Detection of circulating tumor cells as therapeutic markers in patients with penile squamous cell carcinoma: A preliminary study

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ABSTRACT

Background: This study aimed to investigate the presence of circulating tumor cells (CTCs) in patients with penile squamous cell carcinoma (PSCC).

Methods: CTCs were isolated from 14 patients with PSCC, 6 patients with balanoposthitis, and 6 healthy individuals. CTCs were enriched based on cell surface markers and filtered through the IsoFlux device, followed by identification according to cell morphology and immunofluorescence studies.

Results: CTCs were found in all PSCC blood samples but not in balanoposthitis samples and samples from healthy individuals. Immunofluorescence studies confirmed the tumor origin. When the patients with PSCC were stratified according to metastatic inguinal lymph node status, a statistically significant difference was observed in the number of detected CTCs.

Conclusion: Our study showed that CTCs in PSCC may represent a valuable marker for differentiating PSCC from other tumors. Based on the correlation with some clinical parameters, CTC analysis is possibly relevant for noninvasive monitoring of disease progression and prognosis. The results also suggested a potential role of CTCs in preventing overtreatment, such as inguinal lymph node dissection.

Keywords: Balanoposthitis; Circulating tumor cells; Lymph node dissection; Penile squamous cell carcinoma

1. INTRODUCTION

Penile squamous cell carcinoma (PSCC) is a rare and aggressive tumor with a poor prognosis. Early identification followed by penectomy, possibly with pelvic lymph node (LN) dissection or systemic chemotherapy, has been proven to be the best strategy for the treatment of PSCC.[1] The mean 5-year survival rate is approximately 67% (https://www.cancer.org, American Cancer Society), although it drops to 12% in patients with metastatic disease. Among the patients with PSCC, 58% had inguinal masses and 40% had metastasis despite the small size (T2 and T1c) of the tumors.[2] Extended inguinal lymphadenectomy is the most widespread and useful approach for staging and treating inguinal metastasis in these cases. Although it is a commonly used technique, postoperative complications are often observed.[2] Since early and appropriate preoperative staging is crucial to the prognosis, identifying specific, sensitive, and noninvasive biomarkers is necessary to significantly improve the clinical treatment decision, survival rate, and quality of life in patients with PSCC.

Circulating tumor cells (CTCs) are shed from primary or metastatic tumors. They subsequently travel through the circulation to distant organs and lead to the formation of distant secondary tumors. CTCs are responsible for the initiation of metastasis and hold important information for cancer prognosis and diagnosis.[3,4] The detection of CTCs in peripheral blood can represent a readily accessible "liquid biopsy" and is a reliable tool for disease progression monitoring and follow-up in several solid cancers.[3,5–7] However, its application in patients with PSCC has not been analyzed to date.

In the present study, we explored the presence of CTCs in the blood samples obtained from 14 patients with PSCC, 6 patients with balanoposthitis, and 6 healthy individuals using the IsoFlux (Fluxion Biosciences, Alameda, CA) platform to isolate the CTCs. Subsequently, immunofluorescence characterization was performed with the same markers used in the tumor tissue for PSCC diagnosis. IsoFlux utilizes immunomagnetic beads targeting antigens expressed on the cell surface, which allows the enrichment of the CTCs in the microfluidic device. We also evaluated the correlation between the number of CTCs detected in the samples and clinical parameters of PSCC.

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2. METHODS

2.1. Patients and blood sample collection

All patients provided written informed consent to participate in the study. The study was approved by the local ethics committee (TSGHIRB NO: 2-107-05-167). The study included 26 participants evaluated at the Tri-Service General Hospital. Altogether, 7.5 mL of blood was collected from each participant in ethylenediaminetetraacetic acid (EDTA) tubes. Blood sampling was performed in our clinic. All samples were processed within 4 hours of collection and evaluated for CTC analysis.

2.2. Preparation of immunomagnetic beads for CTC isolation

The original IsoFlux CTC enrichment protocol was modified to enable the replacement of IsoFlux beads with CELLection Dynabeads (Invitrogen). Antibodies are attached to the surface of the Dynabeads via a DNA linker, which provides a cleavable site to release and remove the beads from the cells after isolation. This process is not possible with the IsoFlux beads. CELLection Dynabeads coated with human antimouse immunoglobulin (Ig) G (Invitrogen) was utilized for cell enrichment. Dynabeads were incubated with each antibody (0.02 μg antibody/μL bead suspension) (EpCAM [Ber-EP4], Cambridge, United Kingdom) at room temperature. After incubation, the beads were washed twice with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) and stored at 4°C.

