial 100% methylated DNA and 100% unmethylated DNA were prepared after the bisulfite conversion. The qPCR assays detected the methylated *SEPT9* up to 625 pg of input methylated DNA (left) and the unmethylated *SEPT9* up to 1,250 pg of input unmethylated DNA (right). (B) 1-D amplitude plot of a representative droplet digital PCR experiment showed the detection of methylated *SEPT9* (left) and unmethylated *SEPT9* (right) in a two-fold serial dilution of input DNA. A threshold was manually set for FAM and HEX dyes to select positive droplets. Positive droplets for methylated *SEPT9* were blue; positive droplets for unmethylated *SEPT9* were green; negative droplets were dark grey. Both assays detected positive droplets as low as 78 pg of input DNA. (C) In mixed samples containing methylated DNA and unmethylated DNA in different percentages (20 ng of total input DNA for each well), an MS-qPCR assay detected the levels of methylated *SEPT9* up to 30% of methylated DNA. The graphs represent the mean of Ct detected in qPCR; bars are the standard deviation. The red dotted line indicates the cut-off (Ct=35) above which the samples should be excluded from the analysis. MS-qPCR, methylation-specific quantitative PCR; *SEPT9*, septin 9; Ct, cycle threshold; U, undetermined cycle.

Among the 18 patients who were followed up longitudinally, 10 reached the time point of 12 months after the treatment (T_3) at the time of the writing of the present study. For the *SEPT9* analysis, 2 out of 18 patients were not included due to undetectable methylation levels at all time points. By monitoring the longitudinal methylation levels of *SEPT9*,

