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► **To cite this version:**

David Veyer, Maxime Wack, Marion Mandavit, Sonia Garrigou, Stéphane Hans, et al.. HPV circulating tumoral DNA quantification by droplet-based digital PCR: A promising predictive and prognostic biomarker for HPV-associated oropharyngeal cancers. *International Journal of Cancer*, 2019, 147, pp.1222 - 1227. 10.1002/ijc.32804 . hal-04301283

HAL Id: hal-04301283

<https://hal.science/hal-04301283>

Submitted on 22 Nov 2023

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Short Report

HPV circulating tumoral DNA quantification by droplet-based digital PCR: A promising predictive and prognostic biomarker for HPV-associated oropharyngeal cancers

David Veyer¹, Maxime Wack^{2,3}, Marion Mandavit⁴, Sonia Garrigou⁵, Stéphane Hans⁶, Pierre Bonfils⁶, Eric Tartour^{4,7}, Laurent Bélec^{1,3}, Shu-Fang Wang-Renault⁵, Pierre Laurent-Puig⁵, Haitham Mirghani⁶, Bastien Rance^{2,3}, Valérie Taly⁵, Cécile Badoual^{4,8} and Hélène Péré^{1,4}

¹Laboratoire de Virologie, Hôpital Européen Georges Pompidou, and Assistance Publique - Hôpitaux de Paris, Paris, France

²Département d'Informatique Médicale, Biostatistiques et Santé Publique, Hôpital Européen Georges Pompidou, and Assistance Publique - Hôpitaux de Paris, Paris, France

³INSERM, Centre de Recherche des Cordeliers, UMRS 1138, Université Paris Descartes, Sorbonne Paris Cité, Université Paris Descartes, Paris, France

⁴INSERM U970, PARCC, Hôpital Européen Georges Pompidou, Faculté de Médecine Paris Descartes, Université Paris Descartes, Paris, France

⁵INSERM UMR-S1147, eDIAG platform, CNRS SNC5014, Université Paris Descartes, Equipe labellisée Ligue Nationale Contre le Cancer, Paris, France

⁶Service d'ORL et Chirurgie Cervico-Faciale, Hôpital Européen Georges Pompidou, and Assistance Publique - Hôpitaux de Paris, Paris, France

⁷Laboratoire d'Immunologie Biologique, Hôpital Européen Georges Pompidou, and Assistance Publique - Hôpitaux de Paris, Paris, France

⁸Laboratoire d'Anatomo-Cytopathologie, Hôpital Européen Georges Pompidou, and Assistance Publique - Hôpitaux de Paris, Paris, France

We aimed to determine whether pretherapeutic assessment of HPV circulating tumoral DNA (HPV ctDNA) by droplet-based digital PCR (ddPCR) could constitute a predictive and prognostic biomarker for HPV-associated oropharyngeal squamous cell carcinoma (OPSCC). A mono-institutional prospective biomarker study on 66 patients with p16+/HPV16-positive oropharyngeal squamous cell carcinoma (OPSCC) was conducted in European Georges Pompidou Hospital, Paris, France. Blood samples were collected at the time of diagnosis before any treatment. Optimized digital PCR assays were used to quantify HPV16 ctDNA. Forty-seven (71%) patients showed a positive pretherapeutic HPV ctDNA at time of diagnosis. Interestingly, the quantity of HPV16 ctDNA at baseline, as assessed by ddPCR, was significantly correlated with the T/N/M status or OPSCC stages according to the 2018 new staging criteria for high-risk human papillomavirus (HR HPV) related OPSCC from American Joint Committee on Cancer (AJCC). Moreover, all recurrences and the majority (83%) of death reported events occurred in patients with positive HPV16 ctDNA at baseline. Finally, when posttreatment blood samples were available ($n = 6$), the kinetic of pretreatment/posttreatment HPV16 ctDNA was clearly associated with treatment success or failure. HPV ctDNA monitoring by ddPCR could constitute a useful and noninvasive dynamic biomarker to select HR HPV-related OPSCC patients eligible for potential treatment de-escalation and to monitor treatment response.