2.3. Sample preparation

Red blood cells (RBCs) were lysed in 45 mL RBC lysis buffer containing 0.01 M potassium hydrogen carbonate, 0.155 M ammonium chloride, and 0.1 mM EDTA. After centrifugation, cell pellets were washed with PBS. The cells were resuspended in Roswell Park Memorial Institute (RPMI) medium containing 1% fetal bovine serum (FBS), 1 mM $CaCl_2$, and 5 mM $MgCl_2$, followed by addition of antibody-coated beads according to the original blood volume. The cells were then incubated with magnetic beads at 4°C.

2.4. IsoFlux and CTC enrichment

IsoFlux utilizes immunomagnetic beads that target antigens expressed on the cell surface. The beads are magnetic cores surrounded by a polymeric layer coated with a monoclonal human anti-mouse IgG antibody. In combination with primary mouse IgG antibodies, CTCs are enriched in microfluidic devices. The sample was positively isolated from the sample using an immunomagnetic capture reagent, while the cells flowed through a microfluidic cartridge designed for cell isolation.

2.5. Sample isolation and collection

Beads containing cancer cells were retrieved by running the enrichment protocol on the IsoFlux machine. Isolated cells were then recovered in 200 μL RPMI medium containing 1% FBS, 1 mM $CaCl_2$, and 5 mM $MgCl_2$ and transferred to a low-retention microcentrifuge tube (Fisher). To enable removal of the supernatant, a cylinder magnet was used to pull down the cells bound to the beads toward the bottom of the tube. Cells were then fixed in 4% paraformaldehyde and added onto glass slides. A circle of the same size as that of the magnet was drawn on the glass slide using a water repellent Dako pen. The glass slide was placed on top of the magnet when a buffer was added or removed from the cells.

2.6. CTC analysis and immunofluorescence

Isolated cells were mounted and fixed on the slides and blocked for 5 minutes in 10% normal donkey serum. The cells were then stained with phycoerythrin-conjugated anti-CD45 [5B-1] antibody (1:200) (MACS, Miltenyi Biotec). Subsequently, the cells were permeabilized using 0.2% Triton X-100 in PBS containing 0.5% BSA and 2 mM EDTA, followed by staining with FITC-conjugated anticytokeratin (CK3-6H5) antibody (1:10) (MACS, Miltenyi Biotec). To stain the cell nuclei, the cells were incubated with 4′,6-diamidino-2-phenylindole. Samples were mounted with a Dako Faramount aqueous mounting medium. Images were captured using a fluorescent microscope (Axio Scan.Z1, Zeiss). CTCs were defined as cytokeratin (CK)-positive, CD45-negative, and nucleated cells.

2.7. Statistical analysis

All experiments were performed at least three times independently unless stated otherwise. Continuous variables were presented as mean ± standard deviation unless stated otherwise. Statistical significance was determined using Mann–Whitney *U* test. The data were analyzed using Prism version 7 (GraphPad Software Inc., San Diego, CA). Statistical significance was set at $p < 0.05$.

3. RESULTS

3.1. Patient characteristics

The cohort of 26 enrolled participants consisted of 14 patients with PSCC, 6 with balanoposthitis, and 6 healthy individuals whose characteristics are presented in detail in Table 1. Among the 14 patients with PSCC, the mean age at diagnosis was 77.1 ± 6.1 years. Stages at diagnosis were as follows: stage 1 in three patients (21%), stage 2 in seven patients (50%), stage 3 in four patients (29%), and stage 4 in none of the patients. All patients with PSCC underwent penectomy. Among these patients, seven (50%) had palpable inguinal LNs. The mean follow-up duration from the time of surgery was 11.8 ± 4.9 months after diagnosis. Seven patients with palpable inguinal LNs underwent inguinal LN dissection (ILND). Among the six patients with balanoposthitis, mean age at diagnosis was 67.5 ± 6.4 years.

