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Cancer Diagnostics

Circulating Tumor Cells as a Prognostic Factor in Recurrent or Metastatic Head and Neck Squamous Cell Carcinoma: the CIRCUTEC Prospective Study

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BACKGROUND: This prospective multicenter study evaluated the prognostic value of circulating tumor cells (CTCs) in relapsing nonoperable or metastatic head and neck squamous cell carcinoma (rHNSCC) treated by chemotherapy and cetuximab.

METHODS: In 65 patients suitable for analyses, peripheral blood was taken at day 0 (D_0) D_7, and D_{21} of treatment for CTC detection by CellSearch®, EPISPOT, and flow cytometry (FCM). Progression-free survival (PFS) was assessed with the Kaplan–Meier method and compared with the log-rank test ($P < 0.05$).

RESULTS: At D_0, CTCs were detected with EPISPOT, CellSearch, and FCM in 69% (45/65), 21% (12/58), and 11% (7/61) of patients, respectively. In the patients tested with all 3 methods, EPISPOT identified 92% (36/39), 92% (35/38), and 90% (25/28) of all positive samples at D_0, D_7, and D_{21}, respectively. Median PFS time was significantly lower in (*a*) patients with increasing or stable CTC counts (36/54) from D_0 to D_7 with $EPISPOT^{EGFR}$ (3.9 vs 6.2 months; 95% CI, 5.0–6.9; $P = 0.0103$) and (*b*) patients with ≥1 CTC detected with EPISPOT or CellSearch (37/51) ($P = 0.0311$), EPISPOT or FCM (38/54) ($P = 0.0480$), and CellSearch or FCM (11/51) ($P = 0.0005$) at D_7.

CONCLUSIONS: CTCs can be detected before and during chemotherapy in patients with rHNSCC. D_0–D_7 CTC kinetics evaluated with $EPISPOT^{EGFR}$ are associated with the response to treatment. This study indicates that CTCs can be used as a real-time liquid biopsy to monitor the early response to chemotherapy in rHNSCC.

CLINICAL TRIAL INFORMATION: NCT02119559 (ClinicalTrials.gov Identifier)

Head and neck squamous cell carcinoma (HNSCC)[12] is the seventh most common cancer globally and can develop at different anatomical sites *(1)*. Uncontrolled HNSCC (i.e., metastatic or locoregional relapsing cancer) after first-line treatment is frequent and has poor prognosis *(2)*. Since 2008, chemotherapy plus cetuximab [anti-epithelial growth factor receptor (EGFR) monoclonal antibody] has significantly improved the median progression-free survival (PFS) (2.3 months) and overall survival (OS) (2.7 months) *(3)* of such patients. However, because of a lack of objective biological response criteria and biomarkers, poor responders are undergoing unnecessary treatment. Moreover, the development of alternative therapeutic strategies and inclusion of patients in controlled trials have been limited.

"Liquid biopsy" has been introduced for the analysis of circulating tumor cells (CTCs) in the blood of patients with cancer, and a considerable amount of research has focused on this new diagnostic approach over the past decade *(4)*. CTCs are derived from primary tumors and metastatic lesions and harbor important information on the tumor molecular characteristics that are relevant for

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[12] Nonstandard abbreviations: HNSCC, Head and neck squamous cell carcinoma; EGFR, epithelial growth factor receptor; PFS, progression-free survival; OS, overall survival; CTCs, circulating tumor cells; CK, cytokeratin; CS, CellSearch; rHNSCC, relapsing head and neck squamous cell carcinoma; FCM, flow cytometry; D, day; EMT, epithelial to mesenchymal transition.

the prognosis and therapy of solid cancers *(5, 6)*. CTCs may also play a diagnostic and prognostic role in HNSCC *(7–9)*.

Here, we carried out an exploratory prospective multicenter study (CIRCUTEC) in patients with relapsing HNSCC (rHNSCC) treated by chemotherapy plus cetuximab to evaluate CTCs as a prognostic factor and as an early response criterion to treatment. The main objective was to assess whether CTC detection/enumeration is a PFS or OS predictor in rHNSCC, as previously reported in breast, colon, and prostate cancer with the EPISPOT assay *(10–12)*. To this aim, we used 3 complementary technologies for CTC detection: (*a*) the EPISPOT technique, which is based on the detection of cytokeratin (CK) 19 and EGFR (in this study) released and shed, respectively, by viable CTCs *(13, 14)* combined to a negative selection (leukocyte depletion) of CTCs, (*b*) CellSearch (CS), which targets CK8, CK18, CK19, and EGFR, combined with a positive (EpCAM-based) selection of CTCs, and (*c*) flow cytometry (FCM; to study epithelial and mesenchymal markers) combined with a negative selection (leukocyte depletion).