D.V. and M. W. contributed equally to this work

Stéphane Hans's current address is: Service d'ORL et de Chirurgie Cervico-Faciale at Hôpital Foch, UFR Simone Veil, université Versailles, Saint Quentin-en-Yvelines, France

Key words: HPV16 ctDNA, droplet-based digital PCR, HPV OPSCC staging, clinical outcome

Abbreviations: AJCC: American Joint Committee on Cancer; ddPCR: droplet-based digital PCR; HNSCC: Head and neck squamous cell carcinoma; HPV ctDNA: HPV circulating DNA; HR HPV: high-risk human papillomavirus; OPSCC: oropharyngeal squamous cell carcinoma; WHO/IARC: World Health Organization's International Agency for Research on Cancer

Conflict of interest: No conflict of interest has to be declared by the authors.

Grant sponsor: Institut National de la Santé et de la Recherche Médicale; **Grant sponsor:** Centre National de la Recherche Scientifique

DOI: 10.1002/ijc.32804

History: Received 5 Aug 2019; Accepted 12 Nov 2019; Online 22 Nov 2019

Correspondence to: Hélène Péré, E-mail: helene.pere@aphp.fr

What's new?

Circulating tumor DNA represents a tantalizing avenue for non-invasive diagnostic and prognostic testing. Here, the authors investigated whether HPV16 ctDNA correlated with disease stage and outcome in oropharyngeal squamous cell carcinoma (OPSCC). Using digital PCR, they tested 66 blood samples collected at diagnosis, before treatment began. 71% of the patients tested positive for HPV ctDNA, and those patients tended to have more advanced disease than those with undetectable HPV ctDNA. Outcomes also appeared linked with the marker, as 83% of deaths occurred among patients with HPV ctDNA. Finally, post-treatment assessment of ctDNA levels in 6 patients correlated with treatment response.

Introduction

Head and neck cancers, mainly represented by squamous cell carcinomas (HNSCC), are estimated to be the sixth most common cancers worldwide, with 600,000 new cases per year.¹ Since 2007, the infection by high-risk human papillomavirus (HR HPV) in head and neck cancer patients has been considered as an independent risk factor for HNSCC, particularly oropharyngeal cancers, by the World Health Organization's International Agency for Research on Cancer (WHO/IARC).² Different HR HPV have been incriminated in oropharyngeal squamous cell carcinoma (OPSCC), including HPV16 in 85% of cases.³ Finally, OPSCC is currently considered as an epidemic viral-induced carcinoma, since the incidence of HPV-positive carcinoma of the tonsil nearly doubled every 10 years.⁴

A recent major modification in the eighth edition of the American Joint Committee on Cancer (AJCC) Staging Manual (Head and Neck Section) was the introduction of a specific staging classification for HR HPV-associated OPSCC.⁵ Furthermore, the 2017-revised edition of the WHO/IARC on head and neck tumors recommended to classify the OPSCC on the basis of HPV status.⁶ HPV-positive OPSCC constitutes a tumor entity with a better prognosis and a distinct epidemiological profile, with specific genetic features, clinical presentations and outcomes.⁷ However, appropriate selection of patients for potential treatment de-intensification is critical.⁸ Therefore, biomarkers are strongly needed to classify more precisely HPV-associated OPSCC for treatment optimization. Moreover, the recent development of immunotherapy for HNSCC treatment requires the identification of biomarkers which could help patient selection and therapeutic response monitoring.

The liquid-biopsy approach using the detection of circulating tumor DNA (ctDNA) released from tumor cells and detectable in blood has garnered growing interest⁹ particularly in HNSCC.¹⁰ Various mechanisms including apoptosis, necrosis, phagocytosis as well as an active secretion by vesicles could be involved in the release of ctDNA from tumor cells. However, the physiopathological and clinical factors that account for the differences in ctDNA load observed are still poorly understood.^{11,12} Detection of ctDNA has demonstrated its interest in lung or colorectal cancer with the detection of *EGFR* and *KRAS* mutations for noninvasive tumors genotyping, treatment response follow-up and relapse prediction.¹³

