

# Prognostic Impact of Residual HPV ctDNA Detection after Chemoradiotherapy for Anal Squamous Cell Carcinoma



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## Abstract

**Purpose:** Chemoradiotherapy (CRT) is the current standard of care for patients diagnosed with locally advanced anal squamous cell carcinoma (ASCC), but some patients develop local and/or distant relapse during follow-up. This study was designed to monitor human papillomavirus (HPV) circulating tumor DNA (ctDNA) levels during CRT in patients with ASCC.

**Experimental Design:** We analyzed samples from patients with HPV16- or HPV18-positive locally advanced ASCC. Blood samples were collected before and after CRT. HPV16 or HPV18 ctDNA detection was performed by droplet digital-PCR.

**Results:** HPV ctDNA was detected before CRT in 29 of 33 patients with stages II–III ASCC [sensitivity: 88%; 95% confidence interval (CI), 72–95]; ctDNA positivity rate was associated with tumor stage (64% and 100% in stages II and III,

respectively;  $P = 0.008$ ). Among ctDNA-positive patients at baseline, ctDNA levels were higher in  $N^+$  than in  $N^-$  tumors (median 85 copies/mL, range = 8–9,333 vs. 32 copies/mL, range = 3–1,350;  $P = 0.03$ ). ctDNA detection at baseline had no significant prognostic impact. After CRT, three of 18 (17%) patients displayed residual detectable HPV ctDNA; ctDNA detection after CRT was strongly associated with shorter disease-free survival ( $P < 0.0001$ ).

**Conclusions:** This is the first proof-of-concept study assessing the prognostic value of ctDNA after CRT in locally advanced ASCC. In most patients, HPV ctDNA can be detected before CRT and becomes undetectable during CRT. In this study, we show that residual ctDNA levels after CRT are associated with very poor outcome. *Clin Cancer Res*; 24(22); 5767–71. ©2018 AACR.

## Introduction

Anal squamous cell carcinoma (ASCC) is a rare, although increasingly prevalent (1), cancer due to human papillomavirus (HPV) infection; HPV DNA sequences can be found in cancer cells of about 90% of ASCC cases, most commonly the HPV16 genotype (2, 3). Definitive chemoradiotherapy (CRT) is the current standard of care for patients diagnosed with locally advanced ASCC. Abdominoperineal resection is a salvage approach, which

is reserved for patients with local relapse or persistent disease after CRT (4). However, no sensitive blood biomarkers are currently available to predict relapse in patients, as squamous cell carcinoma (SCC) antigen demonstrated low clinical validity in this setting (5), although the interpretation of conventional imaging can be challenging after CRT (4).

In many tumor types, circulating tumor DNA (ctDNA) has demonstrated a good correlation with tumor response or progression and usefully complements standard tumor imaging (6–10), and is detectable in ASCC (11). In breast and colorectal cancers, ctDNA was able to detect early relapse after treatment in the nonmetastatic setting and to detect a relapse several months before conventional imaging (6, 12–15).

In HPV-related cancer, HPV viral genomes are usually integrated into the tumor cell genome or episomal DNA (16). Our team has previously validated that droplet-digital PCR (ddPCR) is able to detect and quantify tumor-derived HPV DNA sequences in patient blood (HPV ctDNA) with high sensitivity and specificity (17, 18), and can be used to monitor the efficacy of immunotherapy (19). Importantly, no HPV ctDNA was detected in healthy controls and in women treated for HPV16-associated highgrade cervical intraepithelial neoplasia (17, 18).

This proof-of-concept study was designed to investigate the sensitivity and prognostic impact of HPV ctDNA detection in a cohort of patients with locally advanced ASCC treated by CRT.

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### Translational Relevance

HPV circulating tumor DNA (ctDNA) was detected in 88% of patients before starting chemoradiotherapy (CRT) for nonmetastatic anal squamous cell carcinoma (ASCC). HPV ctDNA was detected in only three of 18 (17%) patients after CRT; all three patients experienced rapid metastatic relapse. Among patients with no residual ctDNA detected after CRT, only one of 15 (7%) experienced local relapse. HPV ctDNA detection by droplet digital PCR appears to be a rapid, noninvasive, and affordable prognostic marker for patients with ASCC treated by CRT.