Patients and Methods

PROTOCOL REGULATORY ISSUES

The CIRCUTEC study was carried out in centers belonging to the French Group of Oncology Radiotherapy Head & Neck (GORTEC) and the Southwest Canceropole (Canceropole GSO). The human investigations were performed after approval by a local Human Investigations Committee (Nîmes University Hospital bioethics committee, number: 2012.03.02) and in accordance with an assurance filed with and approved by the Department of Health and Human Services. The coordinators (R.G. and C.A.P.) took full responsibility for the data quality and for the analyses. According to the ethics committee's rules, all enrolled patients were fully informed and they signed a written informed consent. As a control group for the EPISPOT$^{CK19/EGFR}$ assay, 15 healthy donors were enrolled from the Etablissement Français du Sang of Montpellier, France.

PATIENT TREATMENT AND BLOOD SAMPLE COLLECTION

The prospective, observational, prognostic, open CIRCUTEC study was carried out at 8 clinical centers in France and enrolled 80 consecutive patients treated for rHNSCC (clinicaltrials.gov identifier: NCT02119559). Peripheral blood samples were collected before (day 0, D_0) and at D_7 and D_{21} after treatment initiation. At each time point, 3 blood samples were collected: 10 mL in a specific CellSave tube, 15 mL in an EDTA tube for the EPISPOT assay, and 10 mL in an EDTA tube for FCM. All blood samples were sent to the Laboratory for the Detection of Rare Human Circulating Cells, Montpellier University Medical Center, for CTC detection.

Treatment was based on the protocol described by Vermorken and colleagues *(3)*: cisplatin (100 mg/m^2) at D_1 and 5-fluorouracil (1000 mg/m^2/day) for 4 days (optional) every 3 weeks for 6 cycles at most, and cetuximab (400 mg/m^2 initial dose, then 250 mg/m^2 weekly until critical side effects or radiological-proven progression). Patients who continued cetuximab for more than 6 months after the chemotherapy endes were considered long-term responders.

CTC DETECTION USING THE CS SYSTEM

Blood samples were stored at room temperature and were processed within 72h after collection. As recommended by Menarini, 7.5 mL of blood in each CellSave tube was processed using the CellTracks Autoprep and the CS CTC Kit. Briefly, fixed CTCs were first enriched on the basis of epithelial cell-adhesion molecule (EpCAM) production, and then isolated EpCAM-positive intact cells were stained for CK and CD45 (a marker of normal hematopoietic cells). EpCAM$^+$ DAPI$^+$ CK$^+$ CD45$^-$ cells were identified as CTCs. EGFR expression was also assessed in the fourth channel of the CS system to improve CTC detection, by using an antihuman EGFR antibody (CellSearch Tumor Phenotyping Reagent EGFr RUO Ref 7900011, Menarini; final concentration of 8 μg/mL) (see Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol65/issue10).

DETECTION OF VIABLE CTCS BY THE DUAL FLUORESCENT EPISPOT$^{CK19/EGFR}$ ASSAY

Currently, the EPISPOT (EPithelial ImmunoSPOT) assay is the only functional test to detect single viable CTCs. In addition, this technology has been validated in different cohorts of patients with different tumor types *(10–12)*, allowing the detection of viable, clinically relevant CTCs. The EPISPOT assay is based on the in vitro short-term culture of CTCs (24–48 h) after an enrichment step based on a leukocyte depletion *(13–16)*. Briefly, blood samples in EDTA tubes were stored at room temperature until processing (<24 h after collection). To separate erythrocytes and leukocytes from viable CTCs (negative selection), the RosettSep™ (STEMCELL technologies) reagent (20 μL/mL) was added to 15 mL of blood, following the manufacturer's instructions. The dual fluorescent EPISPOT$^{CK19/EGFR}$ assay was developed specifically for this study to target CTCs that release or shed CK19 and/or EGFR by use of anti-CK19.1 (Progen; 6 μg/mL) and anti-EGFR (panitumumab; 1.5 μg/mL and anti-EGFR-AF488 R&D Systems 2 μg/mL) antibodies. The EPISPOT$^{CK19/EGFR}$ assay was considered positive when at least 1 marker (CK19 or EGFR) was detected. Representative images of immunospots detected

with the dual fluoro-EPISPOT$^{CK19/EGFR}$ assay are shown in Fig. 2 in the online Data Supplement.

CTC DETECTION BY FCM

FCM was performed after the same RosetteSep™ enrichment step with a 3-laser (405 nm, 488 nm, and 640 nm) cyan cytometer (Beckman Coulter), and data were analyzed with the Kaluza software (Beckman Coulter). A panel of 7 antibodies targeting EpCAM, N-Cadherin, EGFR, CD44, CD45, CD34, CD235 (glycophorin A) (see Table 1 in the online Data Supplement) was chosen to characterize different CTC subpopulations, particularly metastatic cells undergoing epithelial to mesenchymal transition (EMT) as well as LIVE/DEAD to evaluate directly cell viability. To keep CTCs viable, only membrane proteins were directly detected by adding antibodies without fixation and permeabilization.

