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HPV virus and biomarkers of resistance to chemoradiation in circulating tumor cells from patients with squamous cell carcinoma of the anus

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ABSTRACT

Localized anal cancer is mostly represented by squamous cell carcinoma of the anus (SCCA) and is cured in ≥80 % of cases by chemoradiation (CRT). Development of techniques for detection/evaluating circulating tumor cells (CTCs) for diagnosis/ prognosis/response to therapy can change the manner we treat/follow SCCA patients. Objective: to detect CTCs from patients with SCCA and evaluate the presence of HPV virus, p16 expression and markers related to resistance to CRT (RAD23B/ ERCC1/ TYMS) in CTCs at baseline and after CRT. Methods: CTCs were isolated/quantified by ISET®, protein expressions were analyzed by immunocytochemistry and HPV DNA was detected by chromogenic in situ hybridization. Results: We enrolled 15 patients: median age was 61 (43–73) years, the majority was women (10/15). CTCs were detected in all patients at baseline (median= 0.4 (0.4–3.33) CTCs/mL) and in 8/9 patients, after CRT (median= 2.33 (0–7.0) CTCs/mL). DNA from HPV was found in CTCs in 14/15 patients (93.33 %) at baseline and in 7/9 (77.7 %) after treatment. At a median follow-up of 22.20 (1.45−38.55) months, three patients expressed ERCC1 in CTCs after treatment, with one of them having disease recurrence. Conclusion: We showed that detection of HPV in CTCs from patients with non-metastatic SCCA is feasible and appears to be a sensitive diagnostic method. These results may be clinically useful for better monitoring these patients. However, future larger cohorts may demonstrate whether there is any correlation between the presence of HPV and the expression of screening markers for CRT in SCCA.

1. Introduction

Anal canal cancer, mostly represented by squamous cell carcinoma of the anus (SCCA), is a rare solid tumor. Global cancer statistics estimated more 50,000 new cases in 2020, with nearly 19,000 deaths [1]. In Brazil, this cancer was responsible for 893 deaths, 393 men and 500 women, in 2019 [2]. Generally being a human papillomavirus (HPV)-related malignancy, the main risk factors for SCCA are unprotected anal sexual intercourse, and immunosuppressive conditions, such as chronic use of immunosuppressant drugs and HIV infection [3]. The standard treatment for localized SCCA is definitive chemoradiation (CRT) with a fluoropyrimidine and mitomycin. In Brazil, because of the unavailability of mitomycin, cisplatin has been utilized as a substitute for mitomycin, based on the results of similar efficacy of both CRT regimens [4]. Nearly 70–80 % of patients are cured by definitive CRT; yet, a significant proportion will experience recurrence or persistent of disease after CRT. Although CRT reduces the absolute risk of recurrence and decreases the colostomy rate, about 10–30 % of patients experience disease progression and 25–40 % patients have a colostomy within 3 years of diagnosis [5]. Therefore, adding other agents and modifying the treatment strategy may be important to improve disease control, decreasing the need for colostomy and the toxicity of the treatment. In this sense, maybe, the

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addition of immune checkpoint inhibitors can be useful, as shown for many solid tumors, including SCCA [6]. PD-1 inhibitors, as pembrolizumab and nivolumab have been approved for the treatment of metastatic anal squamous cell carcinoma [7]. However, the role of the anti-PD-1 in the locoregional disease of SCCA is still under investigation. Considering that these therapies can be added in this scenario, biomarkers of PD-1 and to resistance to CRT in SCCA need to be studied.

Concerning biomarkers in SCCA, the cyclin-dependent kinase 2 A (CDK2N) gene encodes for the p16 protein, whose expression can be evaluated by immunohistochemical staining (IHC). Positive IHC expression of p16 is often observed in HPV-associated tumors and is considered a surrogate marker of HPV involvement in carcinogenesis. [8] IHC expression of p16 is an independent prognostic marker for overall survival and disease-free survival in patients with SCCA treated with CRT [9]. However, other factors associated with resistance to CRT beyond HPV infection have been poorly investigated in SCCA.

Assessment of circulating tumor cells (CTCs), a component of liquid biopsy, is an emerging technology that can provide valuable insights into tumor biology [10]. The investigation of CTCs has not been performed in SCCA. Protein expression of some drug resistance markers such as RAD23B, TYMS and ERCC1, could be evaluated in these cells. RAD23 homolog B (RAD23B) is a DNA repair protein. Some studies have correlated its expression with radiotherapy resistance [11,12]. Our group had previously shown that baseline RAD23B expression in CTCs from patients with locally advanced rectal cancer was an independent prognostic factor for absence of pathological complete response [13]. Thymidylate synthase (TYMS) is an enzyme that participates in one of the 5-FU metabolism pathways. The amount of TYMS expression in the tumor appears to be a predictor of response in patients treated with 5-FU. However, despite many studies reporting poor overall survival in tumors with high TYMS expression, the real prognostic value of this enzyme has not yet been established [14]. In a previous study of our group, with patients with metastatic colorectal cancer, we showed that the expression of TYMs in CTCs, but not in primary or metastatic tissue, correlated with poor progression free survival [15]. The ERCC1 gene is essential in the nucleotide excision repair of DNA damage that distorts the helix, particularly DNA damage caused by ultraviolet light. The increased expression of ERCC1 in patients when exposed to cisplatin treatment has been described in the literature as a poor prognostic factor, associated with worse overall survival [16]. No data is available concerning the expression of this protein in SCCA.