## Materials and Methods

### Patients and samples

This study included plasma or serum samples from patients with ASCC treated at Institut Curie (Saint Cloud, France). Eligibility criteria were: histologically proven HPV16- or HPV18-positive ASCC (HPV subtype was determined by routine diagnostic procedures on tumor biopsies); absence of metastasis (PET-CT, pelvic magnetic resonance imaging); patient treated by CRT alone; absence of any history of other invasive cancer; samples collected either <30 days before initiation of CRT (baseline samples) or <30 days after completion of CRT (posttreatment samples). Patients who did not start the CRT were excluded. Patients who were eligible but not included have not been tracked. A waiver of patient-informed consent was obtained for samples stored at the Institut Curie biobank (serum), and after 2012 for patients included in the ethically approved prospective study on circulating tumor biomarkers (NCT02220556), a written informed consent to participate was obtained and plasma samples were analyzed, according to ethical regulation and the Declaration of Helsinki. Patients received concomitant chemotherapy with either intravenous mitomycin-C (one dose of 10 mg/m<sup>2</sup> on days 1 and 29) or intravenous cisplatin (one dose of 25 mg/m<sup>2</sup> per day on days 1–4 and 29–32), and intravenous fluorouracil (one dose of 1,000 mg/m<sup>2</sup> per day on days 1–4 and 29–32). Patient characteristics, treatment, and outcomes were prospectively registered in the Institut Curie electronic medical records. Tumor staging was performed according to the 2010 TNM classification (20). External radiotherapy delivered a dose of 60 Gy to the tumor and 45 Gy to the locoregional lymph nodes. Follow-up included a clinical evaluation with anoscopy and digital pelvic examination, PET-CT and pelvic magnetic resonance every 4 months until 1 year after completion of CRT, then thoracic–abdominal–pelvic CT-scan every 6 months until 5 years.

### HPV ctDNA detection

Cell-free circulating DNA (cfDNA) extraction was performed on 4 mL of plasma or 2 mL of serum, by using the QIAamp Circulating Nucleic Acid Kit (Qiagen), according to the manufacturer's instructions. cfDNA was eluted into 40 µL of AVE buffer and stored at –20°C. cfDNA was then subjected to HPV ctDNA detection using ddPCR, as described previously by our group (17), with minor changes. Briefly, 6 µL DNA samples were run in triplicate using the same set of HPV16 E7-

or HPV18 E7-specific primers and TaqMan probe and multiplexed with a commercial human ddPCR assay (i.e., *RPP30* gene, dHSaCP2500350, Bio-Rad) to quantify cfDNA and ensure that >250 human genome equivalents were screened. Data were analyzed using QuantaSoft software (BioRad), which expressed the results in copies/µL. DNA from the HPV16-transformed cell line SiHa and HPV18-transformed cell line IC1 was used as positive controls (21). At least three droplets displaying the same fluorescence amplitude as positive controls were mandatory to call a sample as positive. In positive samples, HPV ctDNA concentration was expressed in copies/mL of serum or plasma. We have previously reported that HPV ctDNA amplification is not significantly influenced by the amount of normal human DNA, that is, that absolute quantification of HPV ctDNA does not differ between serum and plasma (17). Samples were analyzed maximum 2 years after collection.

### Statistical analyses

This hypothesis-generating study had no prespecified power. For nonparametric analysis,  $\chi^2$  or Fisher exact test was used for categorical variables. Quantitative HPV ctDNA levels were compared using the Mann–Whitney test. Disease-free survival (DFS) was defined as the time elapsed between the date of initiation of CRT and the date of cancer relapse or death from any cause, whichever came first. The median point estimate and 95% confidence interval (CI) for DFS were estimated by the Kaplan–Meier method. Survival curves were compared by an unstratified log-rank test.

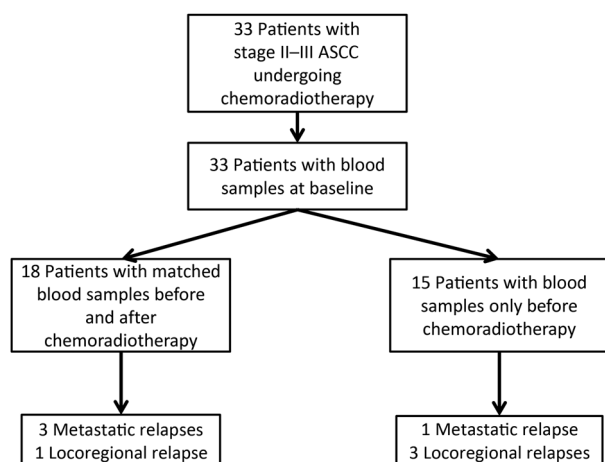
## Results

### Patients and samples

Thirty-three patients with diagnosis of locally advanced HPV16- or HPV18-related ASCC were included in this study. Twenty-two (67%) of these patients were diagnosed with stage III ASCC; other patient characteristics are shown in Table 1. In the 33 patients included, the median interval between pre-CRT blood sampling and initiation of CRT was