HPV-related cancers are an ideal model to monitor ctDNA by detecting HPV oncogenes E6 or E7. Indeed, these HPV oncoproteins are responsible for cell transformation and

carcinogenesis, and have been proven to be indispensable for maintenance of tumor phenotype.¹⁴ To the best of our knowledge, HPV-derived nucleic acids, and particularly the E6 and E7 DNA, have not been detected in blood samples in case of simple HPV infection but only in HPV-related cancer cases.¹⁵ The feasibility and the interest of HPV ctDNA detection in the plasma of HPV-related OPSCC patients using new ultrasensitive molecular tools such as droplet-based digital PCR (ddPCR) assays have been recently reported¹⁵⁻¹⁷ particularly in treatment response monitoring. This quantitative method of ddPCR is characterized by its high-sensitivity, its accuracy and its reproducibility inter-laboratories and intralaboratories.¹⁸

We herein report on the results of pretherapeutic HPV16 ctDNA quantification by ddPCR in a prospective cohort of 66 HPV16-OPSCC patients, at the time of diagnosis, and its correlation with the T/N/M status according to the new staging from AJCC and with clinical outcome.

Materials and Methods**Patients**

We prospectively included OPSCC patients with biopsies that were both positive for p16 and HPV16 molecular detection. Biopsies were collected before treatment at the European Georges Pompidou hospital, Paris, France. HPV detection and genotyping of OPSCC were carried out in our ISO 15189-accredited virology laboratory with the Inno-Lipa technic (Fujirebio, Gent, Belgium). Plasma samples were systematically collected on 5 ml EDTA tubes with the patient's informed consent, in parallel to the collection of biopsies used for diagnosis. OPSCC posttreatment serums were obtained from leftover samples collected during follow-up when available; 4/6 patients sample were collected between 3 and 6 months after the end of treatment and the two left samples were collected more than 2 years after. The mean follow-up time of patients was 2.84 years [0.94; 4.64]. The patients belonged to a cohort declared and approved by the Comité de Protection des Personnes (Ile de France II, no. 2015-09-04) and by the Comité D'éthique de la Recherche AP-HP.5 (#IRB00011928).

HPV16 ctDNA extraction procedures and quantification

After a 200 µl of plasma or serum DNA extraction using Qiaamp Minelute Virus Spin Kit (QIAGEN®, Hilden, Deutschland) following the manufacturer procedure for viral DNA extraction, ddPCR detection of HPV16 E6 gene was performed using RainDrop Digital PCR System (RainDance Technologies, Bio-Rad, Hercules, CA). Simultaneously, the

albumin gene was detected by ddPCR as an internal control for each tested plasma sample. To obtain absolute quantification of HPV16 ctDNA, data analysis was performed using the Raindrop Analyst software (FlowJo, Ashland, Covington, KY). The HPV16 ctDNA load was finally calculated in copies/ml. Regarding the negative control results and the limit of blank of our technic, all detection strictly below five positive droplets (equivalent to <69 cp/ml) were considered as negative samples considering the global limit of blank obtained with negative control at each run.

Statistical analysis

Bivariate comparisons were computed using nonparametrized tests as applicable (Fisher's exact test for categorical variables when possible, Chi-squared test otherwise; Mann-Whitney U test and Kruskal-Wallis one-way analysis of variance for continuous variables). Correlations were assessed using the Spearman's rank correlation coefficient to mitigate nonlinear association between the plasma E6 concentration and the clinical staging.

All *p* values reported were two-sided and a 0.05 threshold was considered statistically significant. Data were analyzed using R version 3.5.2.¹⁹

Data availability

The data that support the findings of our study are available from the corresponding author upon reasonable request.

Results

Study population

Our study included a total of 66 patients newly diagnosed with HPV16-OPSCC. Patients were men in the majority [46 (70%)] with an average age at diagnosis (date of biopsy) of 65 years (IQR: 16.8). The clinical characteristics of the population are shown in Table 1.

Among the 66 OPSCC, a majority was T2 and T4 tumors [(20 (31%) and 32 (49%), respectively], N1 lymph nodes [42 (64%) patients], M0 metastases [42 (64%)], translating to mostly Stages I and III [25 (38%) and 32 (48%) patients, respectively] considering the new 2018 AJCC staging manual. Thirty-six patients (62%) were active smokers, and smoking status was unknown for eight patients. Alcohol consumption was known for only three patients. Eight patients suffered from recurrence and six died during the follow-up period.

