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Lung Cancer

CellSearch® technology applied to the detection and quantification of tumor cells in CSF of patients with lung cancer leptomeningeal metastasis

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ABSTRACT

Objectives: The diagnosis of solid cancer leptomeningeal metastasis (LM) relies on the cytology of cerebrospinal fluid (CSF) and/or imaging evidence of neuraxis, yet both lack sufficient sensitivity. The utility of the CellSearch®, an FDA -approved technology, in assessing CSF tumor cell (CSFTC) was evaluated here in the diagnosis and treatment of patients with lung cancer-related LM.

Materials and methods: In 18 patients with magnetic resonance imaging (MRI) confirmed LM due to lung cancer, 5 mL of CSF were collected in CellSave® preservative tubes, which allow performing the assay within 96 h after sampling. Using a previously adapted CellSearch® method, we detected, visualized and enumerated CSFTCs and compared the results with conventional cytology. In 3 patients, tumor cells were evaluated sequentially to explore the predictive role of CSFTCs enumeration in the treatment response monitoring.

Results: CSFTCs were disclosed in 14 of 18 MRI confirmed LM samples (median 785CSFTCs/5 mL CSF, range 1 to >20,000), yielding a sensitivity of 77.8%, compared with 44.4% for conventional cytology. CSFTC clusters were observed in 12 patients, similar to those previously described in blood as circulating tumor microemboli (CTM), and enumerated sequentially with reproducible results, which did not necessarily correlate with response to treatment.

Conclusion: The CellSearch® technology, applied to limited sample volumes and allowing delayed processing, could be of great interest in the diagnosis of LM in lung cancer patients.

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1. Introduction

Leptomeningeal metastasis (LM) is a devastating metastatic complication of solid cancers with a dismal prognosis and high mortality rate [1]. During the past few decades, the incidence of LM appears to have increased through the improvement of imaging studies and of the better life expectancy of cancer patients receiving more effective treatments [2–6]. Lung cancer (LC) is responsible for

10–26% of LM [3,7], with an estimated incidence of 1% in non-small cell lung cancer (NSCLC) and of 10–25% in small cell lung cancer (SCLC) [7].

The diagnosis of LM, according to the National Comprehensive Cancer Network (NCCN) guidelines [8], can be assessed by the identification of malignant cells in the cerebrospinal fluid (CSF) or by the association of characteristic signs and symptoms and typical magnetic resonance imaging (MRI) findings. However, it can be difficult to confirm a diagnosis of LM, as clinical features can be subtle and may be heterogeneous. The use of specific methodologies, which are not applied in the usual follow-up of solid tumour patients, namely brain and spinal gadolinium enhanced MRI and CSF cytological analyses, is implied in the diagnosis of LM. In fact, CSF cytology remains the gold standard for LM diagnosis with a high specificity (about 95%) but a low sensitivity (55% at the first lumbar

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Fig. 1. Sagittal T1 postgadolinium image of the spine showing typical enhancing subarachnoid nodules considered in this study as unequivocal for the diagnosis of leptomeningeal metastasis (LM).

puncture) [3,7]. Several CSF samples may also be required to finally identify malignant cells. In a retrospective study [9], a large series of patients with LM were evaluated, and it was found that MRI led to the diagnosis of LM in 53% of patients while CSF cytology provided the diagnosis in 23%. As a consequence, more sensitive and precise techniques, preferably employing a robust approach, are needed in order to improve LM diagnosis.

The CellSearch® method provides a semi-automated cell analysis, based on the assessment of nuclear and surface markers, which has greatly improved the sensitivity, reliability, objectivity and accuracy of circulating tumor cell (CTC) detection and enumeration in peripheral blood [10]. With a high sensitivity (detection as low as one CTC in 7.5 mL of blood [11]), this method has been validated by the FDA for the clinical prognostic evaluation of breast, prostate and colorectal cancers [11–15]. Several studies have also demonstrated its potential interest in LC [16]. In recent studies, the CellSearch® has been shown to be performing well in the detection of malignant cells not only in blood samples, but also in fluid samples including CSF, hydrothorax or ascites, allowing for a precise enumeration of malignant cells whatever the cellularity of biological fluid samples [17–20]. In previous studies by our group [21,22], we described an easy and innovative adaptation of CellSearch®, applied to the detection of malignant cells in the CSF of breast cancer patients with LM (CSF tumor cells, CSFTCs) [21] as well as of melanoma cells in the CSF of melanoma patients with LM (CSF melanoma cells, CSFMCs) [22].

In this report, we have continued these pilot studies to validate the feasibility of the CellSearch® technology for the diagnosis of LM in the context of LC. We have also explored the predictive role of CSFTCs in monitoring the response to treatment.

