

Circulating human papillomavirus DNA detected using droplet digital PCR in the serum of patients diagnosed with early stage human papillomavirus-associated invasive carcinoma

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Abstract

Specific human papillomavirus genotypes are associated with most ano-genital carcinomas and a large subset of oro-pharyngeal carcinomas. Human papillomavirus DNA is thus a tumour marker that can be detected in the blood of patients for clinical monitoring. However, data concerning circulating human papillomavirus DNA in cervical cancer patients has provided little clinical value, due to insufficient sensitivity of the assays used for the detection of small sized tumours. Here we took advantage of the sensitive droplet digital PCR method to identify circulating human papillomavirus DNA in patients with human papillomavirus-associated carcinomas.

A series of 70 serum specimens, taken at the time of diagnosis, between 2002 and 2013, were retrospectively analyzed in patients with human papillomavirus-16 or human papillomavirus-18-associated carcinomas, composed of 47 cases from the uterine cervix, 15 from the anal canal and 8 from the oro-pharynx. As negative controls, 18 serum samples from women with human papillomavirus-16-associated high-grade cervical intraepithelial neoplasia were also analyzed. Serum samples were stored at -80°C (27 cases) or at -20°C (43 cases). DNA was isolated from 200 μl of serum or plasma and droplet digital PCR was performed using human papillomavirus-16 E7 and human papillomavirus-18 E7 specific primers.

Circulating human papillomavirus DNA was detected in 61/70 (87%) serum samples from patients with carcinoma and in no serum from patients with cervical intraepithelial neoplasia. The positivity rate increased to 93% when using only serum stored at -80°C . Importantly, the two patients with microinvasive carcinomas in this series were positive. Quantitative evaluation showed that circulating viral DNA levels in cervical cancer patients were related to the clinical stage and tumour size, ranging from 55 ± 85 copies/ml (stage I) to 1774 ± 3676 copies/ml (stage IV).

Circulating human papillomavirus DNA is present in patients with human papillomavirus-associated invasive cancers even at sub-clinical stages and its level is related to tumour dynamics. Droplet digital PCR is a promising method for circulating human papillomavirus DNA detection and quantification. No positivity was found in patients with human papillomavirus-associated high grade cervical intraepithelial neoplasia.

Keywords: circulating HPV DNA; ddPCR; cervical carcinoma; anal carcinoma; carcinoma of the head and neck

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Introduction

One of the major concerns in clinical oncology lies in the ability to detect carcinomas at their earliest

stages of development, including primary, local relapses or metastases. One method of growing interest, preferred over biopsies of solid tumours, uses the less invasive, and potentially highly sensitive, 'liquid

biopsy' approach to detect circulating tumour DNA (ct-DNA) in the blood [1,2]. ct-DNA is described as liberated tumour DNA upon apoptosis or necrosis of tumour tissues that circulates in the blood [3]. It is known that ct-DNA carries specific bio-marker information from the tumour from which it is derived and, therefore, accurate detection of ct-DNA levels can be highly responsive to the tumour status, in particular allowing the detection of tumours at sub-clinical stages, before clinical and radiological lesions are detected [4]. Therefore, ct-DNA detection has great capacity for predicting early stages of cancer and for estimating minimal residual disease and relapses. Beyond its ability to identify the presence of tumours at sub-clinical stages, ct-DNA marker detection provides major advantages at several steps of medical management [2]: (I) for prognostic assessment: the quantity of ct-DNA is proportional to the tumour mass and thus represents a molecular marker indicating the severity of the disease; (II) as a predictive surrogate in response to tumour treatment: the levels of ct-DNA should provide excellent surrogate markers to sensitively reflect the status of the tumour during the course of treatment; (III) to provide markers for patient follow-up care: as ct-DNA is very specific, its detection should indicate a high risk of tumour relapse whereas its absence justifies avoiding deleterious adjuvant chemotherapy in patients without objective risk of tumour progression. However, before ct-DNA detection can be used in clinical practice, at least three major difficulties must be overcome: (I) the need for prior analysis of the primary tumour as a prerequisite to define specific molecular motifs, maintained during the duration of the disease; (II) the development of an assay with sufficient specificity and sensitivity; and finally (III) the need for a prospective study designed to correlate positive ct-DNA detection with subsequent clinical events.