In this study, we aimed to evaluate the presence of CTCs in the blood of patients with localized SCCA treated with CRT, particularly the presence of HPV virus in these cells, and to evaluate the expression of cell markers that could be associated with resistance to CRT. The markers examined were the inflammatory PD-L1 protein, and proteins potentially associated with resistance to radiotherapy (RAD23B), cisplatin (ERCC1) and fluoropyrimidine (TYMS) in this setting.

2. Patients and methods

2.1. Patients and study design

This was a prospective study carried out at A.C Camargo Cancer Center, a large comprehensive cancer center located in São Paulo, Brazil. Eligible patients had histologically-proven SCCA of localized stages (clinical staged I to III) and were candidates to initiate definitive CRT with cisplatin and a fluoropyrimidine. Two serial blood samples were collected in EDTA tubes (10 mL) from patients who gave written informed consent to this study. The first sample was collected within 10 days of the first day of chemotherapy associated or not to radiotherapy (CT or CRT) (baseline/T1) and the second one, after 6–8 weeks after the end of CT or CRT (T2), when patients had radiological and clinical evaluation of treatment response.

The following patients' clinicopathological data were obtained prospectively: gender, age, HIV infection status, clinical tumor stage, ECOG *performance status*, treatment response (complete versus incomplete response or progression) and follow up.

This study was approved by the local research ethic committee (protocol 2669/19).

2.2. Isolation of circulating tumor cells by ISET®

Blood samples were drawn and assembled in EDTA tubes (BD Vacutainer®) with immediate gentle agitation after blood collection. Samples were processed in ISET® (Isolation by SizE of Tumors, Rarecells, France) as manufacturer's instructions. Eight mL of whole blood was diluted in up to 80 mL with buffer containing 0.02 % formaldehyde, incubated for 10 min in room temperature and filtered through a membrane with an 8 μm pore size. To preserve cell integrity, the filtration pressure was optimized to −10 kPa. The membrane was then washed once with phosphate-buffered saline (PBS). After processing, filters were dried, wrapped in an aluminum sheet and stored frozen at −20 °C until use.

2.3. Immunocytochemistry (ICC)

The spots membranes of ISET blocks were submitted to dual color immunocytochemistry (ICC) (DAB+/Permanent Red; DakoTM) on 24 wells plate. Antigen retrieval was then performed using the Antigen Retrieval Solution (DakoTM). Cells were hydrated with tris- buffered saline (TBS) 1X for 20 min and permeabilized with TBS + Triton X-100 for 5 min and endogenous peroxides were blocked with 3 % hydrogen peroxide in the dark for 15 min. The spots were incubated with antibodies diluted in TBS 10 % fetal calf serum. To amplify the antibody signal, the spots were incubated with Envision G/2 Double stain System, Rabbit/Mouse (DakoTM) followed by 10 min of incubation with DAB+/Permanent Red (DakoTM). The spots were then washed with PBS between the steps. Cells were stained with hematoxylin and analyzed by light microscope (Research System Microscope, BX61. Olympus, Tokyo, Japan) coupled to a digital camera (SC100–Olympus).

CTCs were characterized based on the following criteria: negative staining for CD45, nucleus size ≥12 μm, hyperchromatic and irregular nucleus, visible presence of cytoplasm, and a high nucleus–cytoplasm ratio [17]. Negative and positive controls were performed for each ICC staining. We used cell lines spiked in healthy blood, which, accordingly to The Human Protein Atlas (http://www.proteinatlas.org/), express or not the analyzed proteins. The cell lines were acquired from ATCC® HTB-43™ (Fig. 1). For all expression analyses, cells were classified as positive (any staining) or negative ICC expression.

2.4. Chromogenic in situ hybridization

To perform the qualitative detection of HPV DNA types 16/18/31/33/35/39/45/51/52/56/58/59/66/68/82 by chromogenic in situ hybridization, we used ZytoDot (REF: T.1140–400) spot on ISET membranes. The membranes were hydrated with TBS 1X for 5 min in room temperature and then with 1 % formaldehyde for 5 min. Subsequent washes between the steps were performed with distilled water. The membranes were incubated in Hydrogen Peroxide for 10 min in room temperature and in the dark. After washing with distilled water, the membranes were incubated with pepsin cytology (ZytoVision) for 5 min also in room temperature. Membranes were washed in 70 %, 90 % and 100 % ethanol for one minute each. After dried, the membranes were incubated with the HPV high-Risk (HR) (10 μL) probe in the hybridizer at 75ºC (wet) for 5 min, and then, incubating overnight at 37ºC. After this, the washings were performed with the Wash Buffer SSC (WB1) for 5 min. Membranes were incubated with Wash Buffer SSC (WB1) at 80ºC (5 min). After washing, membranes were incubated with Anti-DIG/DNP-Mix (AB14) for 15 min at 37ºC (wet) in the hybridizer. After washing, HRP / AP- POLYMER-MIX (AB13) was applied, followed by AP-Red Solution (1–2 drops) in room temperature. Membranes were

