Detection of circulating tumor cells in patients with laryngeal cancer using ScreenCell: Comparative pre- and post-operative analysis and association with prognosis

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Abstract. The presence of circulating tumor cells (CTCs) in the blood of patients with metastatic breast, colorectal and prostate cancer have been widely investigated; however, few studies have examined CTCs in patients with laryngeal cancer. The present pilot study aimed to detect pre- and postoperative CTCs in the blood of patients with laryngeal cancer and evaluate the association with prognosis. Eight patients with laryngeal squamous cell carcinoma (LSCC) at stage III were included in the present study and underwent total or subtotal laryngectomy and radical bilateral neck lymph node dissection. Blood samples were collected from all patients before and after surgery at different time-points. The following processing steps were followed; preoperative blood sampling, surgery, postoperative blood sampling at 3, 6 and 12 month follow-ups, and prognostic association analysis. CTCs were retained on ScreenCell filters for cytological characterization. The presence of CTCs was associated with a less favorable prognosis, whereas a decrease of CTCs in the postoperative sampling was observed in patients who exhibited an improved therapeutic response. The results of the present pilot study revealed a possible association between the presence of CTCs and a less favorable prognosis in patients with LSCC; therefore, these preliminary findings may encourage further research into the incorporation of a liquid biopsy in the management of LSCC, as this may help identify patients with occult metastatic disease earlier and in a non-invasive manner. In addition, this approach may represent novel independent prognostic factor for use in the clinical evaluation of patients with LSCC.

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Introduction

Laryngeal squamous cell carcinoma (LSCC) is one of the most commonly diagnosed malignancies in the head and neck, with an increased incidence rate in middle-aged and elderly males worldwide in the last few decades (1-3). LSCC originates from laryngeal epithelial tissue and may spread directly to adjacent structures, or through lymphatic and blood vessels to lymph nodes and more distant sites (4). Despite considerable improvements in laryngeal carcinoma treatment, which have improved patient quality of life, the global survival rates have remained unchanged throughout the last 3 decades (5-7).

Circulating tumor cells (CTCs) are rare cells that derive from both primitive cancer and metastases, pass through the blood vessels and circulate together with the red and white blood cells. CTCs are absent in healthy patients (8). Several studies have investigated the presence of CTCs associated with solid types of cancer, including head and neck squamous cell carcinoma (9,10), and proposed the use of liquid biopsy in clinical assessment of patients with cancer (11-19). The presence of CTCs has been validated as a prognostic factor in metastatic breast, colorectal and prostate cancers (15,17), and confirmed in previous meta-analyses (20,21); however, only a few studies have examined CTCs in patients with head and neck and laryngeal cancer (9-11,14,18).

Several techniques for the detection and enumeration of CTCs have been developed during the last decade. For example, epithelial antigenic properties of cancer cells are used to detect and isolate cancer cells from blood using immunomagnetic or microfluidic-based enrichment methods (22). The current techniques allow the isolation of CTCs as: epithelial cells (cytokeratin positive), cells in the epithelial to mesenchymal transition (EMT) phase (cytokeratin negative), stem cells and clusters (two or more CTCs together) (23). However, a number of these detection systems are not commercially available and/or economically accessible (23). Previously, it has been demonstrated that the epithelial antigen-based detection of CTCs may underestimate the real number of CTC (24). This may be due to EMT, which represents an integral component

of the metastatic process in which cancer cells downregulate the expression of epithelial markers in favor of mesenchymal markers, inducing the increased stemness of cancer cells and facilitating the development of chemoresistance (25-28). The ScreenCell system, a filtration-based size and antigen-independent technology, has been developed to identify CTCs (29,30), and the rationale of this device is based on the larger size of CTCs compared with hematological cells (31).

The present pilot study aimed to detect CTCs in the blood of patients with laryngeal squamous carcinoma pre- and post-operatively using the ScreenCell system, and to evaluate the association between CTC presence and patient prognosis.

Materials and methods

Patient enrollment. The Ethics Committee of the Sapienza University of Rome approved the present pilot study (approval no. 32/2017). The experimental protocol met the guidelines and the precepts established by the Declaration of Helsinki; experiments were undertaken with the understanding and written consent of each subject and according to the aforementioned principles.