The gating strategy (see Fig. 3 in the online Data Supplement) was optimized as follows. Doublets were excluded with a pulse geometry gate (Width/FS). Living cells were identified with the LIVE/DEAD™ fixable dead cell stain kit, according to the staining intensity. In living cells, the histogram CD235/CD45 was used to select the CD235$^-$ CD45$^\pm$, and then by applying the histogram CD34/CD45, we selected the CD45$^-$ CD34$^-$ cell population. Within the CD45$^-$ CD34$^-$ subpopulation, the CD325 (N-cadherin)/CD326 (EpCAM) histogram was used to identify epithelial (CTC^{epi}) and mesenchymal (CTC^{mes}) CTCs *(17)*. Then, EGFR expression in the CD45$^-$ CD34$^-$ population was tested using the EGFR/CD44 histogram. At the end, we obtained different phenotypes: (*a*) LIVE/DEAD$^-$ CD235$^-$ CD45$^-$; (*b*) CD34$^-$ CD325$^\pm$ CD326$^\pm$; and (*c*) CD44$^\pm$ EGFR$^\pm$.

STATISTICAL ANALYSIS

The patients' characteristics were described with medians and ranges (quantitative variables) or frequencies and proportions (categorical variables). Concordance between the 3 technologies was assessed at D_0 by an intraclass correlation coefficient. CTC presence or absence was analyzed as a binary variable for each method at each time point and by coupling the methods 2 by 2 (EPISPOT-CS, EPISPOT-FCM, and CS-FCM). A Fisher test was used to assess the possible existence of a link between the detection of CTC at D_0 and the presence or not of clinical metastases. Two groups were created according to the changes in CTCs for each method: group 1 (patients without CTCs at D_0 and D_7, or with a decrease in CTC number between D_0 and D_7) and group 2 (patients with the same number, at least 1 CTC, or an increase between D_0 and D_7).

PFS and OS were analyzed by the Kaplan–Meier method. Endpoints were death by any cause (OS), and disease progression was defined according to the RECIST 2.0 criteria or death by any cause (PFS). Survival curves were compared with the nonparametric log-rank test ($P \leq 0.05$ considered as significant). Statistical analyses were performed with SAS, version 9.4 (SAS Institute).

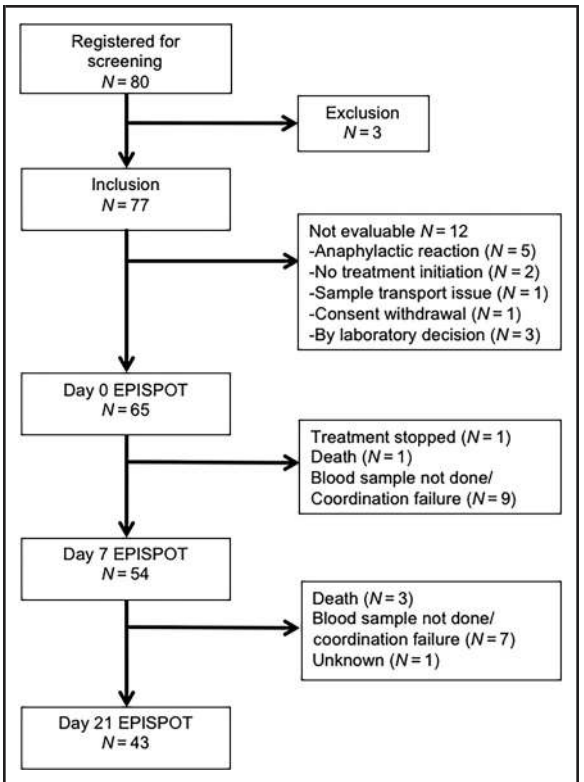

Fig. 1. Flowchart patient.

Study flowchart showing the number of included patients and the number of patients who underwent CTC analysis using the EPISPOT assay at different time points before (D_0) and during treatment (D_7 and D_{21}). The number of patients whose blood samples were analyzed with all 3 technologies (EPISPOT, CS, and FCM) at all specified time points was 54 (D_0), 42 (D_0 and D_7), and 26 (D_0, D_7, and D_{21}).