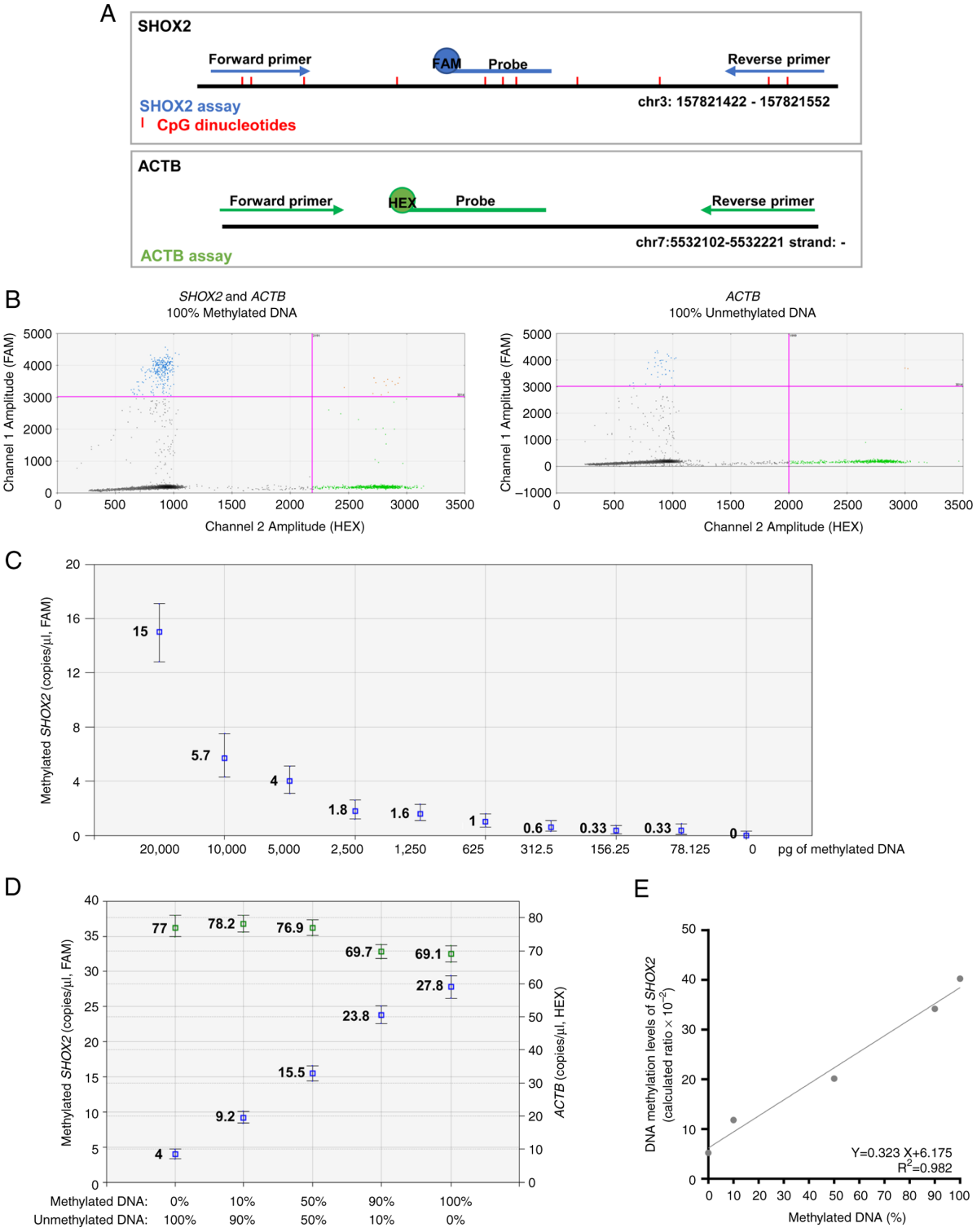

Figure 3. Efficiency of MS-ddPCR assays for the detection of *SHOX2* DNA methylation. (A) Schematic representation of an MS-ddPCR assay used to detect the methylation levels of *SHOX2*. The assay was designed to detect methylated *SHOX2* using methylation-specific primers, a probe (in blue), and a CpG-free region in the *ACTB* on bisulfite-converted DNA. A methylation-specific probe was designed with the FAM fluorescence dye, and the ACTB-specific probe was designed with the HEX fluorescence dye. The vertical red lines represent the CpG dinucleotides; the blue arrows and line are the primers and probe, respectively, used for the detection of methylated *SHOX2*; the green arrows and line are the primers and probe, respectively, used for the detection of *ACTB*; the type of fluorescence dye is indicated as FAM or HEX. (B) Example of a 2D amplitude plot of the multiplex assay for *SHOX2* using commercial methylated DNA (left) and unmethylated DNA (right) converted with bisulfite. A threshold was manually set for FAM and HEX dyes to select positive droplets. Positive droplets for methylated *SHOX2* were blue (Channel 1, FAM), positive droplets for ACTB (sequence without CpG) were green (Channel 2, HEX), and negative droplets were dark grey. (C) Two-fold serial dilutions of commercial 100% methylated DNA converted with bisulfite were prepared. ddPCR detected the methylated *SHOX2* as low as 78 pg of input methylated DNA. (D) Samples were prepared containing commercial methylated DNA and unmethylated DNA in different percentages (20 ng of total input DNA for each well) to verify the ability of the *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) A standard quantification curve was obtained using the *SHOX2* methylation level detected in the function of the percentage values of fully methylated DNA loaded in each reaction. The *SHOX2* methylation level was calculated as a ratio: Concentration (copies/μl) for FAM/concentration (copies/μl) for HEX. MS-ddPCR, methylation-specific droplet digital PCR; *SHOX2*, short stature homeobox 2; *ACTB*, actin beta.