To develop an optimized method of ct-DNA detection, we used the model of human papillomavirus (HPV)-associated carcinomas for ct-DNA detection. HPV is a double-stranded DNA virus of about 8 Kb in size, consisting of numerous polymorphic genotypes [5]. Specific HPV genotypes with oncogenic properties, among which HPV16 and HPV18 are the most prevalent, are able to infect the mucosa of the ano-genital tract [6,7] as well as the oro-pharyngeal region [8] and to induce intra-epithelial lesions that, after a long period, may develop into invasive carcinomas [9]. The physical state of the viral genome is different depending on the stage of the lesions. In intra-epithelial neoplasias, HPV genomes generally replicate as free episomal forms in the cell nucleus

whereas in most invasive carcinomas part of the viral DNA is integrated into the cell genome [10]. Viral mutational insertions, which are clonal and stable over time, are frequently associated with structural alterations of the host genome at the integration site. Co-amplification of viral and flanking cellular sequences may then lead to modified expression levels of nearby genes [11,12]. Thus, most cervical carcinoma cells harbour an integrated proportion of HPV DNA that contain the E6 and E7 oncogenes and/or contain entire viral episomes widely ranging from a few to several hundred copies per cell, that are heterogeneously distributed in the tumour cell population.

These molecular characteristics explain why HPV-associated tumours represent a privileged model for the analysis of ct-DNA in oncology. The extragenic nature of the viral marker, and its abundance upon subsequent amplification and/or presence of multiple episomal copies, facilitates HPV detection. In spite of this favourable context, there is a lack of sensitivity using classical quantitative Polymerase Chain Reaction (qPCR) detection methods. As observed for invasive cervical carcinomas, the rate of positivity reported for circulating HPV DNA (c-HPV DNA) detection ranges from 6.9% to 50% [13–19], corresponding to a global rate of 25.4% (141/556), an insufficient rate for clinical use. In a former study, we tried to overcome this limitation by examining the viral mutational insertion site markers that are highly sensitive and specific for each tumour [20]. For the same cases, we designed specific primers to detect unique viral-host junction sites in ct-DNA, as well as primers for HPV16 and 18 sequences in c-HPV DNA and showed that ct-DNA as well as c-HPV DNA was detectable in the serum of most patients diagnosed with invasive cervical carcinomas, but remained undetectable for those cases containing a limited tumour mass. Therefore, the detection of small amounts of ct-DNA related to minimal residual disease or to subclinical relapses requires an assay more sensitive than the classical qPCR approach. It is thus necessary to optimize the system of detection of c-HPV DNA as well as pre-analytic conditions.

Digital PCR is a new generation of PCR techniques based on the compartmentalization and amplification of a single DNA molecule. Among these new approaches, which offer a high level of sensitivity, droplet digital PCR (ddPCR) is particularly attractive. This technique allows massive partitioning of DNA into thousands of nanoliter-sized droplets, each containing zero or a few copies of DNA, which undergo separate end-point amplification. The fraction of PCR-positive droplets is then determined using a

flow cytometer and absolute quantification of the target DNA into the sample is estimated by modeling as a Poisson distribution. As compared to real time PCR, ddPCR offers an optimized approach for the sensitive detection and quantification of low-target-abundance biological samples [21].

Here, we performed a retrospective analysis to: (I) look for c-HPV DNA in cervical neoplasia at different stages of the disease, including intra-epithelial neoplasias and invasive carcinomas; (II) compare ddPCR versus qPCR methods for the detection of c-HPV DNA; (III) perform quantitative assessment of c-HPV DNA according to clinical and virological criteria; (IV) compare serum versus plasma as a source of c-HPV DNA; (V) compare quality of cryopreservation of c-HPV DNA in sera stored at -20°C versus -80°C ; and (VI) examine c-HPV DNA in other types of HPV-associated tumours, namely in anal canal and oro-pharyngeal carcinomas.

We report that HPV DNA sequences can be detected in the vast majority of HPV-associated carcinomas with this rather simple and sensitive ddPCR approach. Beyond the study of HPV-associated tumours, this work represents an indispensable developmental step for adopting liquid biopsies in standard clinical practice.