Plasma HPV16 ctDNA detection and values

At the time of their OPSCC diagnosis, HPV16 ctDNA could be detected in the plasma of 47 patients (71%) with a median value of 319 cp/ml. Nineteen patients were negative for HPV16 ctDNA by ddPCR (Table 1). Plasmatic DNA was extracted on 200 µl of plasma using QiaAmp DNA Mini Kit (Qiagen).

All plasma samples were positive for albumin, validating the presence of DNA in plasma extracts and the quality of our plasmatic DNA extraction and PCR procedure (data not shown).

Table 1. Clinical characteristics of HPV16-related oropharyngeal squamous cell carcinoma and HPV16 circulating tumoral DNA results

Clinical characteristics	<i>n</i> (%)	Med	Min	Max
Total	66			
Sex				
Women	20 (30)			
Men	46 (70)			
Age at diagnosis	66	65	43	92
Number of recurrences	8 (12)			
Number of death	6 (9)			
T				
1	8 (12)			
2	20 (31)			
3	5 (7,7)			
4	32 (49)			
N				
0	7 (11)			
1	42 (64)			
2	12 (18)			
3	5 (7)			
M				
0	42 (93)			
1	3 (7)			
AJCC staging				
I	25 (38)			
II	6 (9)			
III	32 (48)			
IV	3 (6)			
Tobacco				
No	22 (38)			
Yes	36 (62)			
HPV16 ctDNA results				
HPV16 ctDNA concentrations				
Negative	19 (29)			
Positive	47 (71)			
HPV16 ctDNA concentration cp/ml	66	319,4	0	147,416,7
log[HPV16 ctDNA concentration] cp/ml	66	2,50	0	5,17
DNA µg/ml	66	3,34	0,122	6,93

Pretherapeutic HPV 16 ctDNA values correlation with TNM status, new 2018 AJCC staging and prognosis

HPV16 ctDNA levels at baseline were positively correlated with T and N status and a positive trend was observed for the M status (Fig. 1a). Thus, the higher the T status was, the higher the median value of HPV16 ctDNA loads was (*p* = 0.004; Fig. 1a). The same significant positive correlation was observed between the N status and the increased concentration of HPV16 ctDNA (*p* = 0.03; Fig. 1a). The weak number of patients with metastasis (*n* = 3) did not lead to any significant difference between M0 and M1 group. However, all of