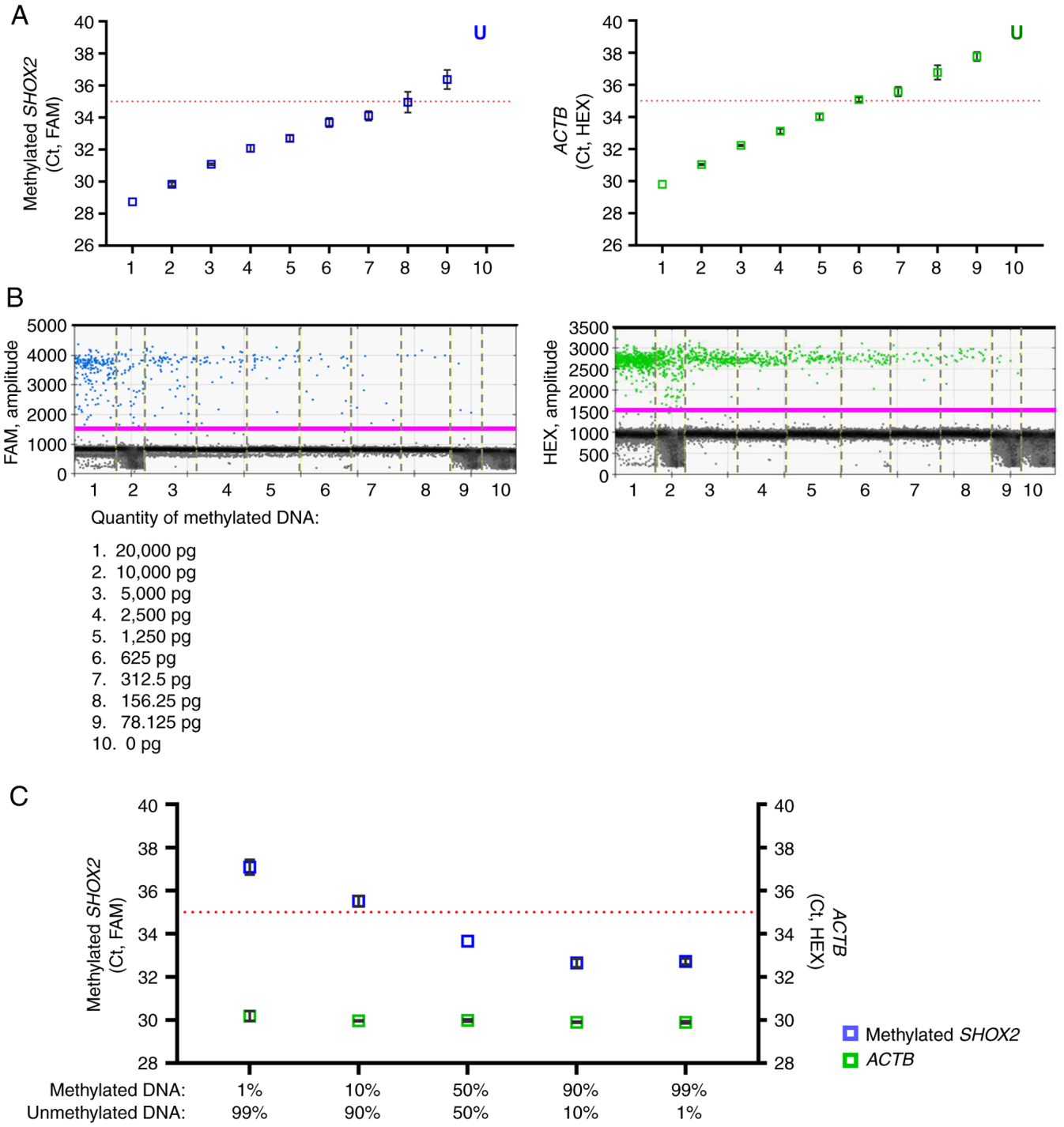

Figure 4. MS-qPCR assay for the detection of *SHOX2* DNA methylation. (A) Two-fold serial dilution of commercial 100% methylated DNA was prepared following the bisulfite conversion. The qPCR assays detected the methylated *SHOX2* up to 156.25 pg (left) and amplified *ACTB* up to 1,250 pg (right) of methylated DNA input. (B) 1-D amplitude plot of a representative droplet digital PCR experiment showed the detection of methylated *SHOX2* (left) and *ACTB* (right) in a two-fold serial dilution of input DNA. A threshold was manually set for FAM and HEX dyes to select positive droplets. Positive droplets for methylated *SHOX2* were blue; positive droplets for *ACTB* were green; negative droplets were dark grey. Both assays detected positive droplets as low as 78 pg of input DNA. (C) In mixed samples containing methylated DNA and unmethylated DNA in different percentages (20 ng of total input DNA for each well), an MS-qPCR assay detected the levels of methylated *SHOX2* up to 50% of methylated DNA. The graphs represent the mean Ct detected in qPCR; bars are the standard deviation. The red dotted line indicates the cut-off (Ct=35) above which the samples should be excluded from the analysis. MS-qPCR, methylation-specific quantitative PCR; *SHOX2*, short stature homeobox 2; *ACTB*, actin beta; Ct, cycle threshold; U, undetermined cycle.

four different groups of patients were depicted according to *SEPT9* methylation levels during follow-up. As shown in Fig. 6A, a decreasing trend was observed for the first group of patients (BS017 and BS019), with a mean of m*SEPT9* in plasma from 4.89 at T_0 to 0 at post-treatment time points (T_1 and T_2). Both of these patients presented oropharyngeal cancer, received the same type of therapy (radiotherapy and chemotherapy), and had no evidence of disease (NED) at T_2/T_3. A total of 4 patients exhibited a decrease in methylated *SEPT9* in plasma at T_1 followed by an increase in