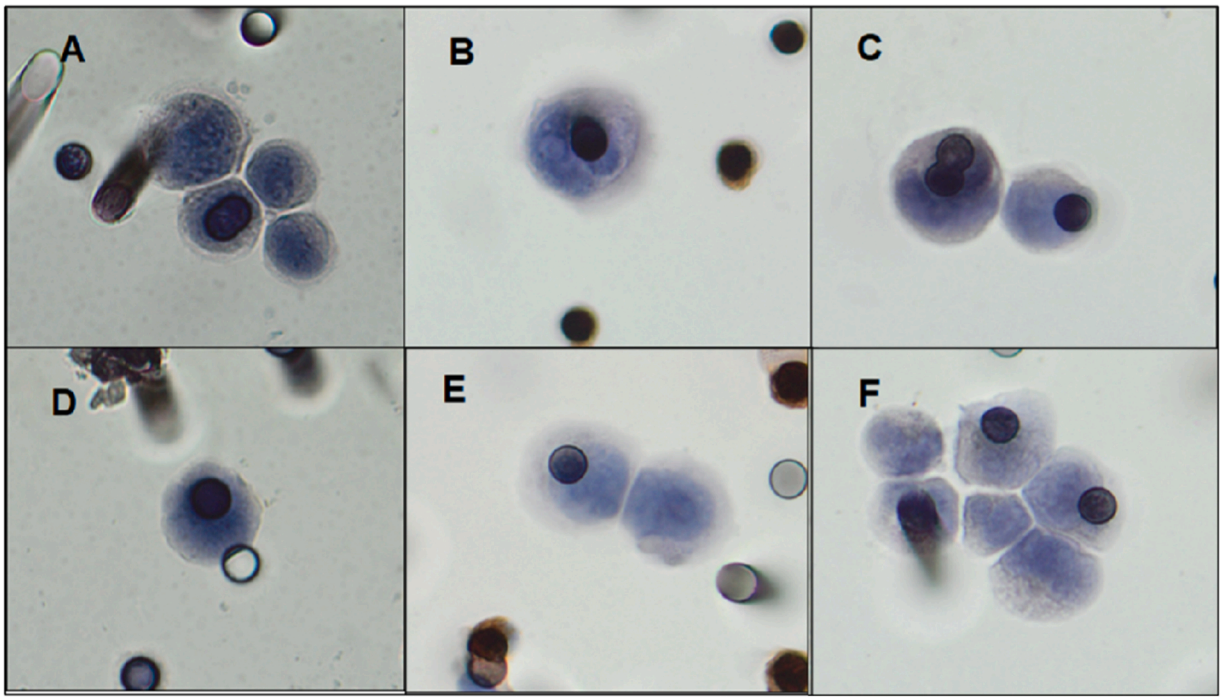

Fig. 1. Immunostaining of positive and negative controls. A) Positive control. MCF7 cell line of breast cancer "spiked" in healthy blood and stained for p16 (x60); B) Negative control. MCF-7 cell line "spiked" in healthy blood, without staining for CD45; C) Positive control. MCF7 cell line "spiked" in healthy blood and stained for ERCC1 (x60); D) Positive control. SKOV (ovarian cancer cell line) "spiked" in healthy blood and stained for TYMS. E) Positive control, A549 (lung cancer cell line) "spiked" in healthy blood and stained for RAD23B. F) Positive control. MCF7 cell line "spiked" in healthy blood and stained for PD-L1. All positive controls were stained with DAB. Photomicrographies were taken using a light microscope (Research System Microscope BX61 - Olympus, Tolyo, Japan) coupled to a digital camera (SC100 - Olympus, Tokyo, Japan).

counterstained with 50 % hematoxylin for 2 min in room temperature after washing. The slides were adhered with aqueous mounting medium (DAKO) and coverslip. The reading was performed under a bright field microscope. For positive controls, we used Ca Ski cell line spiked in healthy blood, which contains HPV accordingly to ATCC® (https://www.atcc.org/products/crl-1550). This cell line was acquired from ATCC® CRM-CRL-1550. For negative control, we used HCT 116 spiked in healthy blood, which do not contain HPV accordingly to ATCC® (https://www.atcc.org/products/ccl-247) and was acquired from ATCC® CCL-247™.