Results

POPULATION

Among the 80 patients enrolled, 65 patients were retained for analysis because 3 did not fulfill the inclusion criteria and 12 could not be evaluated for the reasons listed in Fig. 1. Eight patients were not previously treated. At inclusion, 23 patients had locoregional disease only (38%), and 37 patients had metastatic disease (62%). Data were missing for 5 patients. The median number of complete chemotherapy cycles was 3 (0–6), and the median number of cycles including cisplatin was

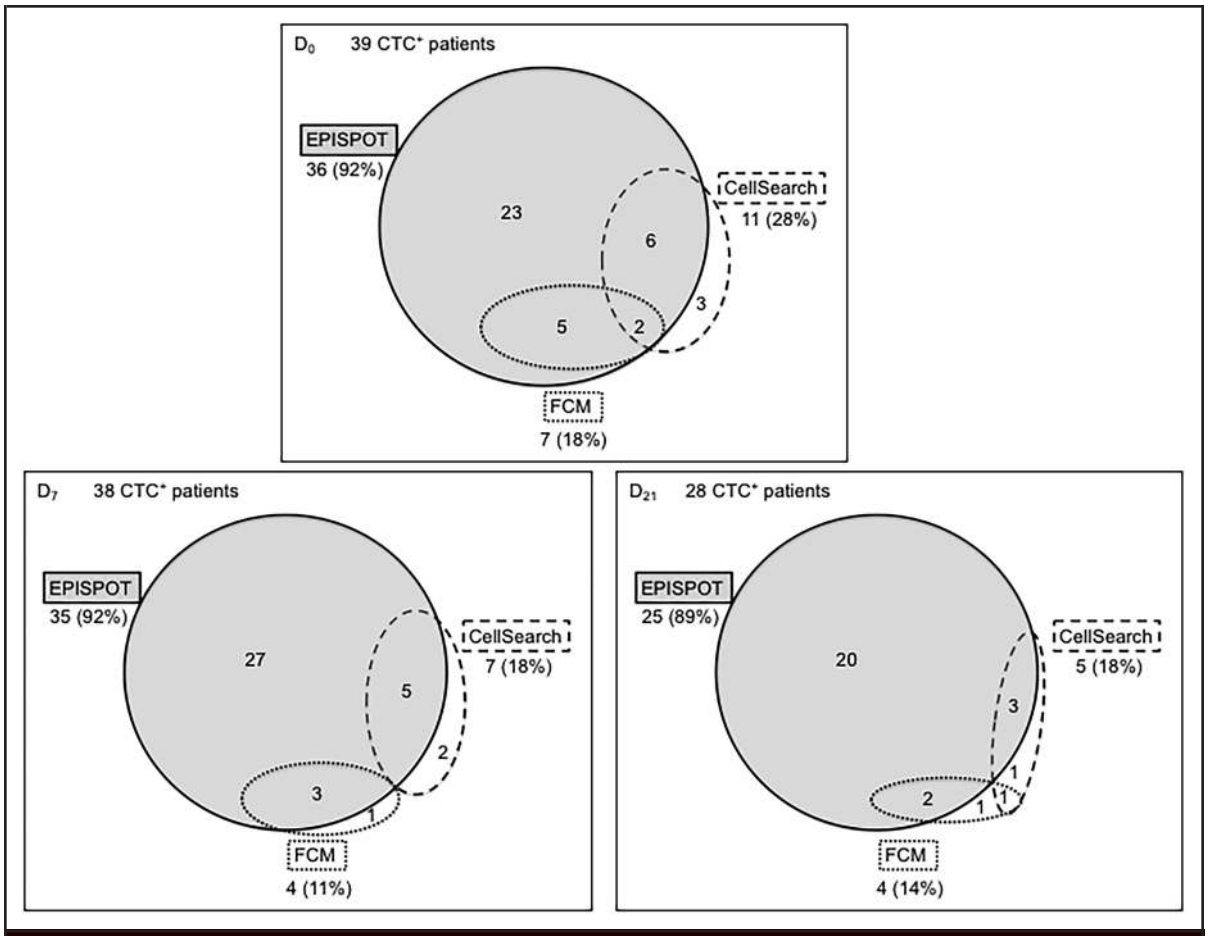

Fig. 2. Distribution of positive results for CTC detection.

Combining the 3 technologies (EPISPOT, CS, and FCM), CTC positive (CTC^+) results are reported at D_0, D_7, and D_{21}. The number at the top of each panel indicates the total number of positive results among the patients whose sample was tested with all 3 methods at D_0 (n = 54), D_7 (n = 51), and D_{21} (n = 41).

4 (1.0–6.0). The median follow-up was 9.3 months, and the median OS was 9.3 months (95% CI, 7.9–13.4) (see Table 2 in the online Data Supplement for the OS analysis in function of the different variables). Six patients were long-term responders.

CTC PREVALENCE AT THE DIFFERENT TIME POINTS

Table 3 in the online Data Supplement summarizes the results obtained with the 3 methods (EPISPOT, CS, and FCM) at the 3 time points. At D_0, CTCs were detected with EPISPOT, CS, and FCM in 69% (45/65), 21% (12/58), and 11% (7/61) of patients, respectively. Intraclass correlation coefficient was −0.05 (95% CI, −0.23 to 0.14). In patients tested with all 3 methods, the EPISPOT assay detected 92% of all positive samples (36/39) at D_0, 92% (35/38) at D_7, and 89% (25/28) at D_{21} (Fig. 2). CTC detection rate was not found associated with the presence of metastases at D_0 (Fisher test $P > 0.1$) (see Table 4 in the online Data Supplement). Concerning the control group, no positive events have been observed with the $EPISPOT^{CK19/EGFR}$ assay in the blood of healthy donors.