3.2. Detection of CTCs

CTCs were enriched and detected in all patients with PSCC after immunofluorescence staining of the cells (Fig. 1). Cancer cells were observed based on the morphology and expression of CK (Fig. 1). CTCs were found in all patients with PSCC before the surgery (Fig. 2A, Table 2) (mean CTCs/7.5 mL = 3.4 ± 2.1, n = 14 patients). The same patients were also tested during the postsurgical period (mean CTCs/7.5 mL = 1.2 ± 1.1, n = 14 patients). The presurgery blood samples were collected at the

Table 1

Characteristics of enrolled patients

	PSCC patients (n = 14)	Balanoposthitis patients (n = 6)	Normal individuals (n = 6)
Age (mean ± SD)	77.1 ± 6.1	67.5 ± 6.4	75 ± 9.1
Gender, male	14 (100%)	6 (100%)	6 (100%)
Stage			
1	3 (21%)		
2	7 (50%)		
3	4 (29%)		
4	0 (0%)		
Follow-up from surgery (mo)	11.8 ± 4.9		
Tumor diameter (cm)	1.9 ± 1.4		
Palpable LN	7 (50%)		
ILND	7 (50%)		

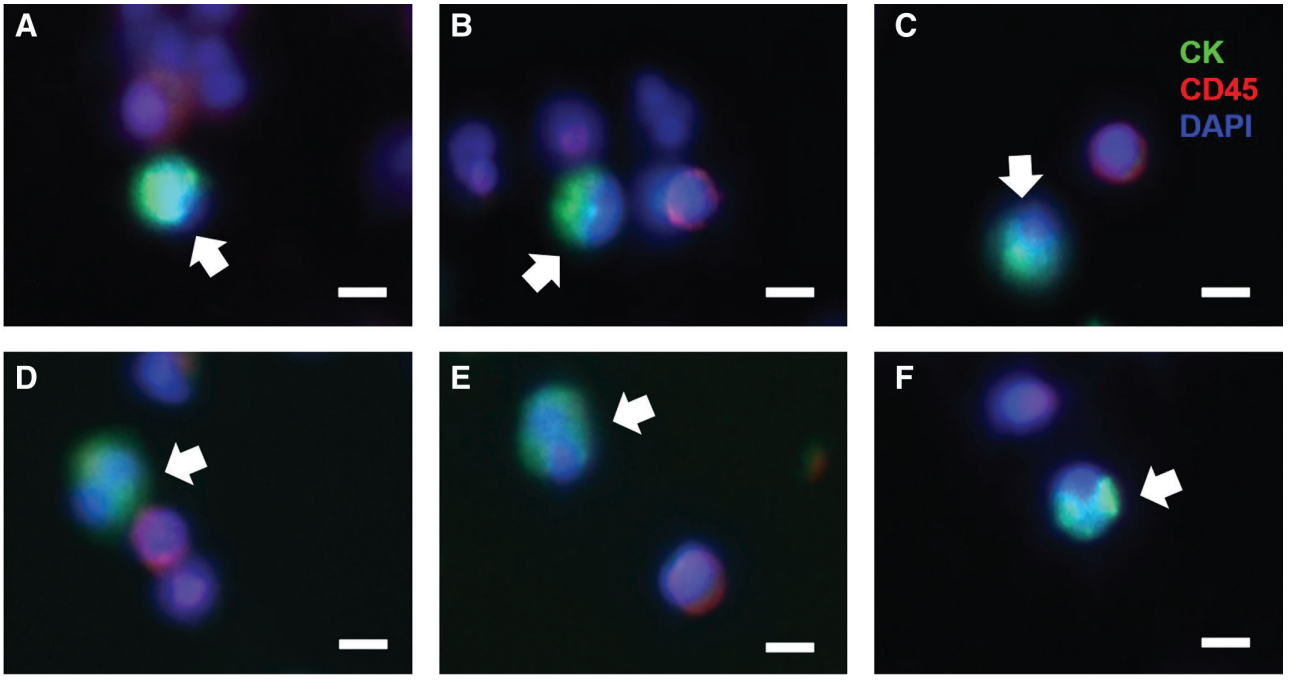

Fig. 1 CTCs were detected in the blood samples from patients with penile squamous cell carcinoma (white arrow). (A–F) Immunofluorescence staining of representative cells obtained from IsoFlux Cancer cells fulfilled the criteria for CTCs including nucleated cells (blue), cytokeratin-positive cells (green), and CD45-negative cells (non-red). Scale bar: 1 μm. CTCs = circulating tumor cells.

hospital 30–60 minutes before the surgery. Postsurgery blood samples were obtained at 2 weeks (mean ± standard deviation) after the surgery. No CTCs were found in the samples from patients with balanoposthitis (Fig. 2A, n = 6), in whom blood samples were collected at 2 weeks after the surgery. The samples were negative for CTCs.