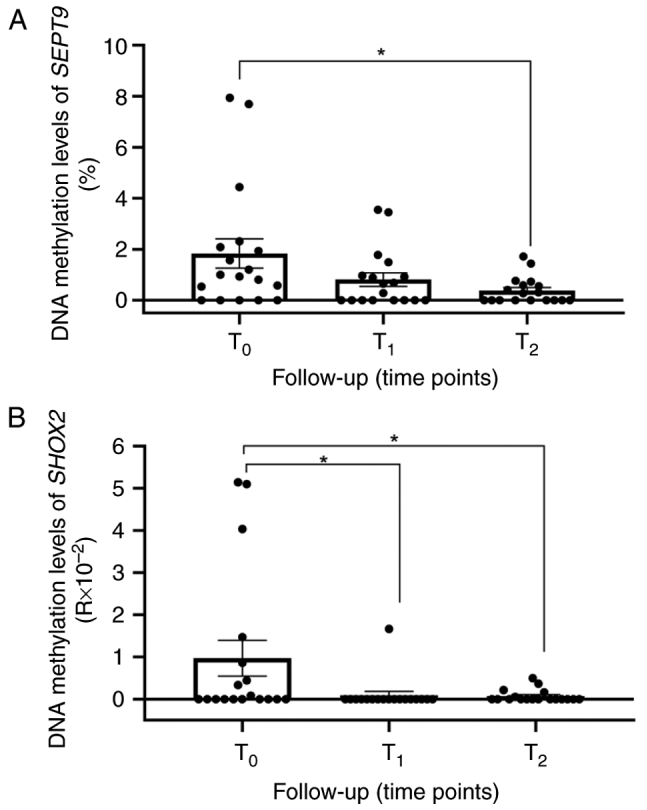

Figure 5. Methylation levels of *SEPT9* and *SHOX2* in circulating cell-free DNA from plasma of patients with HNSCC before and after treatment. Mean methylation levels of (A) *SEPT9* and (B) *SHOX2* in plasma collected at the pre-treatment time point (T_0) and at 3-month intervals (T_1=3 months and T_2=6 months after treatment) during the follow-up of patients with HNSCC (n=18). The histograms indicate the means and bars are the standard error of the mean; one-way ANOVA, followed by Tukey's post hoc test, was used to compare the different groups; *P<0.05. *SEPT9*, septin 9; *SHOX2*, short stature homeobox 2; HNSCC, head and neck squamous cell carcinoma.

methylation levels at T_2 (BS002, BS011 and BS018) or T_3 (BS006) (mean m*SEPT9* T_0=2.4, mean m*SEPT9* T_1=0, mean m*SEPT9* T_2=0.97, mean m*SEPT9* T_3=1.81; Fig. 6B). A total of 3 patients had the same cancer stage (III) and received the same therapy (surgery followed by adjuvant treatment). Furthermore, 5 patients exhibited a decreasing trend of *SEPT9* methylation levels at T_1 and T_2 (mean m*SEPT9* T_0=2.44, mean m*SEPT9* T_1=1.28, mean m*SEPT9* T_2=0.05, mean m*SEPT9* T_3=0.47; Fig. 6C). All these patients underwent surgery resection. The last group of 5 patients showed a significant increase in *SEPT9* methylation levels at T_1, followed by a decrease at T_2 (mean m*SEPT9* T_0=0.31, mean m*SEPT9* T_1=1.66; P<0.05; mean m*SEPT9* T_2=0.52, mean m*SEPT9* T_3=0.67; Fig. 6D). All these patients had stage I or II HNSCC with NED at T_2/T_3. All the patients were divided according to the disease status: NED (n=13) and patients with progressive disease (PD; n=6). In the NED group, a significant decrease in the mean m*SEPT9* was found at T_2 vs. T_0 (Fig. S2A). For *SHOX2* analysis, 6 patients out of 18 were excluded because the methylation levels were undetectable at all time points. In the remaining patients, three different longitudinal trends were observed during follow-up (Fig. 7). In the first group, 5 patients displayed a high methylation level of *SHOX2* at T_0 (mean m*SHOX2* T_0=2.58), followed by a decrease at T_1 (or T_2 for BS013) (mean m*SHOX2* T_1=0.33; T_0 vs. T_2, P<0.05; Fig. 7A). A total of 4 out of the 5 patients shared the following clinical characteristics: Tumor site (oropharynx), cancer stage (I-II), HPV infection, therapy (radiotherapy and chemotherapy), and NED. In the second group, 3 patients exhibited different methylation levels of *SHOX2* at T_0 (mean m*SHOX2* T_0=1.52) followed by a decrease to an undetectable level at T_1 and a slight increase at T_2 (BS011 and BS023) or T_3 (BS016) (mean m*SHOX2* T_2=0.22, mean m*SHOX2* T_3=0.19; Fig. 7B). In the third group of patients, it was revealed that the methylation levels of *SHOX2* were absent at T_0 and T_1 in 4 patients but they increased at T_2 (BS003, BS019 and BS029) and T_3 (BS006) (mean m*SHOX2* T_2=0.16, mean m*SHOX2* T_3=0.03; Fig. 7C). The patients in these two groups did not share any clinical characteristics. No significant variations were found in the methylation levels of *SHOX2* among the different follow-up time points in the NED and PD groups (Fig. S2B).