2. Materials and methods

2.1. Patients

Ruled out the inflammatory or infectious condition, patients with MRI confirmed LC-related LM were enrolled between July 2012 and February 2014 from the Neurology Departments of Nancy University Hospital and Lille Oscar Lambret Center. All patients benefited from lumbar puncture after total spine and brain MRI evaluation. Unequivocal MRI findings were defined as leptomeningeal enhancement with subarachnoid nodules, enhancement in basal cisterns or enhancement/clumping of nerve roots. The CellSearch® technology was applied to the detection and quantification of CSFTCs in CSF samples (recommended volume: 5 mL) tested in parallel with CSF standard cytological analysis for visual cytomorphological examination of tumor cells. Conventional cytology was done on standard sampling tubes transported as quickly as possible to the Pathology Lab. Samples were cytocentrifuged (Cytospin4, Thermo Shandon, Thermo Life Sciences, Cergy Pontoise, France), stained (May Grunwald Giemsa) and examined (Olympus BX-60, Rungis, France) by cytologists. Standard CSF biochemical analyses were also recorded. Additionally, sequential CSF samples were obtained after diagnosis from 3 patients who agreed to CSF sampling during their intrathecal treatment and 1 patient had a concomitant blood sample for CTC detection. Conventional cytology of CSF was performed without knowledge of the results of CSFTC analysis and vice versa. The neuro-oncologist providing treatment and evaluating the patients was blinded to the CSFTC analysis.

All included patients provided written informed consent for an analysis of CSFTCs with the CellSearch® technology.

2.2. Identification of CSFTCs

The CellSearch CTC kit® (Veridex LLC, Raritan, NJ) was used to enumerate tumor cells of epithelial origin firstly by an immunomagnetic enrichment of cells expressing EpCAM (epithelial cell adhesion molecule), followed by nuclear staining with DAPI (4′,6-diamidino-2-phenylindole), and immunofluorescence detection of cytoplasmic cytokeratins (CK) 8, 18, and/or 19. Membrane CD45 staining was also performed for the detection of contaminating leukocytes. Samples were collected in CellSave® preservative-containing tubes which could be stored for up to 96 h at room temperature before processing. When the volume of the samples was less than 5 mL, PBS was used to top the CSF to 5 mL. The standard method of CellSearch® was used to detect CTCs in blood samples, and we applied our previously described method for the detection of CSFTCs [20,21]. Briefly, the CellSearch® technology requires red blood-colored specimens. Therefore, to allow for the analysis of a clear fluid sample such as the CSF, the outside of the tube was simply darkened with a black felt-tip up to the fluid level to mimic the level of sedimented erythrocytes. After deposition of 5 mL of CSF, 5 mL of dilution buffer were added, and the mixture was homogeneized and centrifuged. The "lured-tube" was then placed into the preparation station of CellSearch® and submitted to automated preparation as for blood samples, up to the generation of image galleries of suspect cells for technical validation. Results were reported as numbers of CSFTCs/5 mL CSF. CSFTCs detection was considered positive if at least one CK$^+$/DAPI$^+$/CD45$^-$ cell was identified in the processed sample.

3. Results

This pilot study included 18 LC patients with a MRI-confirmed diagnosis of LM (Fig. 1). Patients' clinical characteristics at the time

of enrolment are described in Table 1. The main histological subtype of LC was NSCLC, with 13 (72.2%) adenocarcinoma. Eight patients (44.4%) had concomitant parenchymal brain metastases.

LM diagnosis was assessed in all patients with both total spine and brain MRI and standard CSF cytology (Table 1). The MRI demonstrated unequivocal images of LM in all the 18 patients (100%). CSF samples volumes varied between 0.5 and 5.0 mL for standard cytomorphological analysis, with a median volume 2.0 mL and tumor cells were detected but not enumerated in 8 patients (44.4%). CSF from these patients contained 0.24 to 3.43 g/L protein and 0.15 to 0.86 g/L glucose.

CSF samples volumes for the detection of CSFTCs varied between 2.6 mL and 5.0 mL (median volume: 4.1 mL). Concomitant CSF analysis with the CellSearch® technology revealed CSFTCs in 14 patients (77.8%) including 6/10 with negative conventional cytology. Meanwhile, CSFTCs were not detected in five LC patients with brain metastases but without LM (data not shown). Table 1 summarizes the quantitative results of CSFTCs enumeration using the CellSearch® technology. The median number of CSFTCs was 785 CSFTCs/5 mL CSF (range 1 to >20 000). The sensitivity of conventional cytology on the first lumbar puncture was 44.4%, (95% confidence interval [CI]: 21.5–69.2%), while that of the CellSearch® technology from the same CSF puncture was 77.8% (95% CI: 52.4–93.6%). The specificity of CellSearch® technology was 100% (95% CI: 47.8–100%).