3.3. Effect of surgery on the number of CTCs

When presurgical and postsurgical samples (2 weeks after surgery) from the same patient were compared, a statistically significant decrease in the number of CTCs was observed (Fig. 2B, Mann–Whitney *U* test, $p = 0.0035$). In all patients, the surgery affected the number of CTCs. The CTC count further decreased when blood samples were obtained at 3 months after the surgery (Fig. 2B). However, no significant correlation was observed between the number of CTCs at different time points after the surgery (Fig. 2B). We did not find any evident correlation between the follow-up duration and the number of CTCs.

3.4. Association of CTCs with clinical prognostic parameters

To determine the correlation between the CTC counts in the blood samples before the surgery and the clinical characteristics of enrolled patients with PSCC, univariate regression analysis was performed between the number of CTCs/7.5 mL and accessible parameters including tumor diameter and age using the samples available at follow-up (11.8 ± 4.9 months). No statistically significant linear correlations were observed (Fig. 3A). Further analyses showed no significant correlation between CTCs and patients' systemic diseases and smoking or drinking habits (data not shown).

When presurgical CTC counts were evaluated according to the patients' clinical stage, there was no statistically significant difference in the median CTC count between stage 1 and stage 2 patients, but a significant difference was observed between stage 2 and stage 3 patients ($p = 0.36$ and $p = 0.0051$, respectively; Table 2, Fig. 3B). Notably, when we stratified the patients according to their clinical inguinal LN status, we observed a statistically significant difference in the median CTC count between PSCC patients with inguinal LN metastasis and those without nodal metastasis (mean CTC count/7.5 mL: 6.3 ± 1.0 in patients with nodal metastasis vs. 2.3 ± 1.1 in those without nodal metastasis, $p = 0.0026$, Fig. 3C). Among the seven patients who underwent ILND due to palpable inguinal LNs, three patients did not have inguinal LN metastasis. Four patients who were proven to have LN metastasis had more than 5 CTCs/7.5 mL of blood sample. A statistically significant difference in the median CTC count was noted between the groups ($p = 0.0249$, Fig. 3D).

4. DISCUSSION

Penile cancer is a urological malignant disease with a low incidence rate in developed countries. Patient survival is high when the disease is diagnosed early and does not spread to the inguinal LNs. Palpable inguinal LNs are present in 28%–64% of the cases of penile cancer at the initial presentation.[2,8–10] Moreover, 47%–85% of the patients have a positive metastatic inguinal LN at diagnosis.[2,8–10] Managing regional LNs with inguinal lymphadenectomy is crucial for long-term patient survival.[1] In cN1/cN2 diseases, ILND is the standard procedure. Although it is a widespread technique, it has significant morbidity (as high as 50%) mainly associated with wound healing and lymphatic drainage.[2,11,12] It is curative, but possibly underused due to the fear of related complications.

ILND is performed occasionally in patients with non-LN metastatic disease to ensure the presence of micrometastatic disease and to avoid unnecessary extensive procedures with a high risk of complications. The probability of micro metastatic disease is approximately 12%–25% in case of clinically normal inguinal nodes.[13,14] Current imaging methods are not reliable for detecting these non-palpable inguinal LNs and micro metastases. However, a previous publication showed that only 17%–45%