2.5. Immunohistochemistry (IHC)

Immunohistochemistry reaction was performed using the following marker: p16 clone (E6H4-Ventana), in a representative section of the neoplasm, in whole slides. Reactions were performed on the VentanachMark XT automated system (Ventana Medical Systems) following the manufacturer's instructions. Briefly, slides were deparaffinized using EZprep solution (cat. 950–102, Ventana Medical Systems, Inc., Tucson, AZ, USA). Antigen retrieval was performed with Cell Conditioning 1 (cat. 950–224, Ventana Medical Systems, Inc.) or Cell Conditioning 2 (cat. 950–223, Ventana Medical Systems, Inc.) reagents at 95 °C. Specification of the detection, recovery and amplification kit for each antibody are described in Table 1. After immunohistochemistry assay, slides were mounted with Entellan mounting medium (cat. 107961, Merck) and developed for histopathological evaluation. Reactions were always positive for the positive control, and known to be for the antibody tested. The slides were read and the results were performed in relation to common positivity, when they presented totally strong and diffuse expression in the cytoplasm or negativity, when they presented negative expression or focal staining.

2.6. Statistical analysis

Descriptive statistics were used to summarize the results. Comparisons between median CTCs counts and binary variables were performed by the Fisher exact test. P value < 0.05 was considered significant. All statistics were performed in SPSS version 24.0 (IBM, Armonk, NY).

3. Results

3.1. Clinical pathological variables

Fifteen consecutive patients with localized SCCA and treated with CT or CRT were enrolled from June 2019 to November 2021. Their median age was 61 years (range: 43–73), all had a HIV negative serology test, 10 were women and 5 were men. Ten patients presented clinical nodal involvement (N1) and 11 had clinically stage III SCCA.

We finished our recruitment in November, 2021 and updated the follow up recently. At 6–8 weeks post CT or CRT, 11 (73.33 %) patients achieved complete clinical response (CCR). One (6.6 %) experienced disease recurrence (DR), one patient died from acute diverticulitis and treatment-related sepsis during CRT, in 1.45 months, two (13.3 %) patients withdrew consent and were lost to follow up before response evaluation. The median follow up in August 2022 was 22.2 months. All patients who achieved CCR at 6–8 weeks remained disease-free until August, 2022. Of both patients with lost to follow up, one showed partial response (PR) with persistent tumor which was confirmed by a biopsy performed 7 months after CRT, and salvage surgery was proposed. The other one also had a positive local biopsy performed and salvage surgery was proposed.

Baseline blood samples (T1) were obtained from all 15 patients; at T2, samples were collected from 9 patients: three patients missed appointments, two withdrew consent and one died before T2.

Table 1
Clinical data, protein expression in CTCs and DNA from HPV presence.

	Baseline collection (T1)								First follow-up collection (T2)							Clinical Data					
Patients	CTC/mL	p16 (primary tumor)	HPV CISH	p16	PD-L1	Rad23B	TYMS	ERCC1	CTC/mL	HPV CISH	p16	PD-L1	Rad23B	TYMS	ERCC1	Clinical Response	Treatment	T	N	M	clinical stage
1	3.33	+	*	–	–	+	+	+	2.66	+	–	–	+	–	–	CCR	CDDP + 5FU cc CRT	3	1	0	3
2	0.33	+	+	–	–	–	–	–	2.66	+	–	–	–	+	+	UNK	CDDP + Capecitabine	3	1	0	3
3	0.33	+	+	–	–	–	–	–	0	–	–	–	–	–	–	CCR	CDDP+ 5FU cc CRT	4	0	0	3
4	1.3	+	+	–	–	–	–	–	0.4	+	–	–	–	–	–	CCR	CDDP+ 5FU cc CRT	3	0	0	2
5	1	+	+	–	–	–	+	–	death	death	death	death	death	death	death	death	CDDP+Capecitabine	2	0	0	2
6	1	+	+	–	+	+	+	–	6,33	+	–	–	–	–	+	DR	CDDP+ 5FU cc CRT	4	1	0	3
7	0.4	+	+	*	*	*	*	*	2,33	+	–	–	–	–	–	CCR	CDDP+ 5FU cc CRT	2	1	0	3
8	0.33	+	+	–	–	–	–	–	0.4	+	–	–	–	–	–	CCR	CDDP + Capecitabine	2	1	0	3
9	0.33	+	+	–	–	–	–	–	7	+	–	–	–	–	+	CCR	Mitomycin + 5FU	1	1	0	3
10	0.4	+	+	–	–	–	–	–	1,6	–	–	–	–	–	–	CCR	CDDP+ 5FU cc CRT	2	0	0	2
11	0.4	+	+	–	–	–	–	–	–	#	**	**	**	**	**	CCR	CDDP+ 5FU cc CRT	2	1	0	3
12	0.33	+	+	–	–	–	–	–	–	#	**	**	**	**	**	UNK	CDDP+ 5FU cc CRT	2	1	0	3
13	0.66	+	+	–	–	–	–	–	–	#	**	**	**	**	**	CCR	CDDP+ 5FU cc CRT	2	1	0	2
14	2.33	+	+	–	–	–	–	–	–	#	**	**	**	**	**	CCR	CDDP + Capecitabine	1	0	0	1
15	0.66	+	+	–	–	+	–	–	–	#	**	**	**	**	**	CCR	CDDP+ 5FU cc CRT	4	1	0	3

3.2. Isolation and identification of circulating tumor cells and protein expression

CTCs were identified in all patients at T1, with a median of 0.4 CTC/mL (0.4–3.33 CTCs/mL). At T2, of 9 patients who had samples collected, the median CTCs was 2.33 CTC/mL (0–7 CTCs/mL); one patient had no CTC at T2.