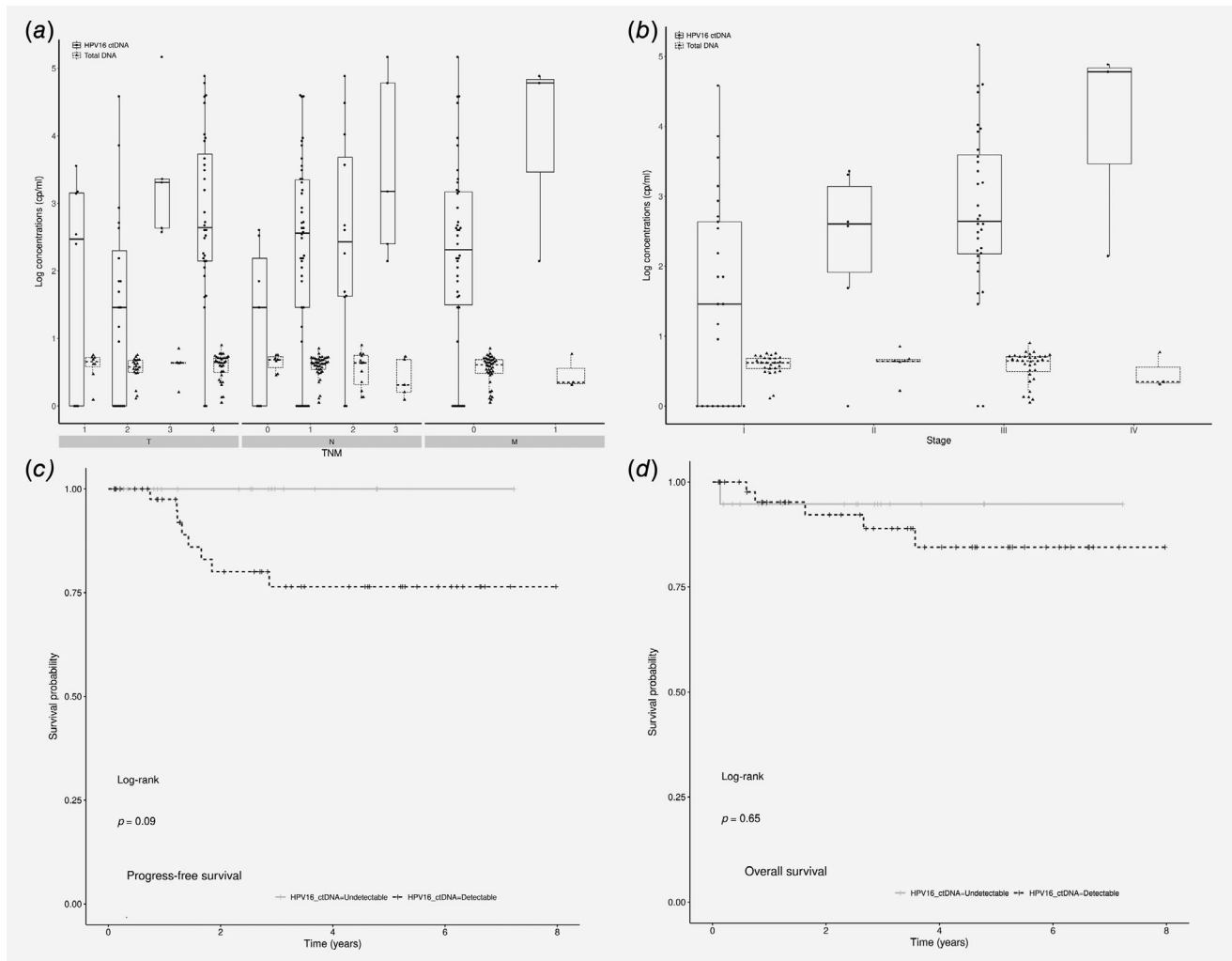


Figure 1. Pretherapeutic HPV 16 circulating tumoral DNA values correlation with TNM status (a), new 2018 AJCC staging (b) and clinical outcome (c). (a) HPV16 circulating tumoral DNA and total circulating DNA values according to T/N/M status of HPV16-related oropharyngeal squamous cell carcinoma. (b) HPV16 circulating tumoral DNA and total circulating DNA according to the American Joint Committee on Cancer (AJCC) Stages defined for HPV-related oropharyngeal squamous cell carcinoma. (c) Progress-free survival depending on baseline HPV16 circulating tumoral DNA status (detectable \rightarrow or undetectable \leftarrow based on optimal cut-off according to limit of blank obtained with negative controls). (d) Overall survival (right panel) depending on baseline HPV16 circulating tumoral DNA status (detectable \rightarrow or undetectable \leftarrow based on optimal cut-off according to limit of blank obtained with negative controls).

the M1 group patients presented positive HPV ctDNA, with a clear trend of increased loads of HPV16 ctDNA.

HPV16 ctDNA loads were also correlated with the 2018 AJCC staging, the median values of HPV16 ctDNA loads increasing from 1.45 to 4.8 log cp/mL from Stages I to IV, respectively ($p = 0.0002$; Fig. 1b) and 1a positive HPV16 ctDNA rate of 46, 83, 88 and 100%, respectively, to Stages I, II, III or IV.

The median value of albumin concentrations, used as internal control, remained stable regardless of the T, N and M status or AJCC stages attesting the specific and isolated increase of HPV ctDNA in patient plasmas.

Furthermore, a clear positive trend was observed between pretherapeutic HPV16 ctDNA detection and poor outcome in terms of progression-free survival ($p = 0.09$; Fig. 1c). Moreover,

a large proportion (83%) of death events reported in our study have occurred in the group of positive pretherapeutic HPV16 ctDNA patients (Fig. 1d). The only death event reported in the group of negative pretherapeutic HPV16 ctDNA patients was associated with heart disease without any link with the OPSCC.