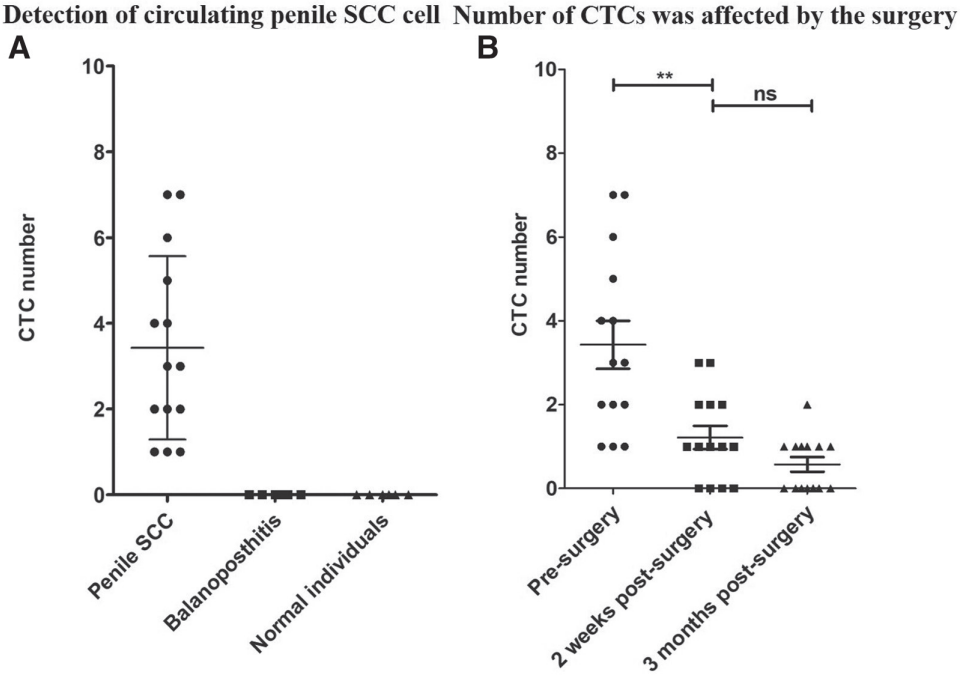

Fig. 2 (A) CTC count in enrolled participants and (B) time course analysis of CTC levels in PSCC. CTC levels were evaluated in 14 patients with PSCC before the surgery and at 2 weeks and 3 months after the surgery. The number of CTCs/7.5 mL are indicated in the plots (Mann-Whitney *U* test, $^{**}p = 0.0035$). CTCs = circulating tumor cells; PSCC = penile squamous cell carcinoma.

of the clinically palpable LNs were proven to be positive for routine prophylactic ILND.[15] Surgery is also limited by associated postoperative complications observed in 24%–87% of the patients and by an estimated mortality rate of approximately 3% associated with the respective technique.[11,12,16] Two other invasive diagnostic procedures are available for clinically palpable nodes: modified ILND and dynamic sentinel node biopsy (DSNB). However, both of these methods may miss micrometastatic diseases.[17] The sensitivity of modified ILND is not known, while that of DSNB can be 90%–94%. However, the false-negative rate of DSNB is still as high as 15%.[17] An ipsilateral radical ILND should be performed if LN metastasis is detected.[1]

To the best of our knowledge, no previous study has reported the clinical significance of CTCs in PSCC. The prognostic value of CTCs has already been identified in several cancer types, such as nonsmall cell lung cancer,[18] prostate cancer,[19] breast cancer,[20] and melanoma.[21] CTCs were also found to have clinical predictive value for progression-free survival, disease-free survival, and overall survival in patients with breast cancer, prostate cancer, and bladder cancer.[22,23] In esophageal squamous cell carcinoma, CTCs can be identified in 8%–100% of the patients.[24–32] Reportedly, the presence of CTCs correlated with staging, treatment response, and patient survival.[24–32] In head and neck squamous cell carcinoma, the detection rate of CTCs is approximately 29%–100%.[32–38] However, it is still questionable whether CTC detection is associated with poorer disease-free survival and overall survival.[32–38] Furthermore, the detection of CTCs correlates with regional LN metastasis in inoperable squamous cell carcinoma in the head and neck region.[33] Various technical platforms have been developed for the detection and isolation of CTCs. The detection and characterization of CTCs in cancer patients has great potential as a minimally invasive approach. Over the past few years, various microfluidic platforms have been developed to isolate CTCs in the peripheral blood, but it is difficult to determine the most effective technique to isolate them.[39] Furthermore, there is no gold standard for the validation.[39] The present study demonstrated the ability of IsoFlux to detect CTCs in PSCC. This process was dependent on EpCAM expression in peripheral blood samples before and after surgical removal of the primary tumor. No CTCs were found in balanoposthitis samples and samples from healthy individuals. These results demonstrate the high sensitivity and specificity of the proposed method. Intriguingly, CTCs were not easily detected after surgery (at 3-month follow-up). Thus, in contrast to the findings in other solid tumors, intraoperative manipulation of the penile mass may not cause tumor cell dissemination.[40,41]