PFS AND CTC KINETICS BETWEEN D_0 AND D_7

At D_0, the median PFS time for the 65 patients was 5.3 months (95% CI, 4.1–6.3), with a survival probability of 0.08 (95% CI, 0.03–0.16) at 12 months and 0.03 (95% CI, 0.006–0.1) at 24 months. Body mass index, World Health Organization Performance Status score, and number of completed chemotherapy cycles were significant clinical prognostic factors of PFS (Table 1).

To assess the effect on PFS estimates of CTC changes between D_0 and D_7, based on the EPISPOT test (n = 54 patients) (Fig. 1), Kaplan–Meier analyses (fol-

Table 1. Univariate PFS analysis.[a]

Variables	Categories	*N.* patient	Median estimate	95% CI		*P*
Body mass index	≤median (21.2)	26	4.1	2.5	5.7	0.0431
	>median (21.2)	28	6.2	5.0	7.2	
WHO Performance Status score	0 and 1	44	5.9	4.7	6.4	0.0001
	2 and 3	11	2.5	1.0	3.0	
Number of cycles of chemotherapy	<6	40	3.7	2.5	4.1	<0.0001
	≥6	25	7.2	6.2	8.7	
EPISPOT$^{CK19+EGFR}$ D_0	neg[b]	60	5.5	4.1	6.4	0.0518
	pos	5	3.8	0.6	5.9	
CS-FCM D_0	CS single pos	9	2.5	1.0	5.0	0.0170
	FCM single pos	5	4.1	0.5	6.2	
	double neg	38	6.0	4.9	6.4	
	double pos	2	13.5	2.9	24.2	
CS D_7	neg	44	5.9	4.1	6.4	0.0246
	pos	7	3.6	0.6	5.9	
FCM D_7	neg	50	5.9	4.2	6.4	0.0049
	pos	4	3.0	2.1	4.1	
EPISPOT-CS-FCM D_7	neg	14	6.6	4.0	10.6	0.0160
	pos	40	5.0	3.8	6.1	
EPISPOT-CS D_7	EPISPOT single pos	30	5.7	4.1	6.4	0.0299
	CS single pos	2	2.8	0.6	5.0	
	double neg	14	6.5	3.0	10.6	
	double pos	5	3.6	1.0	6.9	
CS-FCM D_7	CS single pos	7	3.6	0.6	5.9	0.0008
	FCM single pos	4	3.0	2.1	4.1	
	double neg	40	6.1	4.9	6.5	
EPISPOT-FCM D_7	FCM single pos	1	2.1	–	–	0.0064
	double neg	16	6.3	4.0	8.9	
	double pos	3	3.8	2.1	4.1	
	EPISPOT single pos	34	5.8	4.0	6.4	
EPISPOT-CS D_7	double neg	14	6.5	3.0	10.6	0.0311
	double pos	37	5.0	4.0	6.2	
EPISPOT-FCM D_7	double neg	16	6.3	4.0	8.9	0.0480
	pos	38	5.3	3.8	6.2	
CS-FCM D_7	double neg	40	6.1	4.9	6.5	0.0005
	pos	11	3.6	1.0	5.0	
EPISPOT-FCM D_7	double pos	3	3.8	2.1	4.1	0.0329
	neg or single pos	51	5.9	4.1	6.4	
	double pos	1	2.1	–	–	
EPISPOT D_{21}	neg	18	5.934	4.033	7.180	0.1827
	pos	32	4.820	3.639	6.164	
CS D_{21}	neg	43	5.279	4.131	6.197	0.3304
	pos	5	3.803	2.066	–	
FCM D_{21}	neg	39	5.672	4.131	6.361	0.0980
	pos	5	2.918	2.066	6.361	
EPISPOT ^{EGFR} D_0-D_7	CTC = 0 or decrease	36	6.2	5.0	6.9	0.0103
	CTC > 0 or increase	18	3.9	2.5	5.7	
FCM D_0-D_7	CTC = 0 or decrease	46	5.9	4.2	6.4	0.0043
	CTC > 0 or increase	4	3.0	2.1	4.1	

[a] PFS was analyzed by the Kaplan–Meier method (median in months) with patients divided in 2 groups as a function of the results for the indicated variables (comparison with the log rank test).
[b] neg, negative; pos, positive.