Material and methods

Patients

Serum from patients with HPV16 or HPV18-associated invasive cancer were collected at the time of diagnosis, and retrospectively selected between February 2002 and November 2013. In accordance with French regulation, all patients were informed and did not object to use of their biological specimens for research. Thus, a series of 70 sera were collected from: 47 patients with different stages of cervical cancer; from 15 patients with anal cancer (14 females, 1 male); from 6 patients with tonsillar cancer (3 females and 3 males); and 2 patients with oro-pharyngeal cancer (2 males). Sera from the 8 patients with head and neck cancer, and from 35/47 patients with cervical cancer were stored at -20°C whereas others (27 cases) were stored at -80°C . Plasma samples were also available and jointly analyzed with their matching serum in 27 cases. Serum samples from 18 patients with high grade HPV16-associated cervical intraepithelial neoplasia (CIN3), stored at -80°C , were also analyzed for detection of c-HPV.

Histologically, the cervical cancer cases were mostly identified as squamous cell carcinomas (36 cases), but also included seven adenocarcinomas, two adenosquamous carcinomas and two neuroendocrine carcinomas. Anal and head and neck cancers were identified as squamous cell carcinomas. Patient characteristics are given in supplementary material, Table S1.

DNA serum/plasma analyses

DNA was isolated in duplicate from 200 μl of serum or plasma, using the QIAamp Mini Elute Virus Spin Kit (Qiagen[®], Hilden, Germany) following manufacturer's instructions. The elution step was performed with 25 μl of supplied buffer. Isolated DNAs were assayed for c-HPV detection using two methods, first ddPCR and second real-time qPCR. Ten replicates were performed for each method. Human circulating DNA concentrations were measured using a qPCR system based on long interspersed nuclear element-1 sequence detection [22]. Dilutions of non-tumour liver human DNA were used as standards to quantify circulating human DNA and qPCR was performed as previously described [20]. ddPCR was performed to detect circulating HPV16 or HPV18 using the QX100 ddPCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Basically, 20 μl volumes containing ddPCR[™] Supermix for probes (Bio-Rad Laboratories, Inc.) at the final concentration 1x, HPV16 E7 or HPV18 E7 specific primers at 450 nM each, HPV16 E7 or HPV18 E7 specific TaqMan Probe at 250 nM (Applied Biosystems Life Technologies, Foster City, CA, USA), 7 μl nuclease-free water and 2 μl template were loaded into the middle wells of a droplet generator cartridge and 70 μl of droplet generation oil (Bio-Rad Laboratories, Inc.) was added to the lower wells. The primers for the HPV16 E7 and HPV18 E7 sequence detection were 5'-TCCAGC TGGACAAGCAGAAC-3' (forward primer), 5'-CACAACCGAAGCGTAGAGTC-3' (reverse primer) and 5'-AACATTTACCAGCCCGACGA-3' (forward primer), 5'-TCGTCTGCTGAGCTTTCTAC-3' (reverse primer), respectively. The 2 Taqman MGB probes were 5'-FAM-ACAGAGCCCATTACAAT-3' (HPV16 E7 probe) and 5'-FAM-AACCACAACGTACACAA-3' (HPV18 E7 probe). After droplet generation was complete, 40 μl of droplets were carefully transferred to a 96-well PCR plate, sealed and the following PCR program was used: 95°C for 10 min, followed by 40 cycles of 94°C for 30 s and 60°C for 1 min, followed by 98°C for 10 min, and holding at 10°C on a C1000 thermocycler. After PCR, the droplet reader performed the detection of complete ddPCR reactions in individual droplets. For each well, the data was analyzed