A total of 8/32 patients diagnosed with laryngeal cancer were included in the present study, according to the following sequential inclusion criteria: i) Biopsy specimen positive squamous cell carcinoma (n=32); ii) absence of synchronous and metachronous cancer (n=26); iii) Tumor-Node-Metastasis (TNM)-stage III/IV (n=13); iv) candidates for total or subtotal laryngectomy with neck dissection (n=12); v) no candidates for neoadjuvant therapy (n=10); and vi) patients that provided written informed consent (n=8). In order to increase the uniformity of the population, only patients with homogeneous characteristics for clinical and histopathological parameters were included. All participants were males, smokers, non-alcoholics, age >65 years (age range=61-83; mean age=69 years) with a diagnosis of LSCC. The included patients were waiting for primary laryngeal surgery and were classified, according to the TNM classification (32), as $T3N^{+}M0$. In the total cohort, 1 patient underwent total laryngectomy and 7 underwent subtotal laryngectomy, according to cancer staging. All patients underwent radical bilateral neck lymph node dissection.

The stages of the study were: i) biopsy by microlaryngoscopy and staging by computed tomography (CT) scan, fibro- and micro-laryngoscopy; ii) preoperative blood sampling; iii) surgery; iv) postoperative short- and medium-term follow-up at 3, 6 and 12 months with clinical evaluation and blood sampling for CTC detection; and v) data analysis. The association between CTC detection and prognosis was studied via the comparison between CTC presence with recurrence/lymph node metastasis/death and adjuvant therapy/secondary surgery.

Blood sample collection. A total of 6 ml peripheral blood was drawn from the median cubital vein in K_2-EDTA tube (Thermo Fisher Scientific, Inc.) stored at 4˚C and processed within 3 h of sampling. Four different blood samples were collected from each patient; the first sample was drawn 1 day prior to surgery and the next 3 time-points of collection were at 3, 6 and 12 months after tumor removal.

Depletion of leukocytes. Leukocyte depletion was performed using Dynabeads CD45 (Invitrogen; Thermo Fisher Scientific, Inc.) in order to enrich CTCs from whole blood, following the manufacturer's instructions, as previously described (33). Each blood sample was incubated with Dynabeads (Invitrogen; Thermo Fisher Scientific, Inc.) for 30 min at 2˚C with gentle tilting rotation. The tube was then removed from the mixer and placed on a magnet for 10 min at 20-25˚C. The supernatant was transferred into a new tube and immediately processed for downstream analysis using the ScreenCell device (ScreenCell Ltd.).

CTC detection using ScreenCell. To isolate fixed cells for cytological studies, a ScreenCell Cytokit was used according to the manufacturer's protocol (Caltag Medsystems, Ltd.). A 3 ml leukocyte-depleted blood sample was diluted using 4 ml filtration buffer ScreenCell fixed cells (FC2) dilution buffer (ScreenCell) to fix cells and lyse red blood cells (RBCs). Before use, 30% NaOH was added to the FC buffer until a pH ~7 was reached. After 8 min of incubation at room temperature, 7 ml diluted sample was added into the device tank and filtered under a pressure gradient (determined between the atmosphere pressure and vacuum tube) using a vacutainer tube. Filtration was completed within 3 min. After washing with PBS to remove RBC debris, the filter was left on absorbing paper to dry at room temperature and then stored at -20˚C until Giemsa and immunofluorescence staining were performed. For each patient, the filtration was carried out in duplicate.

Giemsa staining and double immunofluorescence analysis. The filters were stained for 30 min at room temperature with Giemsa stain (1:10; cat. no. 453616; Carlo Erba) and examined using a light microscope (magnification, x400) (Leitz DMRB Camera; Leica Microsystems Inc.) to evaluate the presence of cells adhered to the filter membrane. The double immunofluorescence analysis, using anti-Pan-Cytokeratin (CK) and anti-epithelial cell adhesion molecule (EpCAM) antibodies, was used to identify the adherent cells as CTCs. For this scope, the filters were fixed using 4% paraformaldehyde solution for 5 min at 4˚C and subsequently permeabilized using PBS and 0.01% Tween-20 (Merck KGaA) for 20 min at room temperature. Then, both the filters were incubated with monoclonal mouse anti-Pan-Cytokeratin antibody 2A4 (1:100; cat. no. ab118855; Abcam) for 1 h at room temperature. The filters were incubated with secondary antibody Alexa Fluor 488 goat anti-mouse IgG (H + L) (1:300; cat. no. A11001; Thermo Fisher Scientific, Inc.; green staining). Successively, the filters were incubated with monoclonal mouse anti-EpCAM antibody (clone C-10; 1:50; cat. no. sc-25308, Santa Cruz Biotechnology) for 1 h at room temperature followed by incubation with secondary antibody Alexa Fluor 594 goat anti-mouse IgG (H + L) (1:300; cat. no. A11005; Thermo Fisher Scientific, Inc.; red staining). . The nuclei were counterstained for 10 min at room temperature using DAPI (1:1,000; cat. no. D1306; Thermo Fisher Scientific, Inc.). Immunostaining was examined under a fluorescence microscope (Olympus Corporation) at magnification, x400. For each sample, five randomly selected microscopic fields were evaluated and cells positive for anti-Pan-Cytokeratin (cytoplasmatic green stain) and anti-EpCAM (cytoplasmatic red stain) immunostaining, were counted. The cells positive for anti-Pan-Cytokeratin and anti-EpCAM were considered to be CTCs.