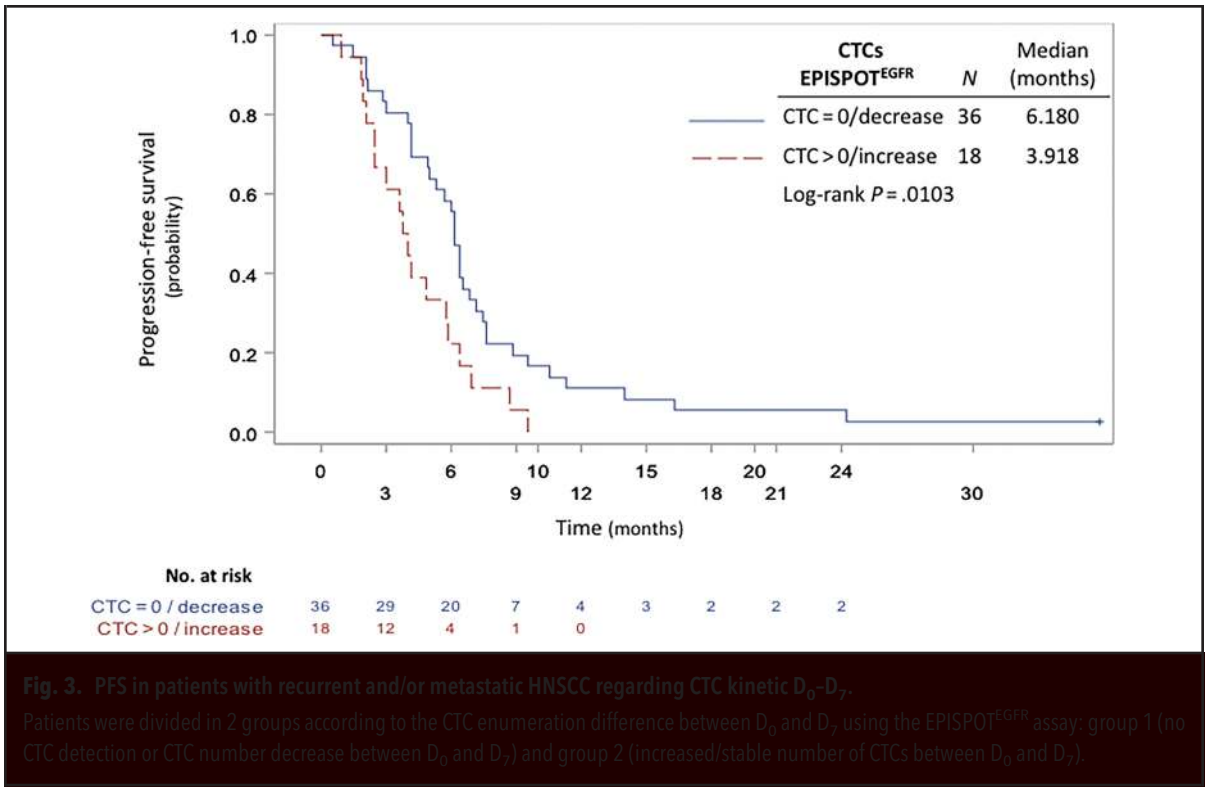

Fig. 3. PFS in patients with recurrent and/or metastatic HNSCC regarding CTC kinetic D_0–D_7.

Patients were divided in 2 groups according to the CTC enumeration difference between D_0 and D_7 using the EPISPOT^{EGFR} assay: group 1 (no CTC detection or CTC number decrease between D_0 and D_7) and group 2 (increased/stable number of CTCs between D_0 and D_7).

lowed by the log-rank test) were performed after having divided patients into group 1 (no CTC or CTC reduction between D_0 and D_7) and group 2 (increased or stable number of CTCs between D_0 and D_7). Only CTC^{EGFR+}, but not CTC^{CK19+} and $CTC^{CK19+EGFR}$ kinetics had a significant effect on survival (6.2 months for group 1, 95% CI, 5.0–6.9, vs 3.9 months for group 2, 95% CI, 2.5–5.7, $P = 0.0103$) (Fig. 3).

Similarly, among the 42 patients whose blood samples were assessed with all 3 techniques (EPISPOT, CS, and FCM) at both D_0 and D_7, median PFS was significantly higher in group 1 than in group 2 (6.1 months, 95% CI, 4.2–6.5, vs 4.1 months, 95% CI, 2.1–5.9; $P = 0.0456$) based on the EPISPOT^{EGFR} assay but not the CS method ($P = 0.080$). For FCM, although only 4 of the 50 patients tested at both D_0 and D_7 were positive for CTC, a significant difference was observed with a median PFS of 5.9 months (95% CI, 4.2–6.4) for group 1 and 3.0 months (95% CI, 2.1–4.1) for group 2 ($P = 0.0043$) (Table 1).

Finally, all the 6 long-term responders were classified in group 1 for CTC^{EGFR+}.