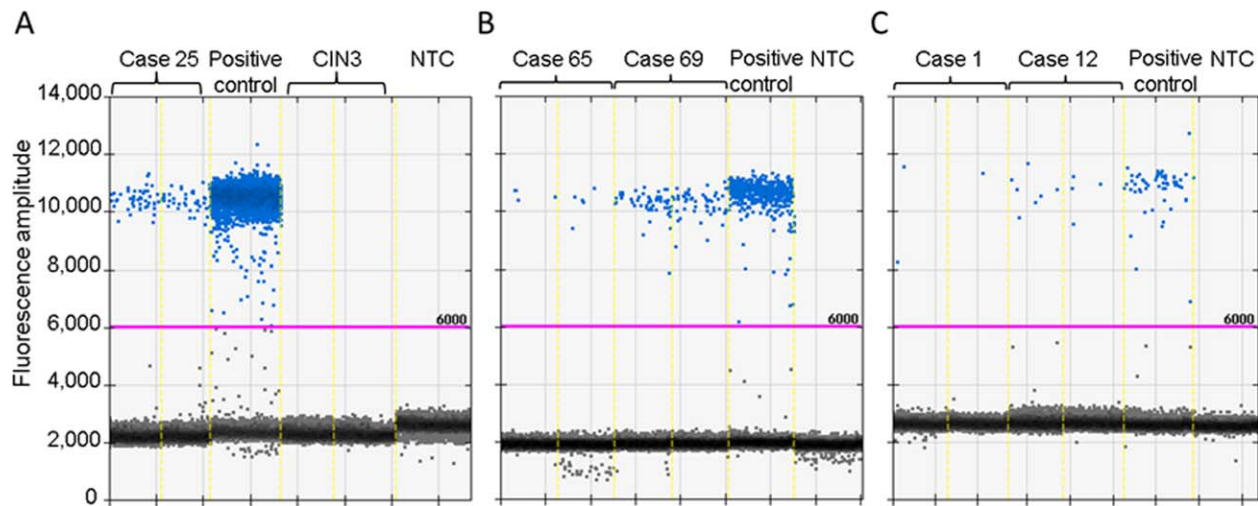


Figure 1. Detection of circulating HPV16 E7 DNA by ddPCR in serum samples from patients with HPV16-associated cervical carcinoma. (A) Stage II invasive cervical carcinoma (Case N°25); Positive plots are represented in blue (4530 copies/ml); No positive droplets detected from patients with CIN3 or from the negative control. (B) Positive detection in the serum of patients with tonsil (case N°65) and oropharyngeal (case N°69) carcinomas (215 and 4605 copies/ml, respectively). (C) Positive detection in the serum of two patients (cases N°1 and N°12) with micro-invasive cervical carcinoma (32 and 249 copies/ml, respectively). Positive DNA controls from HPV16 tumour tissues and non-DNA controls, replaced by elution buffer in no template control (NTC), are indicated.

using QuantaSoft software (Bio-Rad Laboratories, Inc.) which expressed the results in copies/ μ l. DNAs from HPV16 and HPV18 tumour tissues were used as positive controls. Positive HPV16 E7 and HPV18 E7 droplets clustered in a cloud at an amplitude of about 10 000 and 8000, respectively (Figure 1). The threshold for detection was set at 6000 and 5000 for HPV16 E7 and for HPV18 E7, respectively. Ten replicates of the ddPCR assay were performed on each serum specimen for the detection of c-HPV DNA. Serum samples were considered as positive when there was at least one droplet at the same amplitude as the positive control. The concentration of c-HPV was finally expressed in copies/ml of serum after considering that the sum of the HPV copies detected in the ten replicates represented the total amount of c-HPV in 200 μ l of serum sample. qPCR assays, using Sybr[®]-Green (Applied Biosystems Life Technologies), were performed as previously described to specifically amplify HPV16 E7 or HPV18 E7 DNA [20]. To compare the results with ddPCR, the same primers were used, using ten replicates and the HPV copy numbers detected in positive replicates, were summed. Final results were also expressed as copies/ml of serum.

In 27 cases, DNA from plasma samples was also isolated and processed exactly as the matching serum sample, to compare the amount of c-HPV obtained from both samples.

Viral load assessment

Viral loads were estimated by ddPCR in DNA isolated from formalin-fixed tissue blocks of cervical

cancer using the same conditions and the same set of primers and Taqman probes as described for serum analysis. Reactions were performed in duplicate, and the viral load was expressed in copies per cell from the merged value obtained by ddPCR.

Statistical analysis

The GraphPad Prism v-6.02 software (GraphPad Software, San Diego, CA, USA) was used for analyses. Positive rates between ddPCR and qPCR data were compared by the Fisher's test, c-HPV DNA levels between groups were compared by using the t-test with Welch's correction when variances were unequal. Spearman correlation was performed to analyze the relationship between the tumour size and the c-HPV DNA level. Results were considered significant at p value ≤ 0.05 .