Fig. 2A shows representative epithelial tumour cells detected by the CellSearch® technology in a CSF sample. These cells have features similar to those reported for peripheral epithelial tumour cells described as CTCs in LC patients. They are round or oval in shape, with a moderate cytoplasm and a round nucleus. Fig. 2B shows peripheral CTCs from the same patient. Comparison of tumor cells in CSF and peripheral blood from this patient showed that CSFTC was larger, well-stacked, with a stronger fluorescence than CTC. Furthermore, CSFTCs presented as either individual cells in 2 of 14 CSFTC positive patients, or associated with aggregates of tumor cells similar to those described in blood from cancer patients as circulating tumor microemboli (CTM) in 12 patients.

CSFTCs were not detected in 4 LM patients for whom conventional cytology was also negative. Histologically, they were two adenocarcinomas, one large cell neuroendocrine carcinoma, and one small-cell carcinoma.

Among the three patients who benefited from sequential CSF sampling, as mentioned above, during LM treatment (Fig. 3A), two were studied twice and both exhibited a significant decline in the number of CSFTCs following initiation of intrathecal or systemic therapy (respectively from 20 000 to 10 000 CSFTCs/5 mL CSF, and from 600 to 163 CSFTCs/5 mL CSF). The third patient was studied three times, and has decreasing numbers of tumor cells, from 7000 to 683 CSFTCs/5 mL CSF. Fig. 3B shows three results from this patient at different times (before and after the first and second cycles of systemic treatment). Not only the numbers of CSFTCs had decreased, but also the proportions of isolated CSFTCs and aggregated CSFTCs changed after intrathecal treatment. Disappointedly, the decreasing numbers of CSFTCs did not correlate with a favourable treatment response for these patients who showed clinical evidence of worsening.

4. Discussion

In this study, CSF samples from 18 LC patients with MRI-confirmed LM were analysed using the immunomagnetic platform-based CellSearch® technology. The detection of CSFTCs was confirmed to be a robust diagnostic tool in this condition, confirming the diagnosis of LM in 14/18 cases and outperforming the standard diagnostic tool of conventional CSF cytology analysis.

There is an increasing interest in the direct identification of CTCs in peripheral blood as a potential robust marker in cancer diagnosis, prognosis and treatment. Several methods have been reported [23,24], including the FDA-approved CellSearch® technology [11–15], and they should be investigated in the detection of CSFTCs.

The CellSearch® technology vastly improves the sensitivity, reliability, objectivity and accuracy of CTC detection in peripheral blood compared to cytomorphology [14], and has recently allowed a precise enumeration of malignant cells in either rich or paucicellular biological fluid samples [17–20]. Patel et al. [17], by spiking normal blood with CSF, have shown that the CellSearch® technology could be used for the detection of malignant cells in the CSF of breast cancer patients with central nervous system metastases. In this small pilot study of five patients, the number of CSF tumor cells correlated with the tumor response to chemotherapy and was dynamically associated with disease burden while changes in CSF cytology did not necessarily correlate with response. How-

Table 1
CSF characteristics and MRI findings at the time of diagnosis in patients with lung cancer.

Patient no.	Histologic type of cancer	CSF results on first lumbar puncture			MRI findings	Brain metastasis	CSF volume for CellSearch® (mL)	CSFTCs on lumbar puncture (CSFTCs/5 mL)
		Glucose, g/L	Protein, g/L	Cytology				
1	Adenocarcinoma	0.54	1.44	−	+	+	5.0	203
2	Adenocarcinoma	0.42	3.31	+	+	−	3.9	855
3	Adenocarcinoma	0.51	0.24	−	+	+	4.2	1500
4	Adenocarcinoma	0.26	3.43	+	+	+	4.0	500
5	Adenocarcinoma	0.15	3.12	−	+	+	5.0	95
6	Adenocarcinoma	0.71	0.38	−	+	−	5.0	0
7[a]	Adenocarcinoma	0.23	1.36	+	+	−	5.0	7000
8	Adenocarcinoma	0.86	0.30	−	+	−	5.0	0
9	Adenocarcinoma	0.58	1.31	+	+	+	5.0	3000
10	Adenocarcinoma	0.55	0.86	+	+	−	4.5	715
11[a]	Adenocarcinoma	0.32	3.00	+	+	+	3.0	20000
12[a]	Adenocarcinoma	0.45	1.38	+	+	−	3.0	600
13	Adenocarcinoma	0.57	0.89	−	+	−	4.0	380
14	Epidermoid	0.45	1.21	+	+	−	5.0	3200
15	Large cell neuroendocrine carcinoma	0.63	0.59	−	+	+	2.6	0
16	Small cell carcinoma	0.79	0.47	−	+	−	4.0	1
17	Small cell carcinoma	0.55	1.6	−	+	+	2.8	0
18	Unkown	0.27	0.83	−	+	−	4.0	1500