CTC ENUMERATION AT DIFFERENT TIME POINTS

At D_0, the CTC count obtained with any of the 3 technologies was not associated with PFS. However, in patients with $CTCs^{CK19+EGFR}$ (5/65) detected with the EPISPOT method at D_0, the median PFS was slightly shorter than in patients without CTCs (3.8 months, 95% CI, 0.6–5.9, vs 5.5 months, 95% CI, 4.1–6.4; $P = 0.0518$) (Table 1).

At D_7, CTC detection by CS (7/51) and FCM (4/54) was a significant prognostic factor ($P = 0.0246$ and $P = 0.0049$, respectively) (Table 1; see Fig. 4, A–B, in the online Data Supplement), but not with the EPISPOT assay (any marker) ($P = 0.0849$). CTC detection was a significant prognostic factor also when combining the EPISPOT assay (any marker) with CS or FCM ($P = 0.0311$ and $P = 0.0480$, respectively) (Fig. 4, A–B) and when combining CS with FCM ($P = 0.0005$) (Fig. 4C).

CTC enumeration was not significantly associated to PFS at D_{21} (EPISPOT, $P = 0.1827$; CS, $P = 0.3304$; FCM, $P = 0.0980$).

Discussion

Previous studies have demonstrated that CTC enumeration could be a prognostic factor in metastatic breast, prostate, and colorectal cancer *(18–20)*. For HNSCC only a few studies have been reported for CTC detection using the CS system *(21–24)*, and only 1 focused on EGFR production on single CTCs *(23)*. A recent meta-analysis showed that CTC detection in HNSCC, with extremely specific but low-sensitivity assays, also predicts a worse PFS *(8)*. To determine

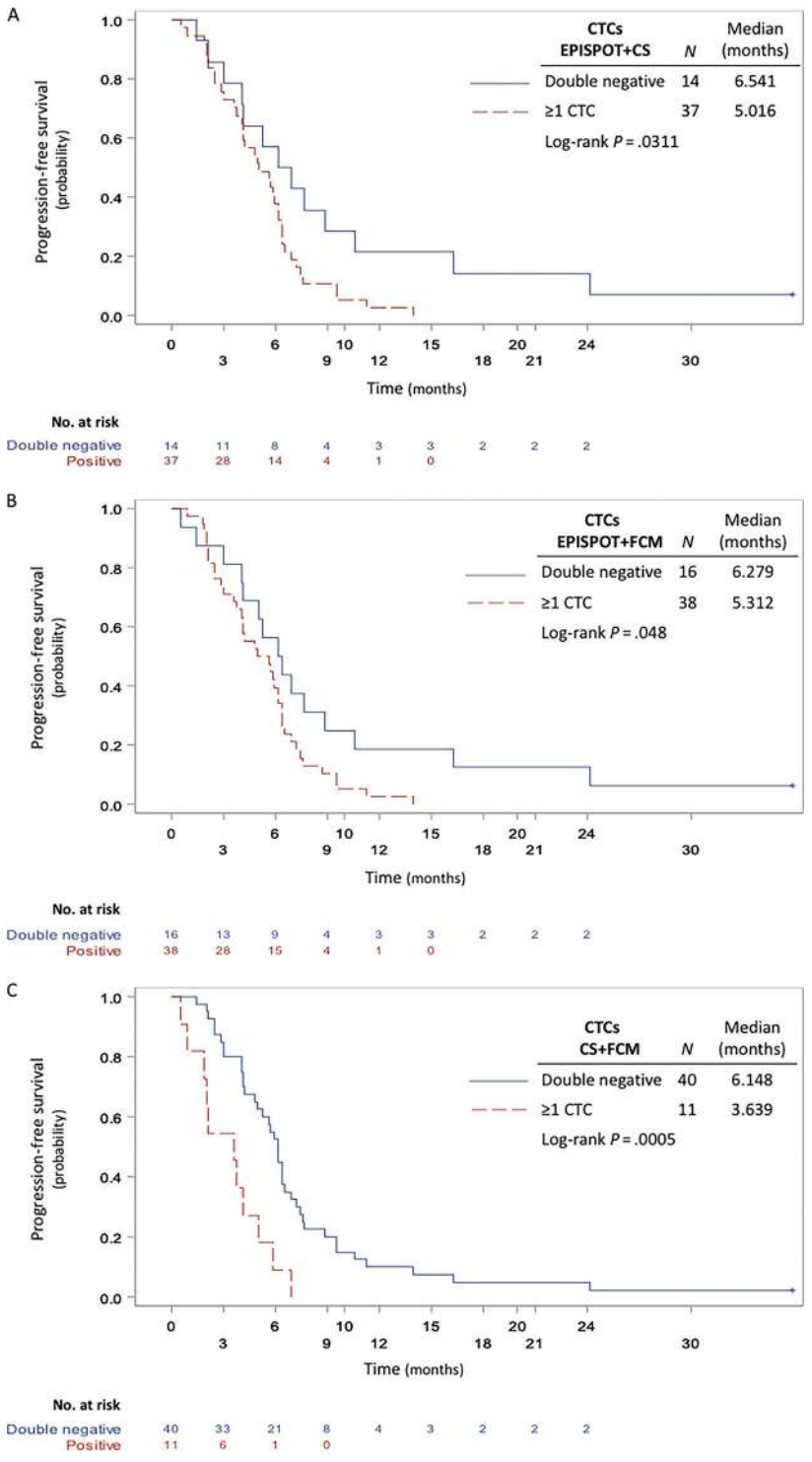

Fig. 4. PFS in patients with recurrent or metastatic HNSCC at D_7.