Results

The first aim of our study was to assess c-HPV DNA levels in patients diagnosed with HPV16- or HPV18-associated invasive carcinomas, developed at various sites including the uterine cervix (47 cases), anal canal (15 cases) and head and neck (8 cases). Globally, ddPCR detection for all HPV-associated carcinomas yields a positive rate of 87.1% (61/70), a rate significantly higher than that obtained using classic qPCR

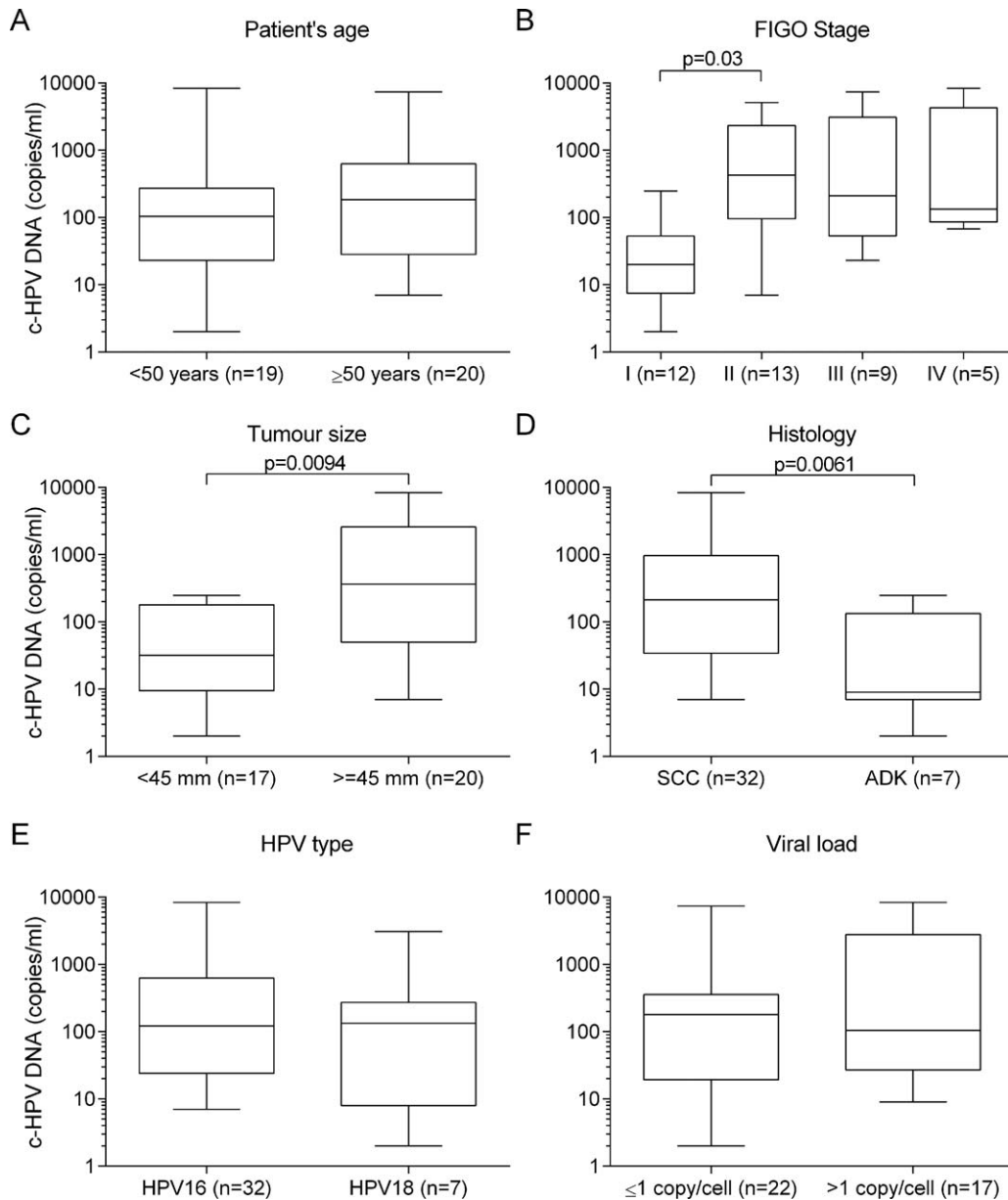


Figure 2. Rate of c-HPV DNA (log scale) detected using ddPCR in patients with cervical neoplasias according to clinical criteria. Statistical analysis using t-test [with Welch's correction for plots (B) to (F)]. In B only stage I and II were compared.