Table 1 describes the characteristics of patients according to CTCs counts, HPV presence and ICC expressions. We observed that patients with clinically involved lymph nodes had higher CTC counts at T2, considering the median of CTCs equal of 2.33 CTC/mL ($p = 0.048$) (Table 2).

Among 9 patients who had CTCs measured at T1 and T2, 7 had a CCR: 3 had decreased and 3 had increased CTCs levels from baseline. The patient that showed disease recurrence had a considerable increase in CTC numbers, to 1 CTC/mL to 6.33 CTCs/mL (Table 1).

The p16 expression was not found in any CTC in any moment. One out of 15 patients showed CTCs which expressed PD-L1 at C1. Correlations between median CTCs counts at T1 ($\geq$ 0.4 CTCs/mL) and RAD23B and TYMS expressions ($p = 0.096$) and between median counts of CTC ($\geq$ 2.33 CTCs/mL) at T2 and ERCC1 expression ($p = 0.119$) were not statistically significant (Table 2). Interestingly, among three patients that expressed ERCC1 at T2, one showed disease recurrence, one was lost to follow up and other achieved CCR. The patient who presented tumor recurrence had positive CTCs at T1 for TYMS, RAD23B and PD-L1 expression (Fig. 2). It is interesting also to observe that one patient who achieved CCR was ERCC1 positive at T1 and ERCC1 negative at T2. No significant associations were found for any other clinical or pathological features (Table 1).

3.3. Identification of HPV in the primary tumor and in circulating tumor cells

Using the CISH technique (Table 1 and Fig. 3), HPV was detected in CTCs of 14 out of 15 (93.33 %) patients (one patient had no CTC in the evaluated spot) at T1 and in 7 out of 9 patients (77.7 %) at T2. HPV was detected at T1 in all patients.

By immunohistochemistry, p16 was analyzed in primary tumor samples from patients included in this study (data form medical records). All patients had p16 expression in their samples (Table 1).

Abbreviations: CDDP: cis-diaminodichloroplatinum; cc: concomitant; CRT: Chemoradiotherapy, CCR: clinical complete response, 5FU: 5-Fluorouracil; PR: partial response, UNK: unknown (lost to follow-up), DP: Disease Recurrence.

no blood collection, * no cell in the spot, * * no collection.

Abbreviations: CR: complete response; ND: non determined; PR: partial response.

4. Discussion

Here, we showed for the first time that it was possible to isolate CTCs from patients with SCCA by filtration method and also to identify the HPV virus in these cells by the CISH technique.

Analysis of CTCs in HPV virus-related tumors has already been reported by others. A study evaluated the expression of HPV16 E6/E7 in CTCs of 22 patients with oropharyngeal squamous cell carcinoma, through a RT-QPCR assay, and its correlation with pre-therapy tumor biopsy results, by immunohistochemistry. The authors showed that it was possible to detect the HPV virus in CTCs, in patients with this type of tumor [18], corroborating with our data, demonstrating that the identification of CTCs as a form of noninvasive diagnosis. Another study isolated CTCs by flow cytometry from patients with anal canal cancer. CTCs were identified in 7 out of 8 patients, however, without protein and viral analysis of these cells [19].

Molecular assessment of cancer-associated viruses is acting as a model for new methodologies for ctDNA analysis and tests with clinical

Table 2-
Correlation among categorized variables and median CTC counts.

	CTC1				CTC2			
Variables	Category	>med n (%)	<med n (%)	*p* value	Category	>med n (%)	<med n (%)	*p* value
T	T1/T2	3 (42.9)	6 (75.0)	0.231	T1/T2	2 (40.0)	2 (50.0)	0.643
	T3/T4	4 (57.1)	2 (25.0)		T3/T4	3 (60.0)	2 (50.0)	
	Total	7 (100.0)	8 (100.0)		Total	5 (100.0)	4 (100.0)	
N	N0	3 (42.9)	2 (25.0)	0.427	N0	0	3 (75.0)	0.048
	N1	4 (57.1)	6 (75.0)		N1	5 (100.0)	1 (25.0)	
	Total	7 (100.0)	8 (100.0)		Total	5 (100.0)	4 (100.0)	
PD-L1	Positive	1 (14.3)	0	0.500	Positive	0	0	
	Negative	6 (85.7)	7 (100.0)		Negative	5 (100.0)	4 (100.0)	
	Total	7 (100.0)	7 (100.0)		Total	5 (100.0)	4 (100.0)	
RAD23B	Positive	3 (42.9)	0	0.096	Positive	1 (20.0)	0	0.556
	Negative	4 (57.1)	7 (100.0)		Negative	4 (80.0)	4 (100.0)	
	Total	7 (100.0)	7 (100.0)		Total	5 (100.0)	4 (100.0)	
TYMS	Positive	3 (42.9)	0	0.096	Positive	1 (20.0)	0	0.556
	Negative	4 (57.1)	7 (100.0)		Negative	4 (80.0)	4 (100.0)	
	Total	7 (100.0)	7 (100.0)		Total	5 (100.0)	4 (100.0)	
ERCC1	Positive	1 (14.3)	0	0.500	Positive	3 (60.0)	0	0.119
	Negative	6 (85.7)	7 (100.0)		Negative	2 (40.0)	4 (100.0)	
	Total	7 (100.0)	7 (100.0)		Total	5 (100.0)	4 (100.0)	
Clinical Response	CR	5 (71.4)	6 (75.0)		CR	3 (60.0)	4 (100.0)	0.278
	death	1 (14.3)	0		death	2 (40.0)	0	
	ND	0	1 (1.5)		ND	5 (100.0)	4 (100.0)	
	PR	1 (14.3)	1 (12.5)		PR			
	Total	7 (100.0)	8 (100.0)		Total			