Several potential molecular markers have been explored to predict disease progression or metastasis in PSCC, but no marker has been used in routine clinical practice. Furthermore, there are no tumor markers for predicting LN metastasis in penile cancer. Our study revealed that CTCs retrieved from the blood of patients with PSCC using minimally invasive techniques could represent a sensitive and reliable marker for the differential diagnosis of inflammatory diseases. Our findings also showed that the presence of 5 CTCs/7.5 mL was significantly associated with clinically positive inguinal LN metastases. Hence, inguinal lymphadenectomy is strongly recommended in patients with palpable inguinal LNs who have more than 5 CTCs/7.5 mL. On the contrary, patients with cN0 status who harbor occult micro metastases could also be identified. Therefore, we might be able to predict a decrease in the number of patients scheduled to undergo inguinal lymphadenectomy in the future. This is a clear advantage in terms of limiting the possibility of postsurgical complications.

In some tumors, tumor diameter has been proven to be an independent predictor of survival and it is one of the best predictors of malignancy.[13,42,43] Furthermore, tumor diameter has also exhibited a correlation with CTC counts from the primary lesion, which represents a good independent parameter.[44,45] However, we did not find a significant correlation between tumor diameter and CTC detection in peripheral blood. The

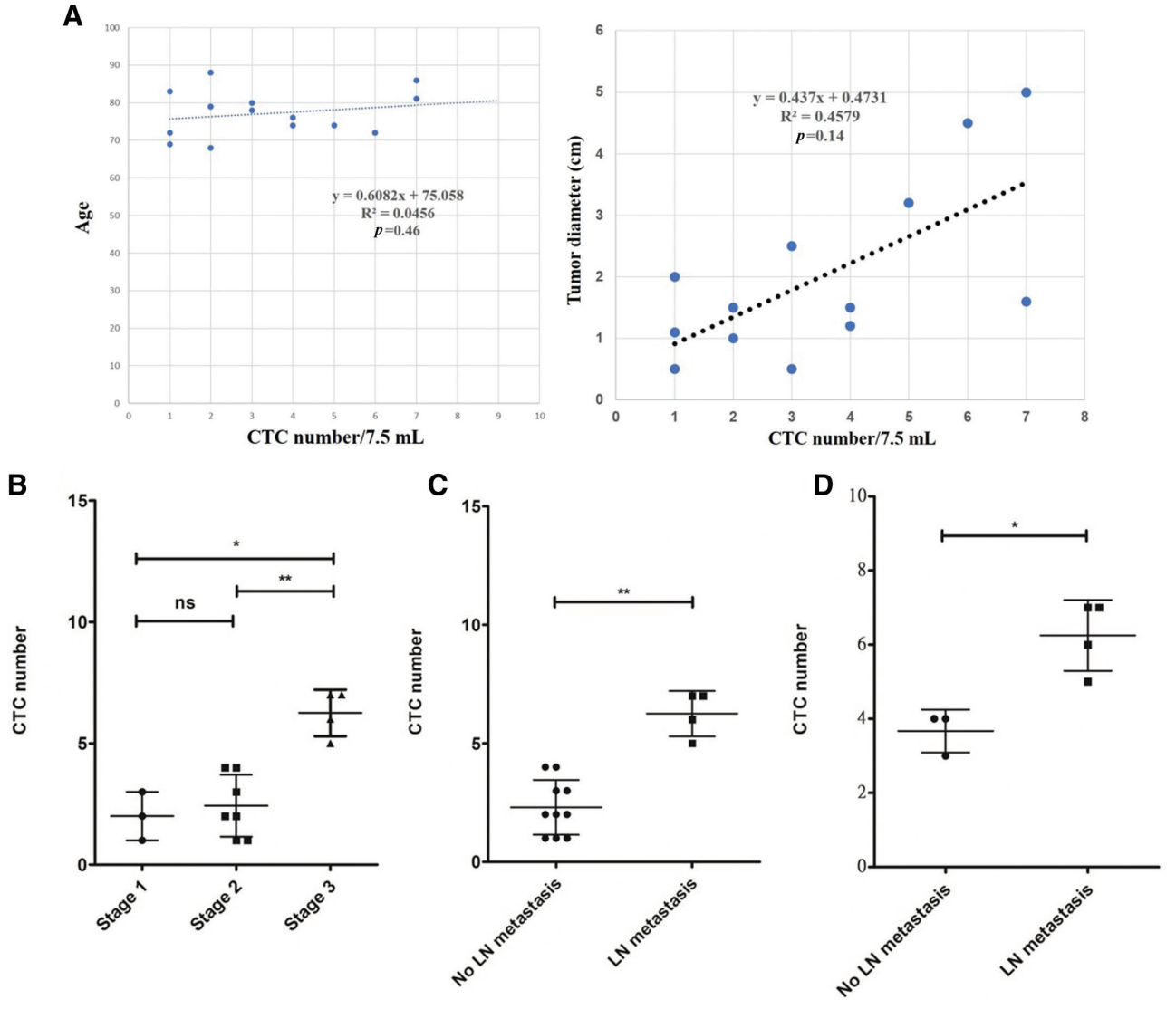

Fig. 3 Correlation between CTC counts and clinical prognostic parameters. (A) Univariate regression analysis between the number of CTCs/7.5 mL and age and tumor diameter; (B) Comparison of median CTC count between tumor stages (*p = 0.36, **p = 0.0051); (C) Comparison of CTC counts between PSCC with inguinal lymph nodes metastasis and PSCC without nodal metastasis (**p = 0.0026); (D) CTC counts in patients with PSCC who underwent inguinal lymph node dissection (*p = 0.0249). CTC = circulating tumor cell; PSCC = penile squamous cell carcinoma.