(48/70; 68.6%) ($p = 0.01$) (Figure 1 and Table 1). Serum samples obtained from patients with anal cancer revealed 14/15 positive cases using ddPCR versus 12/15 cases using qPCR and for head and neck cancers, 8/8 were positive using both PCR methods (Figure 1B). In contrast, none of the 18 serum specimens used as negative controls in women diagnosed with high grade CIN3, were found to be positive (Figure 1A and supplementary material, Table S2), demonstrating the specificity of the approach for detecting invasive cancers (Figure

1A). ddPCR is able to provide an absolute quantification of the molecular target. In the whole population, the mean c-HPV DNA level was 1216 ± 2178 copies/ml, ranging from 2 to 8349 copies/ml. The high SD values are related to the highly variability in c-HPV DNA content among cases. We further asked whether c-HPV DNA levels from the 47 patients with cervical carcinomas were correlated with clinical and biological parameters. We compared c-HPV DNA data obtained from ddPCR with the patients' age, the International Federation of

Table 1. Detection rate of c-HPV DNA using ddPCR and qPCR in patients diagnosed with HPV-associated invasive carcinoma

Tumour localization	Number of cases	Positive cases		<i>p</i> *
		ddPCR N (%)	qPCR N (%)	
Uterine cervix	47	39 (83)	28 (60)	0.02
Anal canal	15	14 (93)	12 (80)	0.60
Head and Neck [†]	8	8 (100)	8 (100)	1.00
Total	70	61 (87)	48 (69)	0.01

*Fisher's exact test.

[†]Tonsil and oropharynx.

c-HPV DNA, circulating HPV DNA; ddPCR, droplet digital PCR; qPCR, real time quantitative PCR.

Gynaecology and Obstetrics (FIGO) clinical stage, tumour size, HPV genotype, histology and viral load in the tumour tissue. We found that the c-HPV DNA levels were mainly correlated with the tumour mass, in other words with the clinical stage and tumour size, and to a lesser extent, with the viral load (Figure 2).

Strikingly, the rise in DNA levels was much higher (more than 20 fold) between stage I (55 ± 85 copies/ml) and stage II (1278 ± 1785 copies/ml) tumours ($p = 0.03$), than between stages II and III (1615 ± 2719 copies/ml) or between stages III and IV (1774 ± 3676 copies/ml) tumours (Figure 2B and supplementary material, Table S2). We further compared the c-HPV DNA levels and tumour size (median 45 mm) evaluated in millimeters by CTscan imaging. c-HPV DNA levels were higher (1627 ± 2614 copies/ml) in tumours ≥ 45 mm, than in tumours < 45 mm (82 ± 89) ($p = 0.02$) (Figure 2C and supplementary material, Table S2). A case-by-case comparison shows the positive correlation between c-HPV DNA levels and tumour sizes ($r = 0.51$; $p = 0.001$) (Figure 3). Importantly, this analysis also reveals that c-HPV DNA is already present at detectable levels in cases with a minimal tumour mass. Therefore, it is striking that ddPCR, unlike qPCR, allows positive c-HPV DNA detection in two cases with micro-invasive carcinomas of the cervix and only 1 mm sized tumours (Figure 1C).

We also detected a trend between the viral load in the tumour tissue and c-HPV DNA levels found in the patient serum (Figure 2F). By comparing HPV16 and HPV18 genotypes, we observed detection of c-HPV DNA more frequently in patients with HPV16 (32/36; 89%) than with HPV18 (7/11; 64%) carcinomas. This might be related to a higher viral load present in HPV16- (4.9 ± 6.9 copies/cell) than in HPV18- (0.6 ± 0.8 copies/cell) tumours ($p = 9 \times 10^{-4}$) (supplementary material, Figure S1). By comparing with histological data, c-HPV DNA detection was also more frequently found in patients with squa-

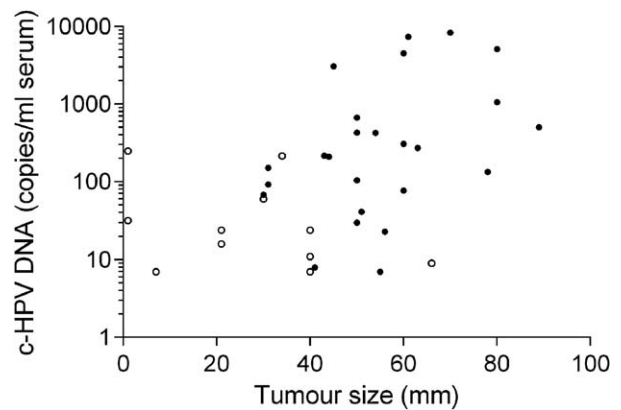


Figure 3. Positive correlation between c-HPV DNA level (log scale) and tumour size in patients with cervical cancer ($n = 37$). Spearman correlation: $r = 0.51$ ($p = 0.001$). (○) Stage I and (●) stage II/III/IV tumours.

mous cell carcinomas than in patients with adenocarcinomas (Table 2).