HPV ctDNA dynamics as a predictor of treatment response and failure

OPSCC posttreatment serum could be obtained for six patients. For five of them, HPV16 ctDNA concentrations were positive at the time of diagnosis. The patient for whom no HPV16 ctDNA was detected remained negative after treatment and four from the five positive patients at baseline markedly decreased their HPV16 ctDNA load (<2 log cp/ml). All of these

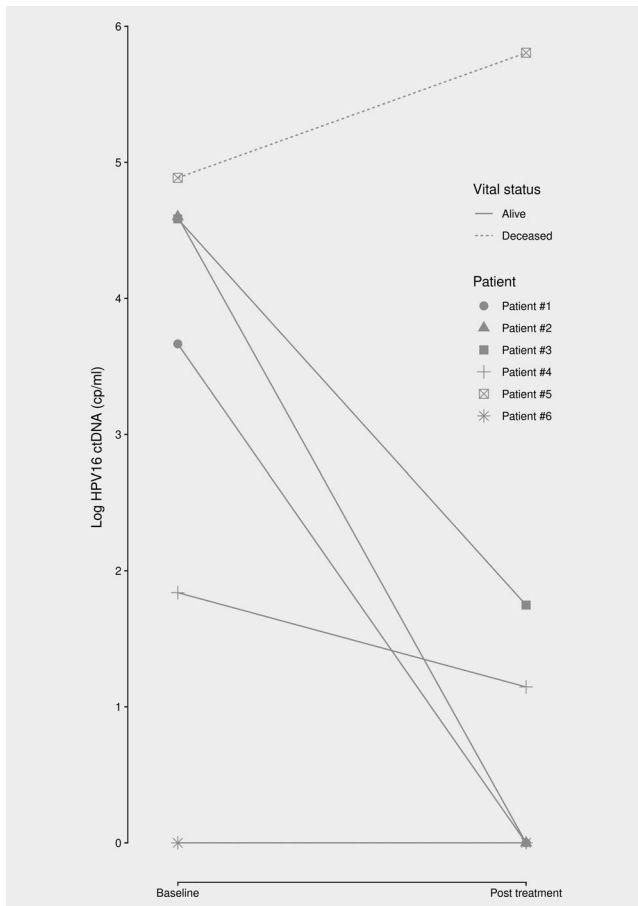


Figure 2. HPV16 circulating tumoral DNA concentration kinetics during antitumoral treatment (baseline and posttreatment) in six patients.

five patients showed a complete clinical response to treatment. The patient presenting an increase (fivefold) of HPV16 ctDNA load after treatment died within days of serum sample collection (Fig. 2) of OPSCC recurrence and distant metastasis.

Discussion

To the best of our knowledge, it is the first time that pretherapeutic HPV16 ctDNA using ddPCR technology was evaluated as a biomarker for OPSCC staging correlated to the new staging algorithm for HR HPV-associated OPSCC⁵ and for patients clinical outcome. In our study, HPV16 ctDNA was detected in most plasma samples tested at the time of OPSCC diagnosis in a large prospective cohort of 66 HPV16-OPSCC patients. After analysis of clinical data at baseline, no significant difference was observed between the two groups concerning the known risk factors of age and tobacco (data not shown). Data concerning alcohol consumption were not sufficient to allow a statistical comparison between the two groups. To the best of our knowledge, it is the first time that a positive correlation was demonstrated between the level of HPV16 ctDNA load quantified by ddPCR and T status, N status and the specific stages of

the new HPV OPSCC staging algorithm. Interestingly, 29% of patients of our cohort showed undetectable HPV ctDNA. As expected, most of them (74%) presented early stage I OPSCC and were all defined histologically as N0/N1 patients. A minority of patients that presented Stages II or III were negative for HPV ctDNA, 16 and 12.5%, respectively. Stage III patients with undetectable HPV ctDNA were all T4 classified with or without nodal invasion (N0, N1 or N2) and were classified in Stage III regarding their T4 status. In addition, all the patients with stage IV OPSCC diagnosis and/or metastasis presented detectable HPV ctDNA. To explain the wide range of HPV16 ctDNA observed in Stage I and rare cases of HPV16 ctDNA negativity in advanced stages (Stages II and III), we investigate precise tumor localization in the oropharynx and tumoral HPV16 viral load of these cases (data not shown). The repartition of the different tumor localization in the oropharynx (tonsil, tonsillar lodge and tongue base) appears to be similar in the two Stage I HPV16 ctDNA positive and negative groups no significant correlation was observed between tumoral HPV16 viral load and HPV16 ctDNA values whatever the stage of the disease. Neither particular tumor localization nor tumoral HPV16 viral load could explain the disparity of HPV16 ctDNA values observed in Stage I and the negative results obtained in some advanced stages cases.