current guidelines do not include tumor diameter as a tumor, node, and metastasis (TNM) staging parameter for PSCC.[1]

Table 2

Enriched CTC numbers

	No. of patients	Baseline CTC numbers/7.5mL (Mean ± SD)	Postsurgery CTC numbers/7.5mL (Mean ± SD)	Tumor diameter (cm, Mean ± SD)
Normal individuals	6	0	–	–
Balanoposthitis patients	6	0	–	–
PSCC patients	14	3.4 ± 2.1	1.2 ± 1.1	1.6 ± 0.7
Stage				
1	3	2 ± 1	0.3 ± 0.6	1.0 ± 0.1
2	7	2.4 ± 1.3	0.9 ± 0.7	1.6 ± 0.6
3	4	6.3 ± 1.0	2.5 ± 0.6	2.2 ± 0.7
4	0	–	–	–

Interestingly, our findings showed that tumor diameter had no clinical predictive value for metastasis; this is consistent with the current TNM staging system.

The present study has some limitations. It failed to show any relationship between CTCs and tumor relapse or the development of metastasis due to the absence of a longer follow-up period. Another limitation was the small cohort size due to the rarity of PSCC. Further longitudinal studies involving large cohorts should be conducted in various medical centers to evaluate the clinical impact of CTCs in PSCC.

In conclusion, our study provided evidence that CTCs may represent a reliable marker to differentiate PSCC from inflammatory diseases. Correlations between CTCs and some clinical parameters of PSCC, such as tumor stage and LN metastasis suggest that this "liquid biopsy" might be a useful minimally invasive tool for predicting prognosis and avoiding unnecessary surgical procedures. Furthermore, CTCs might also be useful markers for monitoring the progression and treatment responses. We have reported the results obtained using IsoFlux and beads with a DNA linker, which enabled us to separate the cells from the beads and to keep the cells alive (data not

shown). Cell viability is critical for further analysis of biological characteristics, sensitivity to subsequent therapy, and metastatic potential. Evaluation of the molecular profile of CTCs in PSCC might help develop tailored therapies in the near future. Studies with larger cohorts and longer follow-up periods are required to validate the prognostic value of this novel potential biomarker in PSCC.

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