Discussion

The methylation levels of *SEPT9* and *SHOX2* in ccfDNA are considered biomarkers of diagnosis, staging, and prognosis for HNSCC and other malignancies (12,40,41). It has been demonstrated that circulating levels of methylated *SEPT9* and *SHOX2* are associated with some clinicopathological features of patients with HNSCC, such as tumor and nodal category, and high methylation levels were associated with an increased risk of death (12). The concentration of ccfDNA ranges from 1 to 15 ng/ml plasma in healthy individuals to 100 ng/ml plasma in patients with cancer (42,43). The total amount of ctDNA can also be <1% of total ccfDNA (44), and these low concentrations make detection challenging. Accurate and precise quantification of the genomic alterations with prognostic and predictive values can be of great importance for clinical management. Therefore, the set-up of a ddPCR-based assay was considered useful and innovative to improve the detection of the methylated *SEPT9* and *SHOX2* circulating levels in the plasma of patients with HNSCC. Additionally, ddPCR is a well-known end-point PCR method that allows absolute quantification of the target template without requiring standard curves. Several studies have previously reported the advantages of ddPCR, including its high sensitivity and great accuracy in assessing DNA methylation levels of low DNA input samples (27,28,39,45). However, for liquid biopsy, there is still limited data on the levels of DNA methylated molecules of cancer-associated genes using ddPCR (32,46). In the present study, the methylation-specific assay with the ddPCR technology (MS-ddPCR or MethyLight ddPCR) was combined to quantify the plasma amount of methylated *SEPT9* and *SHOX2* in HNSCC. Specifically, to detect the *SEPT9* methylation levels in a multiplex ddPCR reaction, two TaqMan probe-based assays labeled with FAM (SEPT9-M) and HEX (SEPT9-U) were designed for the amplification of the *SEPT9* sequence in bisulfite-converted methylated and unmethylated DNA, respectively. For *SHOX2*, an assay with a FAM-labeled probe against the bisulfite-converted methylated *SHOX2* sequence was designed. Due to poor efficiency of assays amplifying the unmethylated *SHOX2*, primers and a HEX-labeled probe were used against a CpG-free sequence in the *ACTB* gene to