CTCs were enumerated after the first chemotherapy cycle (D_7) by (A) EPISPOT (any marker) combined with CellSearch (CS) (n = 51 samples tested with both methods), (B) EPISPOT (any marker) combined with FCM (n = 54 samples tested with both methods), (C) CellSearch combined with FCM (n = 51 samples tested with both methods). Double negative, no CTC detected with both methods; positive, CTC ≥1 with 1 or both methods.

whether CTCs could be an early criterion of response to a combination of platinum, 5-fluorouracil, and cetuximab in patients with rHNSCC, we performed an exploratory prospective multicenter study in which blood samples from such patients were tested with 3 different CTC detection technologies. The studied population is representative because OS and PFS (9.34 months and 5.28 months) were consistent with the results reported in the pivotal study that established cetuximab plus platinum as the standard of care (10.1 months and 5.6 months) *(3)*. Interestingly, CTC presence was independent of the clinical status (i.e., locoregional cancer, metastatic disease, or both). The presence and number of CTCs were not related to the metastatic progression but rather to the prognostic of disease, usually linked to locoregional progression, a well-known observation in HNSCC. CTCs could be assessed in most patients during their routine oncologic follow-up in 8 centers in France, demonstrating the feasibility of these techniques in the clinical practice. Unfortunately, patient accrual had to be stopped at 80 inclusions because a new treatment was proposed for patients with rHNSCC. Indeed, a new clinical trial started in the clinical centers involved in the CIRCUTEC study to evaluate platinum-cetuximab combined with docetaxel or with 5-fluorouracil in rHNSCC (NCT02268695). Thus, because of the limited number of evaluable patients and the repeated measures, we could not perform a multivariate analysis. Despite this limitation, our findings confirm that CTCs can be detected before and during chemotherapy in patients with rHNSCC by use of 3 complementary technologies: the EPISPOT assay that detects only functional CTCs, CS (the only Food and Drug Administration–cleared technology for CTC detection), and FCM (to identify different CTC subpopulations, including CTCs that underwent EMT). The nonconcordance of these 3 technologies highlighted the CTC heterogeneity in rHNSCC. CS showed similar CTC detection rates (up to 21%) as those previously reported (between 16.33% and 28.77%) *(7, 8)*. The EPISPOT assay, which was used for the first time in HNSCC, gave the highest detection rate (up to 69% of the tested blood samples and 92% of all positive patients). Moreover, almost all CTCs detected by the 2 other technologies at any time point were identified also by the EPISPOT assay, suggesting that the main prognostic information could be captured with this assay. During treatment, the early CTC number changes between D_0 and D_7, evaluated with the EPISPOT assay that detects only CTC^{EGFR+}, significantly predicted the response to treatment, whereas no prognostic information was obtained with the 2 other technologies. Conversely, CTC enumeration does not seem to have any prognostic relevance after D_7. These findings suggest that in patients with rHNSCC treated with the anti-EGFR antibody cetuximab, the prognostic value of the early CTC kinetics can be evaluated by a functional test that measures their ability to release EGFR. The EPISPOT assay prognostic value has been already proved in breast, colon, and prostate cancer *(10–12)*, and this is the first study assessing this functional test as an early criterion of response to anticancer treatments. CTC prognostic significance was confirmed over time because all long-term responders were in the favorable CTC kinetics group. Nevertheless, more patients are required to demonstrate CTC prognostic value at baseline before treatment. Pairing 2 different technologies for CTC enumeration allowed an increase in the number of positive samples and of detected CTCs, as previously reported in the CTC field *(7, 8)*. Thus, combining different CTC enumeration approaches might improve CTC detection sensitivity and was associated with a prognostic value at D_0 and D_7.

Finally, we used FCM to try to identify mesenchymal subsets of CTCs that could be missed when focusing exclusively on epithelial markers (i.e., CS method). Because only a few blood samples had a high CTC count, we think that FCM might not be suitable for the detection of rare events, such as CTCs. However, in positive samples, we could observe CTCs that underwent EMT, suggesting that EMT might play a role in HNSCC.

In conclusion, this study confirms that real-time liquid biopsy for CTC analysis is clinically relevant in rHNSCC, particularly to monitor the early response to chemotherapy.

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