**Table 1.** Patient characteristics

Characteristics	Pre-CRT samples <i>n</i> = 33 patients <i>n</i> (%)	Post-CRT samples <i>n</i> = 18 patients <i>n</i> (%)
Age		
Median (range)	64 (47–82)	68 (47–82)
Gender		
Male	4 (12%)	4 (22%)
Female	29 (88%)	14 (78%)
Tumor stage		
Stage II	11 (33%)	4 (22%)
Stage IIIA	9 (27%)	7 (39%)
Stage IIIB	13 (40%)	7 (39%)
HPV genotype		
HPV16	31 (95%)	17 (94%)
HPV18	2 (5%)	1 (6%)
HIV status		
Positive	3 (9%)	1 (6%)
Negative	30 (91%)	17 (94%)
Concomitant chemotherapy		
5FU mitomycin-C	5 (15%)	2 (11%)
5FU cisplatin	28 (85%)	16 (89%)
Sample analyzed		
Plasma	23 (70%)	12 (67%)
Serum	10 (30%)	6 (33%)

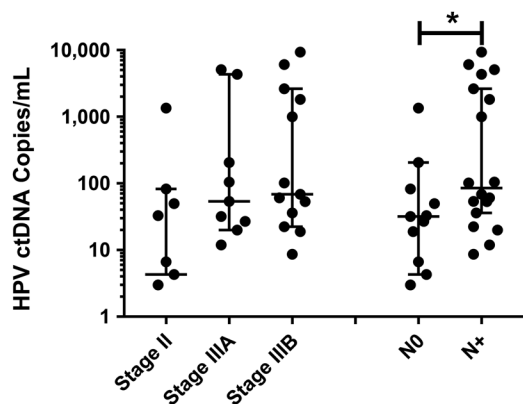


**Figure 1.**  
Flow diagram of patients and events.

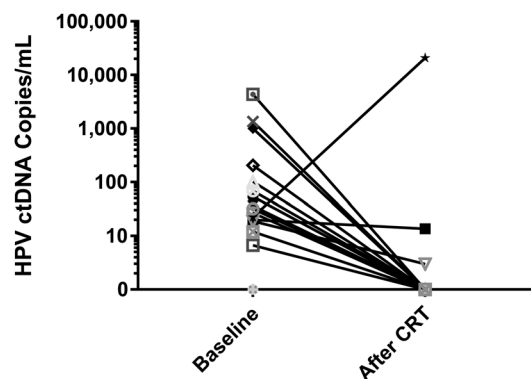
17 days (range = 0–30 days). Paired samples collected after CRT were available for 18 patients, with a median interval of 4 days (range = 0–30 days; Fig. 1). There was no significant association between patient characteristics and blood sample type and availability.

#### HPV ctDNA detection and correlation with patient characteristics

The median baseline HPV ctDNA level was 36 copies/mL (range = 0–9,333; IQR, 12–206). With only four of 33 patients with undetectable HPV ctDNA, this technique displayed a sensitivity of 88% (95% CI, 72–95). The four patients with undetectable ctDNA had stage II tumors (T2N0M0,  $n = 3$  patients; T3N0M0,  $n = 1$ ): detection rates in stage II (64%; 95% CI, 35–85) were significantly lower than in stage III (100%; 95% CI, 85–100;  $P = 0.008$ ). Among the 29 patients with detectable HPV ctDNA, its level was associated with lymph node status: the median ctDNA level was 85.5 copies/mL (range = 8.7–9,333) in node-positive ASCC versus 32



**Figure 2.**  
Baseline HPV ctDNA levels according to patient characteristics. Quantitative HPV ctDNA levels were compared by Mann-Whitney test; \*,  $P = 0.03$ .