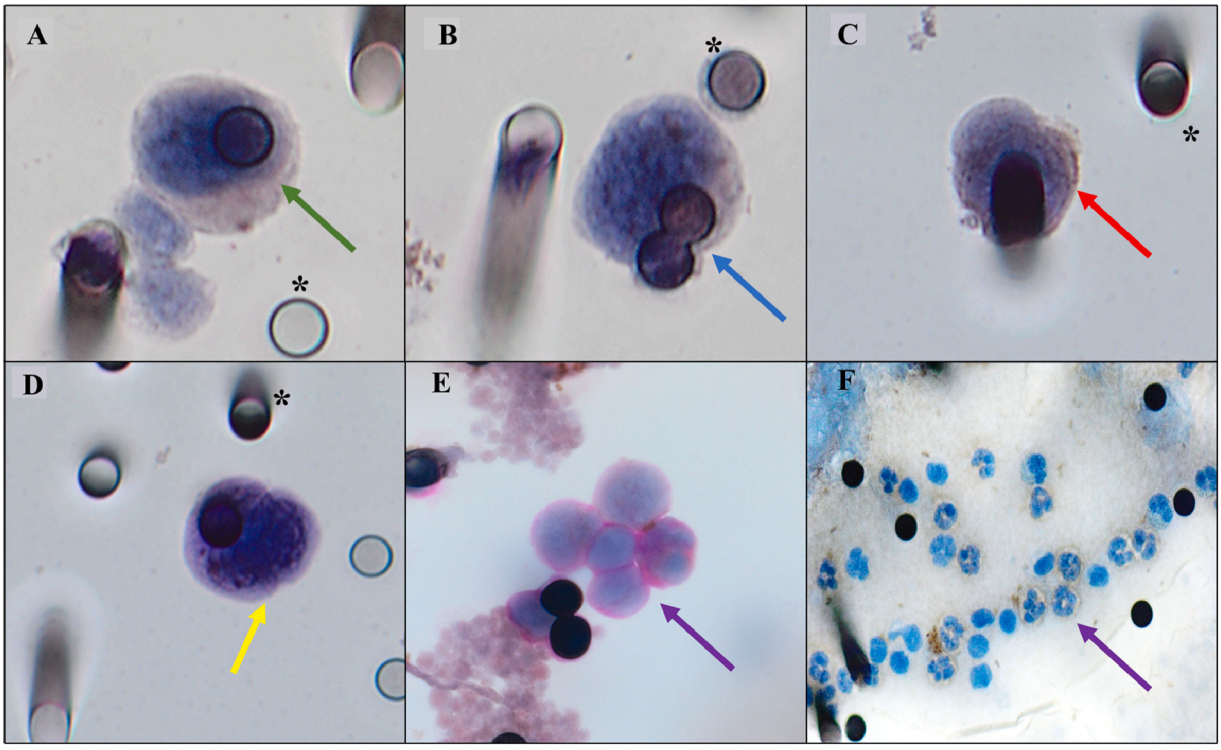

Fig. 2. Immunostaining of CTCs (A–D) non-metastatic anal cancer. A) PD-L1 positive CTC stained with DAB (60x); B) CTC positive for RAD23B (x60); C) Patient CTC for TYMS (40x); D) Patient CTC for ERCC1 (40x). E-F) PBMCs isolated from healthy donors. Staining with CD45 antibody (common leucocyte marker) in Fig. E was visualized with Permanent Red and counterstained with haematoxylin. Photomicrographs were taken with a light microscope (Research System Microscope BX61 - Olympus, Tokyo, Japan) coupled to a digital camera (SC100 - Olympus, Tokyo, Japan). All images were analyzed using a Research System Microscope BX61 (Olympus, Tokyo, Japan) coupled to a digital camera (SC100–Olympus). Thick green, blue, red and yellow arrows indicate CTCs, the thick purple arrows indicate leukocyte and asterisks indicate 8 μm pores of ISET membranes.

applicability [20]. The Epstein-Barr virus (EBV) has been shown to be useful in screening for early asymptomatic nasopharyngeal carcinoma, making early detection possible, with better results in the treatment of patients [21].