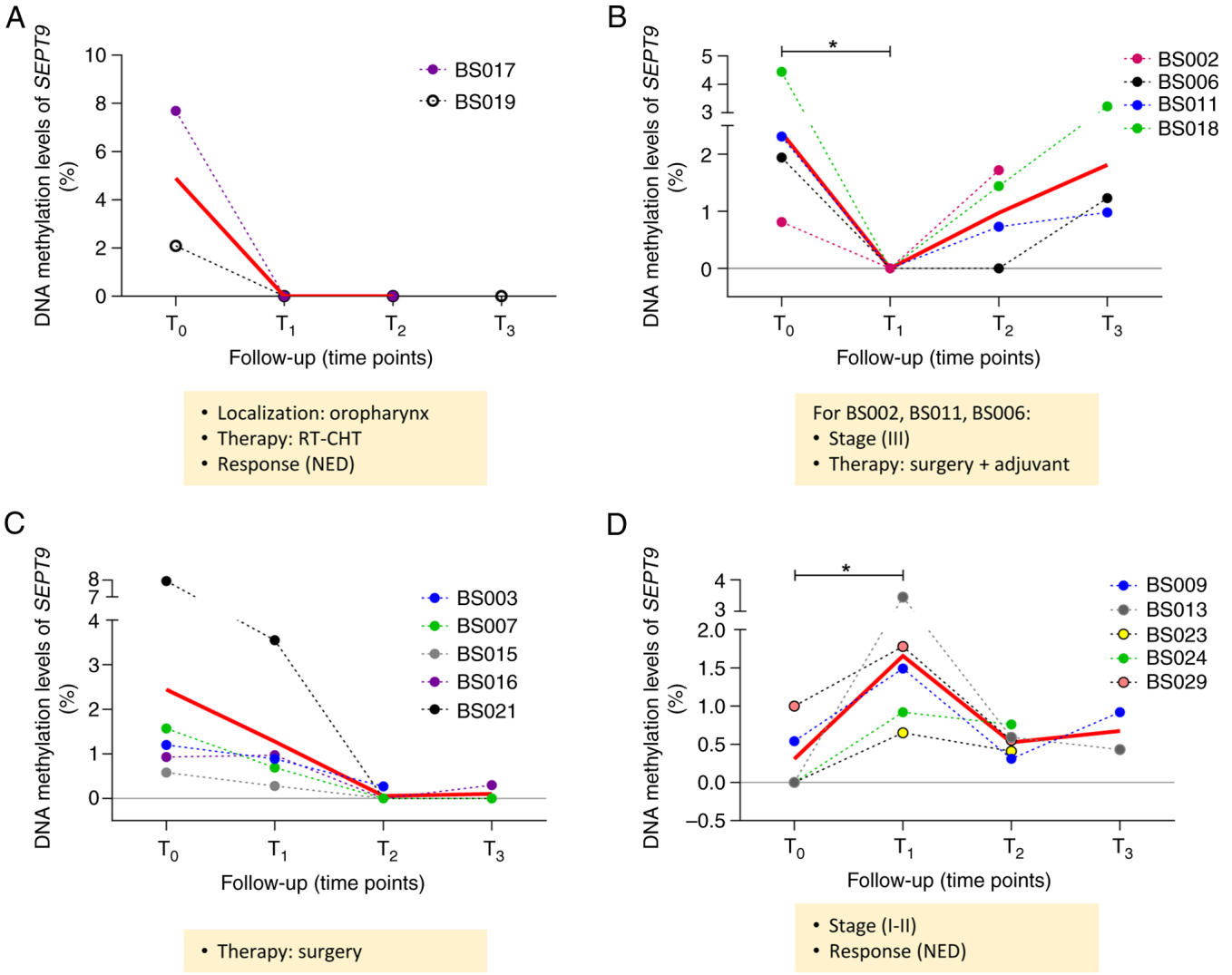

Figure 6. Longitudinal variation of *SEPT9* DNA methylation levels in plasma of patients with HNSCC. The graphs show the amount of methylated *SEPT9* (%) detected in the plasma of patients with HNSCC before (T_0) and after the treatment (T_n). Each line represents a single patient, and the dots indicate 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 mean trend of *SEPT9* methylation for each group; the clinical characteristics shared by grouped patients are reported in the yellow box for each trend; four trends were depicted. (A and B) The *SEPT9* methylation markedly decreased at the first time point post-treatment (T_1) or (C) slightly decreased and remained at low levels till the last follow-up time points. (D) The *SEPT9* methylation levels increased at T_1 and decreased at the following time points. One-way ANOVA followed by Tukey's post hoc test was used to compare *SEPT9* methylation levels among the different follow-up time points; *P<0.05. *SEPT9*, septin 9; HNSCC, head and neck squamous cell carcinoma.

normalize data (38). Using a set of commercial fully methylated and non-methylated DNA, the assays in the present study detected up to 78 pg of methylated DNA and quantified up to 1% of methylated DNA in a non-methylated DNA background. As aforementioned, this amount and relative percentages may reflect those detected in circulation. In the present study, the efficiency of the *SEPT9* and *SHOX2* methylation assays were evaluated using qPCR. The data revealed very low accuracy in detecting small amounts of methylated DNA (as low as 625-312 pg) and low percentages of methylated DNA (as low as 30-50%), making ddPCR the ideal technology for quantifying very low levels of methylated targets.

The ddPCR assay was then assessed on a discovery cohort of 18 patients with HNSCC to determine the methylation levels of *SEPT9* and *SHOX2* in plasma before the initiation of therapies and during monitoring of treatment response at three different follow-up time points. At the time of the writing of the present study, plasma samples up to 1 year (T_3) after the end of treatment (surgical resection of the tumor, chemotherapy and radiotherapy) with 3-months intervals were collected. Most patients are still being monitored, and methylation analysis will be performed at the available follow-up time points.

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, including 3 (T_1) and 6 (T_2) months after the end of treatment, were found. In this context, in a previous study, the post-therapeutic plasmatic circulating *SHOX2* and *SEPT9* methylation ccfDNA levels were decreased in patients with colorectal cancer with localized disease, while there was no decrease in patients with distant metastases (41). Furthermore, high methylation levels of circulating *SEPT9* and *SHOX2* characterized patients with metastatic disease in prostate cancer (47). In HNSCC, the baseline positivity of *SEPT9* and *SHOX2* methylation in plasma was identified in 15 patients (15/20, 75%), and