The second aspect of our approach examined the best pre-analytical procedures for the optimized detection of c-HPV DNA. We separately analyzed the rates of positivity of c-HPV DNA in serum samples stored at -80°C or at -20°C , – for one to 12 years-, and compared serum and plasma as respective sources of c-HPV DNA in patients with HPV-associated carcinomas. A high rate of positivity was obtained when the c-HPV DNA analysis was restricted to serum preserved at -80°C : c-HPV DNA was detected by ddPCR in 25/27 (93%) of these specimens (Table 2). One of the two negative sera corresponded to a cervical carcinoma with a low viral load (< 1 copy/cell) and the second to a serum specimen stored for the longest period (7 years). Using serum preserved at -20°C , a positive ddPCR result was obtained in 33/40 (83%) cases, but, using qPCR, the positivity rate dropped showing 22/27 (81%) positive cases for serum preserved at -80°C and only 23/40 (58%) positive cases for serum stored at -20°C .

We also compared serum and plasma as respective sources of c-HPV DNA in a subset of 27 cases for which both types of specimens, taken the same day

Table 2. c-HPV DNA detected in serum samples stored at -20°C or -80°C in patients with HPV-associated carcinomas

Storage temperature	Number of cases	Number (%) of positive cases		<i>p</i> *
		ddPCR	qPCR	
-20°C	43	36 (84)	26 (60)	0.03
-80°C	27	25 (93)	22 (81)	0.42

*Fisher's exact test.

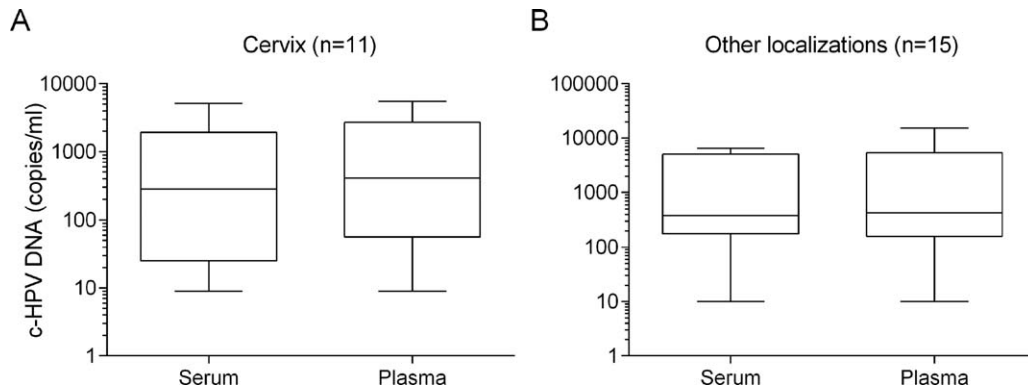


Figure 4. Comparison of c-HPV DNA levels (log scale) detected using ddPCR in serum and plasma samples from patients diagnosed with HPV-associated invasive carcinoma of the uterine cervix (A) or the anal canal or oropharyngeal region (B).

at diagnosis, were available for c-HPV DNA detection and quantification using ddPCR. Qualitatively, this comparative analysis did not provide significant differences. However, quantitative evaluation showed that, in patients with cervical cancer, the mean levels of c-HPV DNA in plasma were slightly higher (1360 ± 2003 copies/ml) than that found in serum (1101 ± 1881 copies/ml) ($p = 0.09$) (Figure 4). This trend was also observed in patients with anal and head and neck tumours (2797 ± 4298 copies/ml in plasma versus 1993 ± 2556 in serum) ($p = 0.16$).