As the HPV virus has a genome without repeated sequences, it is easier to cover the whole and multiple viral genotypes. This observation is consistent with a previous study in locally advanced cervical cancer, which showed that HPV sequencing (HPV-seq) could provide a quantitative and qualitative assessment of HPV ctDNA in low disease burden environments [22].

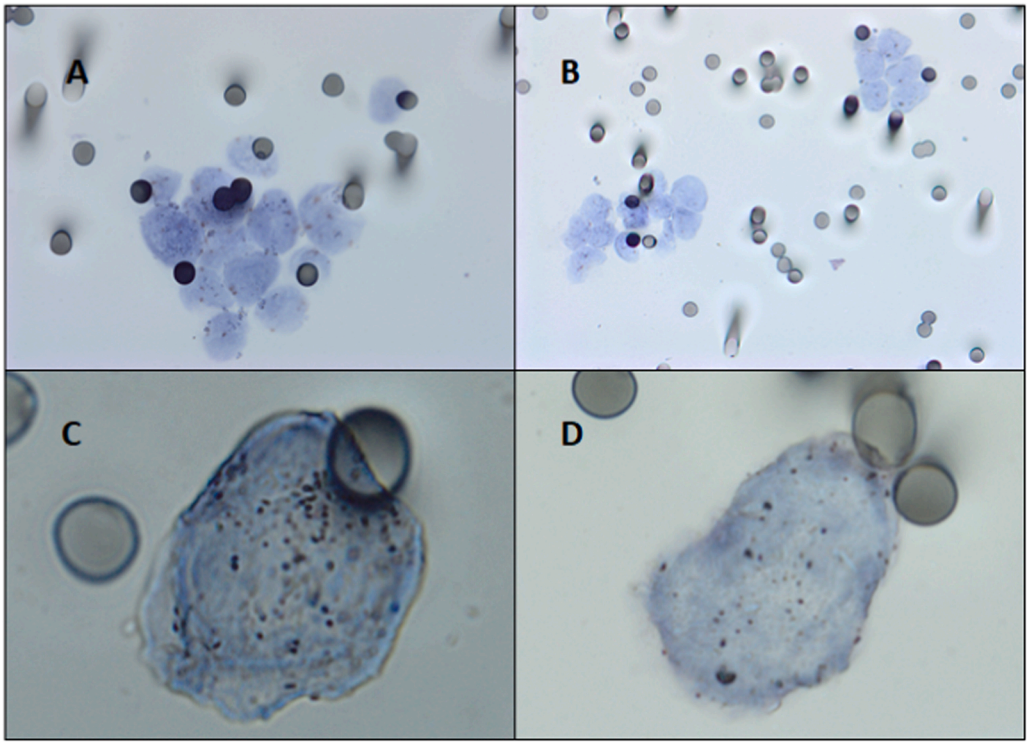

Fig. 3. Chromogenic in situ hybridization (CISH). A-B) positive control, Ca Ski cell line of uterus cancer "spiked" in healthy blood, stained with DNA from HPV, visualized with DAB and counterstained with haematoxylin (×40 magnification). C-D) CTCs from non-metastatic anal cancer patients with a high HPV DNA signal (overexpression) visualized with DAB and counterstained with haematoxylin (×40 magnification). All images were analyzed on a Research System Microscope BX61 (Olympus, Tokyo, Japan) coupled to a digital camera (SC100–Olympus).

The detection of circulating viral DNA can therefore avoid the need to identify changes in tumor DNA. A prospective study of patients with metastatic SCCA enrolled in a clinical trial of a first line triplet chemotherapy regimen (DCF) demonstrated that the levels of HPV ctDNA at baseline had a significant prognostic impact in progression-free survival; persistent of HPV DNA after several cycles of chemotherapy also was associated with shorter post progression disease control and inferior survival at 12 months [23]. Another prospective study found that residual HPV ctDNA after CRT was significantly linked to inferior disease-free survival [24]. These data reinforce our results obtained from the detection of the HPV16 virus in CTCs using the CISH methodology, which can be integrated into the monitoring of treatment efficacy and surveillance of clinical trials, in addition to being an optimizing and easy-to-interpret method.