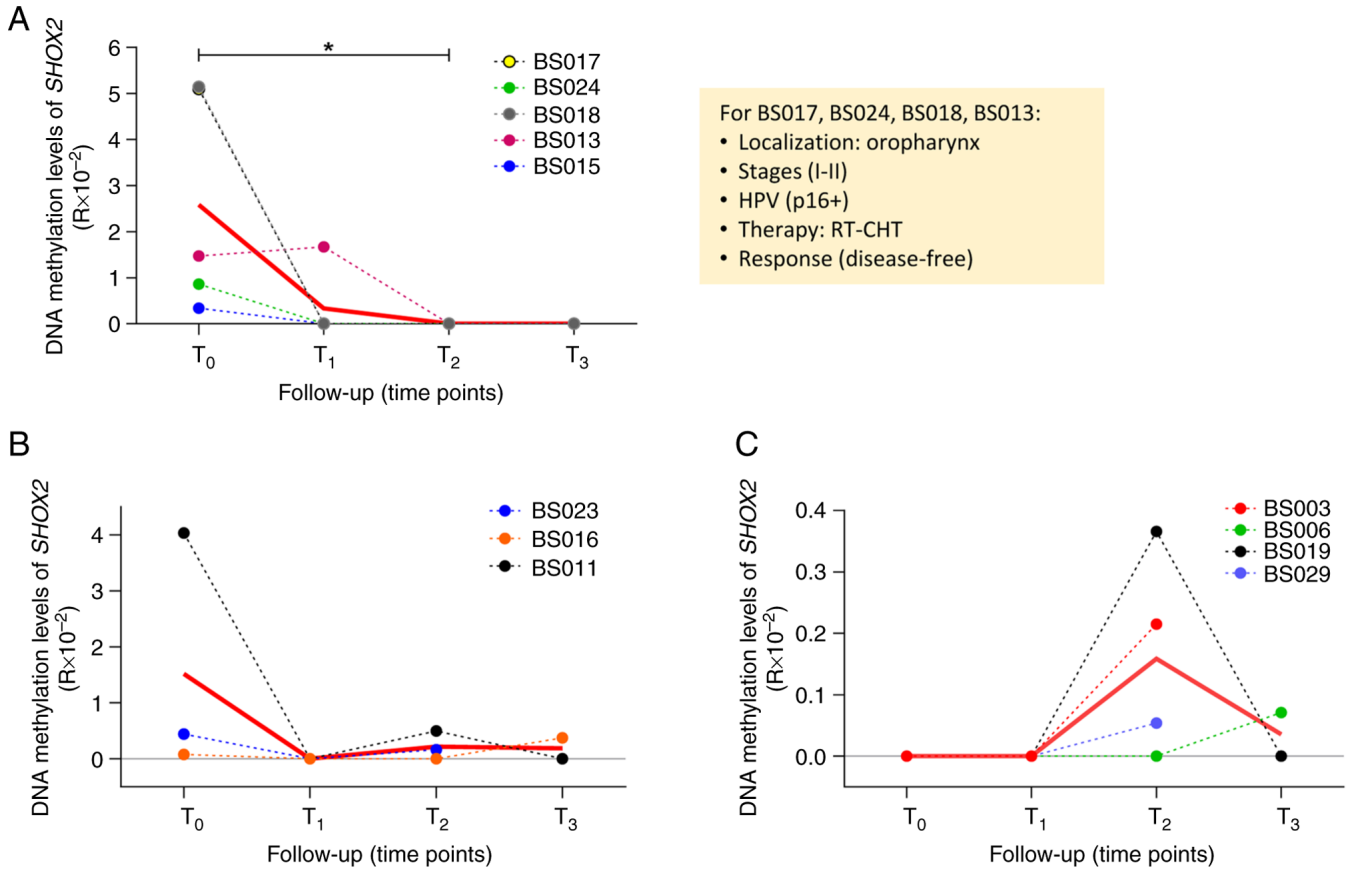

Figure 7. Longitudinal variation of *SHOX2* DNA methylation levels in plasma of patients with HNSCC. The graphs show the amount of methylated *SHOX2* detected in the plasma of patients with HNSCC before (T_0) and after the treatment (T_n). Each line represents a single patient, and the dots indicate 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 mean 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 markedly decreased at the first or second time points post-treatment (T_1 and T_2). (B) The *SHOX2* methylation levels markedly decreased 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 post hoc test was used to compare *SHOX2* methylation levels among the different follow-up time points; $^*P<0.05$. *SHOX2*, short stature homeobox 2; HNSCC, head and neck squamous cell carcinoma.