Discussion

We report improved detection of c-HPV DNA using the sensitive ddPCR approach, in which E7 DNA is identified in the large majority of patients with HPV-associated invasive carcinomas, regardless of the location of the primary tumour. The 87% positivity rate observed in this retrospective study further rose to 93% when using only serum samples stored under optimal conditions (-80°C). Importantly, we also observed c-HPV DNA in the blood of two patients with sub-clinical micro-invasive carcinomas, indicating that release of c-HPV DNA is a concurrent event with the early step of tumour invasion. In these two patients, the blood specimens for c-HPV DNA analysis were taken a few hours following the tumour biopsies performed for histological diagnosis and we cannot rule out the possibility that this sequence favoured the detection of c-HPV DNA. Nevertheless, ddPCR proved also to be efficient for the detection of c-HPV DNA in patients with stage I tumours, independently of any biopsy procedure. In literature, ddPCR was found to be more sensitive than qPCR for the accurate measurement of HIV DNA in blood

specimens [23]. This is in line with our data indicating that, in patients with HPV-associated cancer, the biological detection of sub-clinical tumour mass, such as those corresponding to minimal residual disease or early relapse, can be performed using this sensitive and robust technology. In contrast with the high rate of positivity in patients with invasive HPV-associated carcinoma, none of the patients with HPV16-associated high-grade CINs was found positive. This confirms that no tumour DNA is released at detectable levels in the blood of patients with non-invasive tumours, an observation previously described [13–15]. To our knowledge, no data to date are available for the presence of c-HPV DNA in patients with anal cancers. Recently, a high rate of HPV16 circulating DNA was reported in head and neck carcinomas [24], in which most cases corresponded to advanced stage IV tumours. In large series of non HPV-associated tumours, ct-DNA was detected in 50–73% of the cases [25].

Additional assets of ddPCR include its ability to provide absolute target quantification [23]. Looking at c-HPV DNA levels, we found a positive correlation with the clinical stage and/or tumour size, showing that the dynamics of the tumour marker is related to the tumour mass. A positive correlation was also observed with the viral load in the tumour tissue, indicating that this parameter has to be taken into account when assessing the baseline levels of c-HPV DNA at diagnosis. Regarding its sensitivity and specificity, our methodology should favour prospective studies designed to assess, in clinical practice, the value of c-HPV DNA levels as surrogate markers for tumour dynamics, under treatment and during the patient' follow-up. Negative serology should be observed at the end of treatment whereas, during follow-up, a positive test could precede tumour relapse before the development of any clinical or

radiological evidence of tumour. The efficiency of specific antitumour therapies, such as immunotherapies, would be optimal in cases with a minimal tumour mass, a strategy requiring a test with both high sensitivity and specificity. The HPV genotype is a molecular marker of the primary tumour that is also present in tumour relapses. Furthermore, in our previous work, we have shown that HPV integration mutations can be used for the highly specific detection of ct-DNA in patients with cervical cancer [20]. However, regarding the large heterogeneity of the HPV status in tumour cells (physical state, pattern of integration including viral and cellular sequences), this approach requires detailed molecular characterization of the tumour. Technologies based on the direct capture/sequencing of HPV DNA are able to provide extensive characterization of genetic changes related to HPV integration [26]. This characterization should help to determine, for every patient, the molecular features that can be used as highly specific surrogate markers for an optimal serological follow-up using PCR approaches. NGS-based technologies can also be applied to the isolation and characterization of c-HPV DNA and numerous bio-clinical applications should derive from such emerging approaches, among which the detection of invasive carcinomas associated with any HPV genotype using only a blood test. From this perspective, our present work showing the presence of c-HPV DNA in the vast majority of carcinomas at all clinical stages and tumour localization sites represents a significant contribution.

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Author contributions

XSG conceived and supervised the study, EJ and XSG designed experiments, EJ, MC, MAC and EL performed experiments, EJ, VB, MC, MAC and EL analyzed data, SS and ER collected serum specimens, EJ, VB, ER, SS, AH, DB and XSG interpreted data and EJ, AH and XSG wrote the manuscript. All authors reviewed and provided editorial comments on the manuscript.

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SUPPLEMENTARY MATERIAL ONLINE

Table S1. Clinical and biological data

Table S2. Rate of c-HPV DNA detected by ddPCR in patients with cervical neoplasia according to clinical criteria

Figure S1. Viral load and HPV type. Higher average HPV16 load (4.9 ± 6.9 copies/cell) than HPV18 load (0.6 ± 0.8 copies/cell) in cervical carcinomas ($p = 0.0009$) (t-test with Welch's correction)