Table I. Detection of CTCs pre- and postoperatively in patients with laryngeal squamous cell carcinoma.

		Postoperative follow up time, months					
		3		6		12	
Case	Preoperative CTC detection	Follow-up status	CTC detection	Follow-up status	CTC detection	Follow-up status	CTC detection
#1	[+]	Disease-free	(+)	CH-RT for metastasis	False negative for CH	Dead	(-)
#2	[+]	Disease-free	(+)	Dead	(-)	(-)	(-)
#3	[+]	Disease-free	(+)	Dead	(-)	(-)	(-)
#4	[+]	Disease-free	(+)	Disease-free	(+)	Disease-free	(-)
#5	[+]	Disease-free	(+)	CH for metastasis	False negative for CH	Dead	(-)
#6	[-]	Disease-free	(+)	Disease-free	(-)	Disease-free	(-)
#7	[+]	Disease-free	(+)	Secondary surgery for recurrence	(+)	Disease-free	(+)
#8	(-)	Disease-free	(+)	Disease-free	(-)	Disease-free	(-)

CTC, circulating tumor cells; (+), positive for CTCs; (-), negative for CTCs; NA, not available; CH, chemotherapy; RT, radiotherapy.

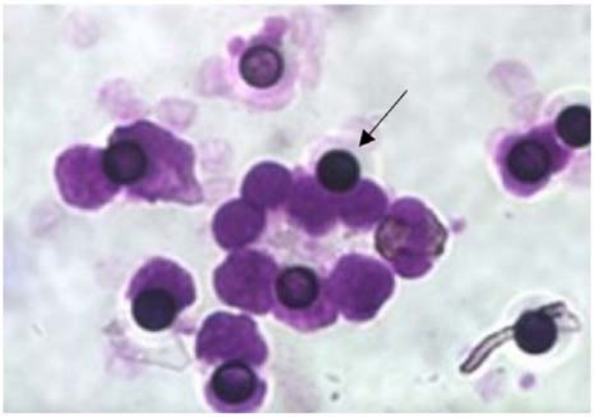

Figure 1. Circulating tumor cells in patients with laryngeal cancer. Giemsa staining of circulating tumor cells in a patient (patient #4) with laryngeal squamous cell carcinoma. ScreenCell filters were stained with Giemsa and examined using a light microscope to evaluate the presence of cells adhered to the filter membrane. Arrow shows the micropore of the filter. Magnification, x400.