methylation levels decreased with systemic therapy (40). All the patients except one (BS002) (n=17) were disease-free at the T_2 monitoring time. In the observational cohort of the present study, different trends were observed in *SEPT9* methylation levels by representing the data according to the longitudinal quantification for each patient during monitoring (as detailed in Results and shown in Fig. 6). Among them, 5 patients with I-II tumor stages treated differently (Fig. 6D and Table I) did not display tumor progression, at least until T_3 monitoring time, and exhibited a decrease of *SEPT9* methylation at T_2. Furthermore, 3 patients with the same cancer stage (III) undergoing the same treatment (surgery followed by adjuvant treatment) displayed a significant decrease of *SEPT9* methylation at T_1 monitoring time followed by an increase at T_2 (Fig. 6B). It appears that the changes of the *SEPT9* methylation level detectable at T_2 may be relevant, but it is necessary to expand the cohort to attribute clinical significance to this observation. For *SHOX2* (as detailed in Results and shown in Fig. 7 and Table I) 5 patients displayed a significant decrease in methylation levels at the T_2 monitoring time compared with T_0. A total of 4 patients out of 5 had the same clinicopathological features in terms of tumor localization (oropharynx), tumor stage (I-II), HPV p16 infection, type of treatment (chemotherapy and radiotherapy) and they were all disease-free. Therefore, this could be promising because it is known that high circulating levels of methylated *SHOX2* are correlated with a worse prognosis in patients with HNSCC (48). The authors of the present study are aware that the results obtained in this study are derived from the analysis of circulating *SEPT9* and *SHOX2* methylation levels in small groups of patients, however promising longitudinal changes during the time points of the follow-up of the patients were revealed. In an ongoing larger study (Identify project), it will be thoroughly investigated whether these variations are potentially associated with the clinical features of the patients or response to treatments.

At present, at least to the best of the authors' knowledge, three studies have evaluated the impact of circulating *SEPT9* and *SHOX2* in post-therapeutic monitoring as epigenetic biomarkers of prognosis and early diagnosis of tumor recurrence in HNSCC. *SEPT9* and *SHOX2* methylation levels determined by qPCR were revealed to be correlated with diagnosis, prognosis, staging and monitoring of patients with HNSCC (12). The impact of *SEPT9* and *SHOX2* DNA methylation in the diagnosis of HNSCC and treatment response was evaluated by relative and quantitative determinations using qPCR (24,40).

The main limitation of the present study is related to the number of patients. It will be necessary to expand the cohort

to confidently define the translational implication of the study. In addition, it is difficult at present to associate a clinical significance to the longitudinal DNA methylation variations detected in small groups of patients. Another limitation may be the heterogeneity of the patients recruited thus far according to major clinical features (such as tumor site, type of systemic treatment, and tumor stage). There is a lack of an association between the main risk factors for HNSCC (including smoking, alcohol abuse, and HPV infection) and methylation levels of circulating *SEPT9* and *SHOX2*. Furthermore, the methylation levels of *SEPT9* and *SHOX2* in solid biopsies to compare with the corresponding circulating levels, were not analyzed.

In conclusion, a sensitive assay based on ddPCR technology was developed by the authors to detect the methylation levels of circulating *SEPT9* and *SHOX2* DNA. At least to the best of the authors' knowledge, the use of a performant ddPCR assay for these two epigenetic markers has not yet been developed. The use of ddPCR to detect small amounts of circulating methylated *SEPT9* and *SHOX2* and monitor their dynamic changes at multiple pre-established time points during clinical monitoring represents an advancement in the HNSCC field. The diagnostic accuracy of either methylated *SEPT9* and *SHOX2* has been previously demonstrated leading to their use as diagnostic biomarkers for lung cancer (Epi proLung) and colorectal cancer (Epi proColon) (49). For future clinical practice, the identification of the circulating methylation levels of *SEPT9* and *SHOX2* in patients with HNSCC in the post-treatment phase may allow for earlier diagnosis of recurrence/second primary malignancy and the definition of a personalized follow-up based on patient risk stratification. Extensive validation of *SEPT9* and *SHOX2* as circulating methylated biomarkers capable of stratifying groups of patients with HNSCC based on homogenous clinicopathological characteristics is necessary. For this purpose, the authors are continuing with a multicenter study, to collect liquid biopsies from eight different hospitals, to investigate the methylation levels of *SEPT9* and *SHOX2* in a large cohort of Italian patients.

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Availability of data and materials

The data obtained and analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

PB, GDP and AS conceptualized the study. AS and IG developed methodology. AS, IG and IAP conducted investigation. CA, LL, DS, CG, SG, AP, DM, CP and PB acquired and interpreted data. AS and IG wrote the original draft. AS, IG, IAP, GDP and PB wrote, reviewed and edited the manuscript. AS and PB supervised the study. PB, GDP and AS acquired funding. AS and IG confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript. All authors have agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Spedali Civili of Brescia (Protocol Identify; Ethics Committee approval no. NP 4551). Written informed consent was obtained from each patient.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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