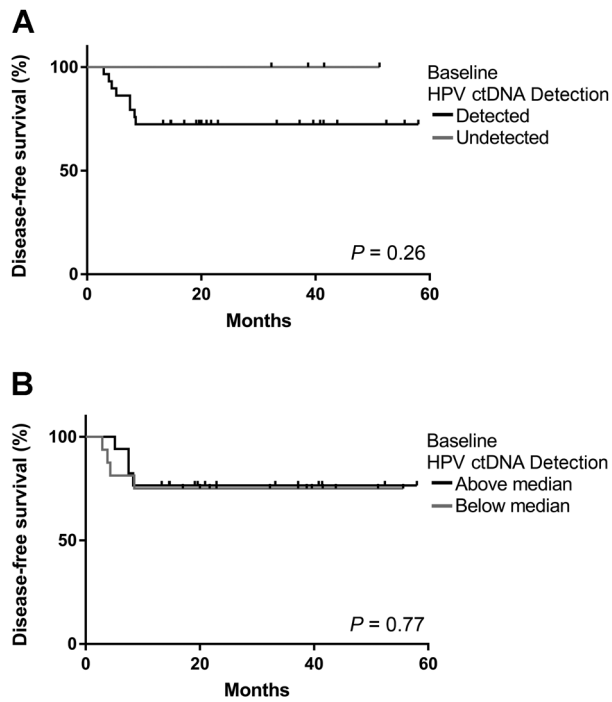
**Figure 3.**  
HPV ctDNA level changes during therapy.

copies/mL (range = 3–1,350) in node-negative ASCC ( $P = 0.03$ ; Fig. 2). Three patients displayed a higher HPV ctDNA copy number than the number of genome equivalent (estimated by *RPP30* gene level, used as haploid genome reference), suggesting a high number of HPV copies per cancer cell.

Paired plasma or serum samples after CRT were available in 18 patients with nonmetastatic ASCC. Only three of these patients had detectable residual HPV ctDNA levels (detection rate = 17%; 95% CI, 5.8–39; comparison versus baseline detection rate;  $P < 0.001$ ). HPV ctDNA levels decreased during CRT in two of these three patients (Fig. 3).

#### HPV ctDNA detection and correlation with patient outcome

With a median follow-up of 30 months (range = 8–60 months), four metastatic relapses (time of relapse: 2.9, 3.8, 4.3, and 7.5 months), four locoregional relapses (time of relapse: 3.5, 7.5, 8.3, and 8.5 months), and three deaths (secondary to metastatic ASCC relapses) were observed in the total cohort (Fig. 1). At baseline, neither ctDNA detection status (positive vs. negative) nor ctDNA levels (dichotomized between baseline HPV ctDNA copies/mL equal to or below median and above median) were associated with relapse ( $P = 0.26$  and  $0.77$ , respectively; Fig. 4A and B). Although not statistically significant due to the small number of patients, no relapse occurred in patients with baseline undetectable ctDNA. After CRT, residual HPV ctDNA detection was dramatically associated with patient outcome, as the three patients with residual HPV ctDNA levels experienced rapid metastatic relapse (at 2.9, 3.8, and 4.3 months; Fig. 1). Among the 15 HPV ctDNA-negative patients, only one experienced local relapse at 8.5 months (7%; 95% CI, 1–30). Altogether, DFS was strongly associated with HPV ctDNA status after CRT ( $P < 0.0001$ ; Fig. 5). In the only patient who experienced local relapse, HPV ctDNA was not detected after CRT, but was again detected at the time of local relapse, and this patient had a low baseline HPV ctDNA level (12 copies/mL plasma). Because of the limited number of events and the fact that all HPV ctDNA positive patients experienced a relapse, multivariate analysis was not considered relevant. In an exploratory analysis, nine samples collected per standard of care in nine patients during follow-up (90–150 days after CRT) were also subjected to HPV ctDNA detection. None of these patients experienced a cancer relapse and HPV ctDNA was undetectable.



**Figure 4.** DFS according to baseline HPV ctDNA status. Survival curves were compared by a log-rank test. **A**, Detectable versus undetectable HPV ctDNA; **B**, HPV ctDNA  $\leq$  median versus  $>$  median.