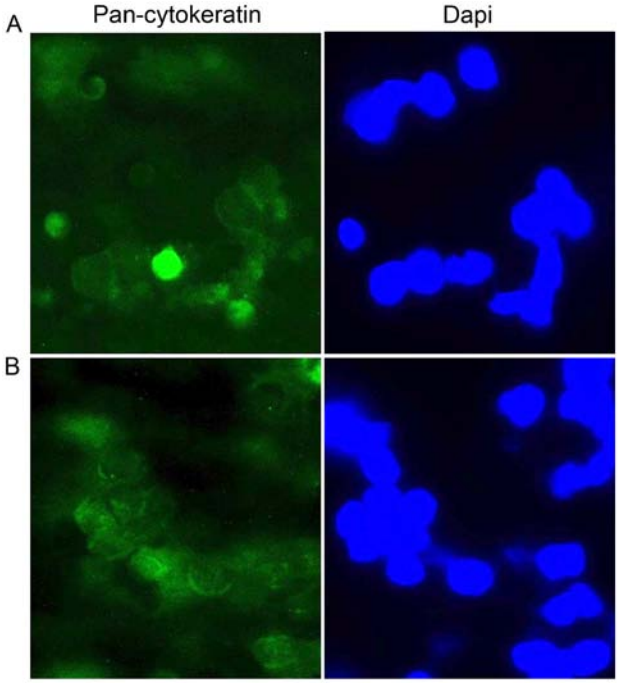

Figure 2. Immunofluorescence analysis. Immunofluorescence analysis with anti-Pan-Cytokeratin antibody used to characterize tumor circulating cells of patients with laryngeal squamous cell carcinoma adherent to the filter membrane (A) pre- and (B) post-surgery. The nuclei were counterstained using DAPI. Magnification, x400.

Results

Patient follow-up. CTCs were characterized by positivity for CK and EpCAM. Fig. 1 shows Giemsa staining of circulating tumor cells in patients with laryngeal squamous cell carcinoma. Fig. 2 shows immunofluorescence analysis with anti-Pan-Cytokeratin antibody used to characterize tumor circulating cells of patients with laryngeal squamous cell carcinoma before and after surgery. Preoperatively, 6 patients had evidence of CTCs and 2 patients were negative. At the 3-month follow-up, all participants were disease-free and CTC^+. At the 6-month follow-up, 3 patients were disease-free (1 CTC^+ and 2 CTC^-), two patients had died (CTC^+), 1 patient had recurrence and 2 had metastasis (1 CTC^+, 1 underwent secondary laryngectomy and 2 were false negatives (due to chemo/radiotherapy for liver/pulmonary metastasis). At 12-months follow-up, 4 patients were disease-free (1 CTC^+ and 3 CTC-) and 4 had died (CTC+) (Table I).

Association between pre-operative CTC detection and prognosis. Preoperatively, 6 patients had evidence of CTCs and 2 patients were negative. The data revealed that the presence of CTC before the surgery may be associated with a

less favorable prognosis, whereas a negative result for CTCs preoperatively was associated with a favorable prognosis. The majority of patients who were CTC^+ (83.3%) exhibited a poor prognosis (4 died and 1 underwent secondary surgery), while all patients who were CTC^- preoperatively were disease-free at all follow-up visits (Table I).

Association between post-operative CTC detection and prognosis. Three months after surgery, all patients were CTC^+ and disease-free. Patients exhibiting increased or overlapping values of CTCs, had a poor prognosis (4 deceased and 1 relapsed). Conversely, if there was a reduction or zeroing of CTC value, this was associated with a more positive prognosis (Table I).

The analysis of the data at medium-term (6 months after surgery) revealed that: i) Negative matching of CTC in the pre- and post-operative at 6 months may be a positive prognostic factor (25% of patients CTC^- in the pre- and post-6-months were disease-free at all follow-ups); ii) patients who underwent chemotherapy were negative for CTCs; and iii) 50% of patients with detectable CTC both in pre- and post-6-months follow up experienced a progression of disease (Table I).

Data collected 12 months after surgery indicated that: i) CTC negativity preoperatively and at the 12-month follow up were associated with a more favorable prognosis; and ii) CTC positivity preoperatively and at the 12-month follow up was associated with a less favorable prognosis. In fact, all patients that were CTC^+ both pre- and post-operative died, except the patient that exhibited a decrease in CTC levels in the long-term (Table I).

Discussion

In the last two decades, comprehensive treatment measures, such as surgery, radiotherapy, chemotherapy and gene therapy, have resulted in a higher 5-year survival rate globally for patients with laryngeal cancer; however, 30-40% of patients still succumbed to the disease due to tumor recurrence or metastasis (34). An improved understanding of the metastatic processes underlying LSCC is needed to identify novel prognostic factors and treatment methods.

The present pilot study evaluated whether the presence of CTCs in patients with laryngeal cancer may represent a novel independent prognostic factor and may be quantified in a liquid biopsy to aid clinical evaluation. The techniques used for CTC detection in the present study were immunological and morphological; CTCs were isolated from the blood of patients with LSCC using the ScreenCell system, a technology based on polycarbonate filter with 8 μm diameter pores able to isolate CTCs from other blood cells. An advantage of this technology is the possibility to detect CTCs using Giemsa histochemical staining and to identify the presence of CTC markers by immunofluorescence staining (29). Leukocyte depletion was performed prior to the CTC isolation in order to improve the CTC detection (35).