p16 IHC expression is a marker widely used in the diagnosis associated with HPV, especially in cervical cancer. Its expression in anal lesions is less studied. A work published by Liu et al. evaluated p16 immunoreactivity of HPV-associated anal intraepithelial neoplasia in 1000 biopsies, providing 91 % sensitivity and suggested that its overexpression can be used as an indicator of biological behavior of progression in HPV-induced lesions [25]. The association of p16 status with better prognosis in patients with ASCC was observed in a meta-analysis published recently. The three-year overall survival (OS) for patients p16^{+} was better than for negative ones (84 %; 95 % CI, 81–88 % vs 49 %; 95 % CI, 40–58 %) respectively. The combined analysis of p16 and hrHPV DNA status showed that patients with p16^{+}/hrHPV DNA^{+} had superior 3y-OS (86 %; 95 % CI, 82–90 %) compared to p16^{-}/hrHPV DNA^{-} patients (OS= 39 %; HR= 0.26; 95 % CI, 0.14–0.50). Also, hrHPV DNA^{+} status alone was associated with better prognoses. OS for hrHPV DNA^{+} and hrHPV DNA^{-} patients were 84 % (95 % CI, 80–88 %) vs 52 % (95 % CI, 38–64 %) respectively [26]. Here, 15 samples of T1 and 9 samples of T2 were negative for p16 expression in CTCs. The real reasons why we did not identify p16 expression in CTCs of SCCA, even if most had CTCs which were positive HPV DNA by CISH, are unknown. Yet, a plausible reason is that the antibody used to examine ICC expression of p16 in CTCs was distinct from that utilized in IHC tissue samples, and likely, less sensitive. Also, it is known that an HPV-induced cancer will not necessarily express p16 antibody, although T cells directed against p16 can be isolated [27]. Further research with antibodies from other companies and used in both samples, tissue and CTCs, might confirm this hypothesis.

There are currently 2369 clinical trials testing PD-L1 as a monotherapy or in combination in cancer treatments, according to the ClinicalTrials.gov, [28] the vast majority in lung, colorectal and breast cancers. In line with this, we thought about using this protein to evaluate the potential of this biomarker in patients with anal canal cancer. Unfortunately, as we worked with a small cohort, we were unable to evaluate it, and further investigations are needed to assess the clinical significance of PD-L1 expression. in CTCs from canal anal cancer.

Studies in non-small cell lung cancer and ovarian cancer have shown that the expression of ERCC1 has a negative influence on the efficacy of cisplatin-based treatment, making ERCC1 a potential independent predictive biomarker for platinum resistance and poor prognosis [29,30]. In our study, ERCC1 was observed in CTCs from three patients in T2, 6–8 weeks after the end of CT/CRT. One patient was positive for ERCC1 at T2 and for RAD 23 B, PD-L1 and TYMS at T1, showing disease recurrence. Another patient was positive for ERCC1 at T1 and negative at T2, and achieved CR. While, likely to our small sample, the results were not statistically significant, they suggest that ERCC1 may be a useful predictive marker of response to CT/CRT with cisplatin.

The ability to identify tumors that do not respond to chemo and radiotherapy is useful to help patients and aid in possible responsive strategies. Many studies discuss these considerations. We have shown previously that CTCs from patients with metastatic colorectal cancer express markers related to resistance to 5-fluorouracil (thymidylate synthase/TYMS) and irinotecan (Multidrug resistance protein 1 /MRP-1) [15,31] in CTCs and that those expression was related to disease progression. In another study, we demonstrated that the expression of RAD23B in CTCs from patients with locally advanced rectal cancer was directly associated with lack of response to radiotherapy, suggesting that the qualitative measurement of CTCs can be used as an additional tool in the identification of non-responders' patients, before the initiation of CRT [13]. Here, the patient with disease recurrence was positive for TYMS and RAD23B at T1.

It is important to mention that in some patients (n = 3) we observed the raised of CTC numbers even with complete response. We believe that this increase in CTCs numbers in the second collection (6–8 weeks after treatment) reflects the positive effect of chemoradiotherapy (2/3 patients). The cell death caused by radiation can lead to abscopal effect, stimulating the immune system through the release of tumor antigens, making treatment also effective in distant sites. This effect may have contributed to the increase of tumor cells in the circulation. However, these CTCs may just be cellular debris without potential harm to the patient. For this reason, although there was an increase of the CTCs count, the patients had a complete response. We need, in future studies, to distinguish the harmful CTCs from residual CTCs.

In summary, the identification of HPV inside CTCs of patients with localized SCCA treated with CRT is feasible and an interesting liquid biopsy method to monitor minimum residual disease in this tumor type. Predictive markers of CRT efficacy and the presence of HPV in CTCs can help in the design of new therapeutic strategies, as well as understanding the molecular and immunological characteristics of SCCA. This was a proof-of-concept study that needs to be confirmed by a larger patient cohort.

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CRediT authorship contribution statement

A.P.C.R: data analysis and interpretation, collection and/or assembly of data, manuscript writing. A.C.B: data analysis and interpretation, collection and/or assembly of data. D.J.F.C: data analysis and interpretation. JAT: data analysis and interpretation, manuscript writing.GB: IHC analysis. PHPB, EAA, CB: collection and/or assembly of data. V.S.S: data analysis and interpretation. MECB: final review of manuscript. R.P. R: data analysis and interpretation, manuscript writing, final approval of manuscript. VSA: collection and/or assembly of data. L.T.D.C: conception/design, data analysis and interpretation, manuscript writing, final approval of manuscript.

Conflicts of interest

All authors declared that there are no conflicts of interest.

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Ethical approval and consent to participate

Approved by the ethics committee: (CEP 2669/19).

Consent for publication

Not applicable.

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