The median value of total DNA concentrations used as internal control remained stable regardless of the T, N and M status or AJCC stages, attesting that the increase of HPV16 ctDNA loads was specific and not due to a global increase of total cell-free circulating DNA. Altogether, these results confirmed that this molecular biomarker is correlated with tumor cells invasion at the time of OPSCC diagnosis. Therefore, our observations extend and complete three previous recent studies showing the feasibility to monitor HPV ctDNA by ddPCR in HPV-related OPSCC and evaluating its correlation with treatment response in such cancer^{15–17} by suggesting the potential interest of the detection of pretherapeutic HPV ctDNA by ddPCR as an eventual complementary diagnostic tool in addition to histological and clinical diagnosis.

Moreover, in our series, we observed a positive but not significant correlation between HPV16 ctDNA detection by ddPCR and patient clinical outcome. This is in accordance with the correlation of HPV16 ctDNA to the AJCC stages. The absence of significance could be explained by a lack of power in our study regarding an insufficient sample size. Further evaluation of larger cohorts of HPV-related OPSCC patients would be needed. This could be of potential interest to select patients for whom treatment de-escalation could be offered.

HPV ctDNA detected by ddPCR has been shown to constitute a biomarker to monitor treatment response^{16,17} in OPSCC. In our study, we were able to assess HPV16 ctDNA quantification in six available posttreatment samples, clearly confirming the potential interest of this biomarker to predict the treatment response. Indeed, a dramatic decrease or clearance of HPV16 ctDNA was observed after treatment in four

of the five positive pretherapeutic HPV16 ctDNA patients, and, at the same time, a five-fold increase of HPV16 ctDNA was observed in the only patient who died from OPSCC recurrence and metastasis shortly after his posttreatment biological sample was obtained. Thus, HPV16 ctDNA monitoring by ddPCR could constitute a noninvasive dynamic biomarker for HPV-related OPSCC treatment response monitoring. This is of particular importance regarding the recent development of new targeted and expensive antitumor therapeutic for OPSCC treatment (specific therapeutic vaccine, immunotherapy) for which biomarkers are needed for the selection of eligible patients and for response monitoring.

Our protocol included only HPV16 positive patients but it is more than likely that working on different HPV genotypes involved in OPSCC would give similar results. Data to confirm this hypothesis are needed.

The feasibility of detecting HPV ctDNA in the plasma of patients with HPV-related cancer using ddPCR, a new highly sensitive method, has been already shown.^{16,17} The ddPCR technology showed high efficiency for the detection of rare events, something particularly relevant for ctDNA detection.²⁰ Moreover, its high tolerance to PCR inhibitory molecules makes it an attractive alternative to qPCR for medical

applications including viral diagnostics and cancer research. It has also been shown that women with cervical invasive cell carcinoma have detectable HPV ctDNA by ddPCR even in early-stage cases with microinvasive carcinomas while this detection was strictly negative in the plasma of patients with high-grade cervical intraepithelial lesion.¹⁵ Additionally, two recent studies performed in advanced anal cancer suggested that HPV ctDNA could be an interesting biomarker to monitor the efficacy of cancer therapy^{21,22} in such cancer too.

Herein, our results showed that HPV16 ctDNA could be an interesting biomarker for OPSCC patient management, not only for posttreatment patient monitoring but also at the time of diagnosis, as a complementary tool for patient pretreatment classification and emphasize the need for further validations on larger multicentric cohorts.

Acknowledgements

This work was supported by the Ministère de l'Enseignement Supérieur et de la Recherche, the Université Paris-Descartes, the Centre National de la Recherche Scientifique (CNRS), the Institut National de la Santé et de la Recherche Médicale (INSERM), the SIRIC CARPEM, the Ligue Nationale Contre le Cancer (LNCC) and the CRC AP-HP.

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