**Discussion**

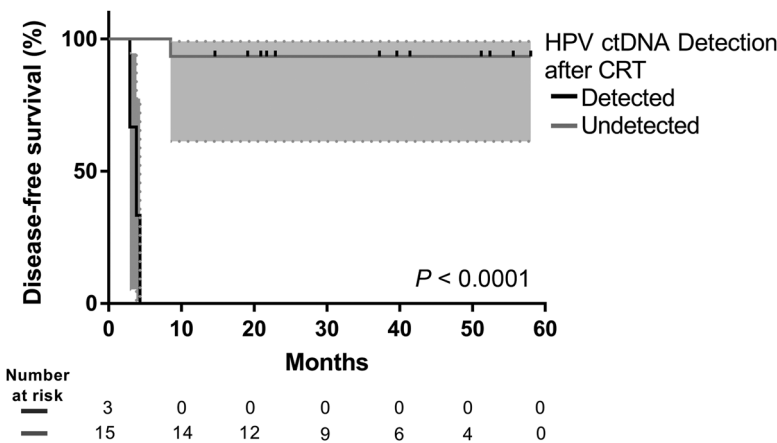
This proof-of-concept study shows, for the first time, that HPV ctDNA detection by ddPCR is a very sensitive assay in the context of untreated nonmetastatic stages II–III ASCC. The 88% detection rate before therapy appears to be higher than that reported in other stages II–III cancer types (22), in which ctDNA detection is usually based on detection of point mutations in cancer genes. In contrast with point mutations, which are present at 1 copy per cancer cell, multiple HPV DNA sequences are present in a given ASCC cell (16). These numerous copies are released into the blood following cancer cell death and contribute to the higher sensitivity of HPV-based ctDNA detection. The number of HPV copies per

ASCC cell is known to vary between patients (and possibly between ASCC cells in a given patient), as confirmed in our study, as some patients had even more HPV sequences in their plasma than the actual total number of human genome equivalent (one copy per haploid genome). Of note, very satisfactory detection rates have been previously reported with HPV ctDNA in other HPV-induced head and neck (23) or gynecologic cancers (17, 18), and even with EBV ctDNA detection in nasopharyngeal cancers (24). This heterogeneity (and the limited number of patients) may explain the lack of overt prognostic significance of baseline HPV ctDNA levels observed in this study.

The most striking results of this study are that HPV ctDNA levels drop markedly in most patients during CRT and that residual detectable ctDNA levels after CRT were associated with short-term metastatic relapse, although all patients underwent a metastasis workup by PET-CT scan before CRT. Similar results have been reported after surgery for localized colorectal (12) and breast cancers (13, 14). The question of whether residual ctDNA levels detected after therapy reflect the presence of a minimal residual disease (which may be quiescent) or metastases that are already growing (but not initially detected by imaging) remain unresolved. However, as one patient with undetectable HPV ctDNA after CRT experienced local relapse, we hypothesize that, as previously shown in triple-negative breast cancer patients (15), only short-term relapses may be predicted by residual ctDNA detection.

The main limitation of our proof-of-concept study is the low number of patients analyzed. Pending confirmation of its prognostic value, quantitative HPV ctDNA monitoring might become clinically useful to tailor the use of supplementary post-CRT therapies, such as immune therapy — currently investigated as adjuvant therapy in stages II–III ASCC (NCT03233711). In such post-CRT adjuvant setting, not only ctDNA may define the group at the highest risk of relapse after CRT, but longitudinal quantitative ctDNA monitoring may also provide an early and reliable assessment of immune therapy efficacy (19, 25, 26).

Beyond the specific clinical context of this study, HPV ctDNA detection studies should also be set up in other HPV-induced malignancies. Owing to the limited cost of ddPCR, potential uses of HPV ctDNA detection and quantitative monitoring are cancer screening (as in EBV-induced cancers; ref. 24), modulation of local and/or systemic treatment and surveillance after therapy. The validity of HPV ctDNA detection during surveillance of HPV-induced pelvic cancers will be assessed in a large



**Figure 5.** DFS according to HPV ctDNA detection after CRT. Survival curves are shown with their 95% CIs and were compared by the log-rank test.

multicenter prospective study in France (CirCA-HPV, NCT number pending).

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Authors' Contributions

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**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** L. Cabel, E. Jeannot, I. Bieche, S. Vacher, W. Chémali, A. Schnitzler, F.-C. Bidard, W. Cacheux

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**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** L. Cabel, J.-Y. Pierga, F.-C. Bidard, W. Cacheux  
**Study supervision:** L. Cabel, A. Vincent-Salomon, C. Proudhon, F.-C. Bidard, W. Cacheux

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