To the best of our knowledge, the present study is the first to focus exclusively on CTCs in laryngeal cancer using the ScreenCell system. No other studies have been published on the use of ScreenCell in laryngeal cancer and these preliminary results may present an incentive for further studies on this topic. The ScreenCell system differs from other systems such as the CellCollector system, used by Zhang *et al* (36) in which CTCs from laryngeal cancer were evaluated as it is a filtration-based size and antigen-independent technology (29). CTC isolation using the ScreenCell system is promising due to its simplicity, speed and the benefit that it eliminates any antibody bias that may be introduced by other techniques (37,38). Although no complex instruments or training are needed to use this system, the costs are high.

The development of semiautomatic technologies, such as the CellSearch system, has allowed evaluation of the prognostic role of CTC status in patients with other types of cancer, such as lung and breast cancer, with promising results (37-49). A recent study from Chudasama *et al* (38) evaluated the efficacy of the ScreenCell filtration system, to capture, isolate and propagate CTCs from patients with primary lung cancer. The results suggested that the ScreenCell system had the potential to be used as a CTC isolation tool following further work, adaptations and improvements to the technology and validation of results. Another study from Hashimoto *et al* (48) concluded that there was an increase in the CTC count of pulmonary vein blood following surgical manipulation of a tumor. Hou *et al* (49) identified an association between an increased CTC count and less favorable patient survival in small cell lung cancer.

The preliminary results of the present study indicate that, in laryngeal cancer, the absence of CTCs may predict a more favorable prognosis, while high levels of CTCs in the peripheral blood may be associated with a less favorable prognosis. A decrease of CTCs in postoperative sampling may suggest an improved response to surgical treatment, and the early detection of CTCs may predict recurrence or metastasis.

The results of the present study are in accordance with other studies investigating CTCs in solid cancers, including the head and neck, which revealed that the presence of CTCs may influence prognosis (11-17,44-57). Zhang *et al* (36) and He *et al* (58) revealed that CTCs have a role in the progression and metastasis of head and neck squamous cell carcinoma. Nichols *et al* (59) isolated CTCs in 6/15 patients with advanced head and neck carcinoma using CellSearch and demonstrated an association with lung nodules >1 cm. Winter *et al* (60) tested 16 patients with advanced head and neck squamous cell carcinoma and demonstrated that almost all (15/17) patients had circulating cells at the time of surgery, similar to what was observed in the patients in the present study (6/8 were positive to CTC preoperatively). A recent meta-analysis comprised of 17 studies confirmed the significant prognostic value of CTCs in patients with head and neck cancer, wherein positive CTCs were significantly associated with poor overall, disease-free and progression-free survival (61). Patients who were CTC^+ tended to have higher recurrence and regional lymph node metastasis rate and a more advanced tumor stage. The authors concluded that the presence of CTCs may be used as a monitoring tool for tumor status of head and neck cancer, especially for the early detection of tumor recurrence and progression, advanced disease and node metastasis.

The primary limitation of the present study is the small number of patients included. Such small sample did not allow reliable statistical analyses to be performed. Further studies aimed at investigating CTCs in laryngeal cancer using the ScreenCell system in a larger cohort of patients are necessary to improve the definition of the sensitivity and specificity of the ScreenCell filtration system and to confirm the preliminary results.

In conclusion, the results of the present study revealed a possible association between the presence of CTCs and a less favorable prognosis in patients with LSCC. The current preliminary findings may encourage more research into the incorporation of a liquid biopsy test in the management of LSCC, as it may help identify patients with occult metastatic disease earlier and in a non-invasive manner. This may also represent an independent prognostic factor which may help in clinical evaluation. Further studies aimed at investigating the role of CTC using the ScreenCell system in a larger number of patients with laryngeal cancer are necessary to confirm these preliminary results.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MIR and MR conceived and designed the study. CN and AGra collected and analyzed the samples and interpreted the data. CN, CDG and RC prepared the first draft of the manuscript. CDG, AGre and RC made substantial contributions to acquisition and analysis and interpretation of data. MR and MDV performed the clinical evaluations and surgical procedures. AGre and CDG reviewed and revised the manuscript critically for important intellectual content. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The Ethics Committee of the Sapienza University of Rome approved this pilot study (approval no. 32/2017). All patients provided informed written consent.

Patient consent for publication

Signed informed consent for publication has been obtained from the patients.

Competing interests

The authors declare that they have no competing interests.

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