Abbreviations: LM: leptomeningeal metastasis; CSF: cerebrospinal fluid; CSFTC: CSF Tumor Cell. [a] patients with sequential CSF samples after treatment.

ever, this spiking method requires blood samples from "healthy donors", which renders this approach more difficult to standardize and therefore less suitable for a routine setting. Nayak et al. [18] have evaluated the utility of the CellSearch® technology in the diagnosis of LM from 51 solid tumors patients, including LC, through the identification of CSFTCs. In this study, CSF samples were analyzed using the "control" mode of the CellSearch® instrument. The "control" mode is customarily designed as quality control for this technology, using a specific breast cancer cell suspension with known quantity. Under this mode, CSF can be treated as a cell suspension with a definitive volume of 3.5 mL. Ultimately 15 patients fulfilled LM criteria, and 16 presented with CSFTCs, achieving a sensitivity of 100% as compared to 66.7% for conventional cytology and 73.3% for MRI. The detection of CSFTCs was confirmed as a robust diagnostic tool in a wide range of epithelial tumor types, including lung cancer [18].

Here, we focused on LM-confirmed LC patients with or without brain metastases, using a newly, easy, adapted CellSearch® method.

Besides a high specificity (100%), we confirmed a better sensitivity of the CellSearch® technology (77.8%) compared to conventional cytology which fared poorly in this setting (44.4%). Several points could explain this superiority. First, in conventional cytology, the tumor cells can die more easily after sampling while we used CellSave® tubes, which contain an appropriate preservative, authorizing a 4-days delay in sample processing with retained cellular features. With CellSave® tubes, samples handling is easier, allowing for multicentre studies with a centralized analysis. Secondly, inadequate volume is an intuitively obvious cause of false-negative CSF cytology, and many recommendations plead for the use of larger volumes for cytology. Theoretically, obtaining 10.5 mL or more of sample is an effective means to increase the sensitivity of conventional cytology [25]. In our study, the median volume of CSF for CellSearch® detection was larger than that for conventional cytology, which could be a possible reason for the higher sensitivity of CellSearch®. Thirdly, this technology has been validated because of its improved sensitivity, reliability, objectivity and accu-
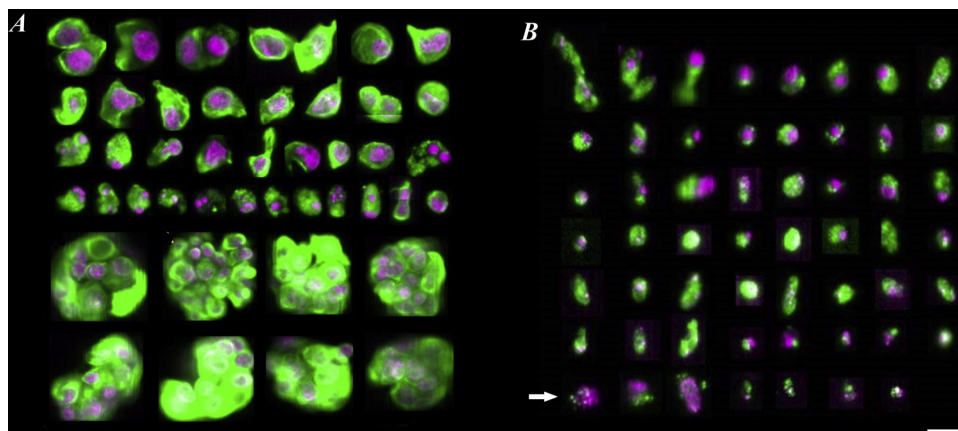

Fig. 2. Gallery of images of tumor cells in CSF (A) and peripheral blood (B) detected by CellSearch® technology. By definition, CTCs are nucleated (purple), express cytokeratin (green), and lack CD45 expression. In the CSF sample, CSFTCs were either isolated or in clusters and their morphology was similar to that of CTCs in the peripheral blood without morphologically apoptotic features, which were present in some of the CTCs in blood sample (arrow, shrunken cell containing CK inclusion). Scale bar 10 μm. CSF: cerebrospinal fluid, CTC: circulating tumor cell, CSFTC: CSF tumor cell, CK: cytokeratin (For interpretation of the references to colour/color in this figure legend, the reader is referred to the web version of this article.)
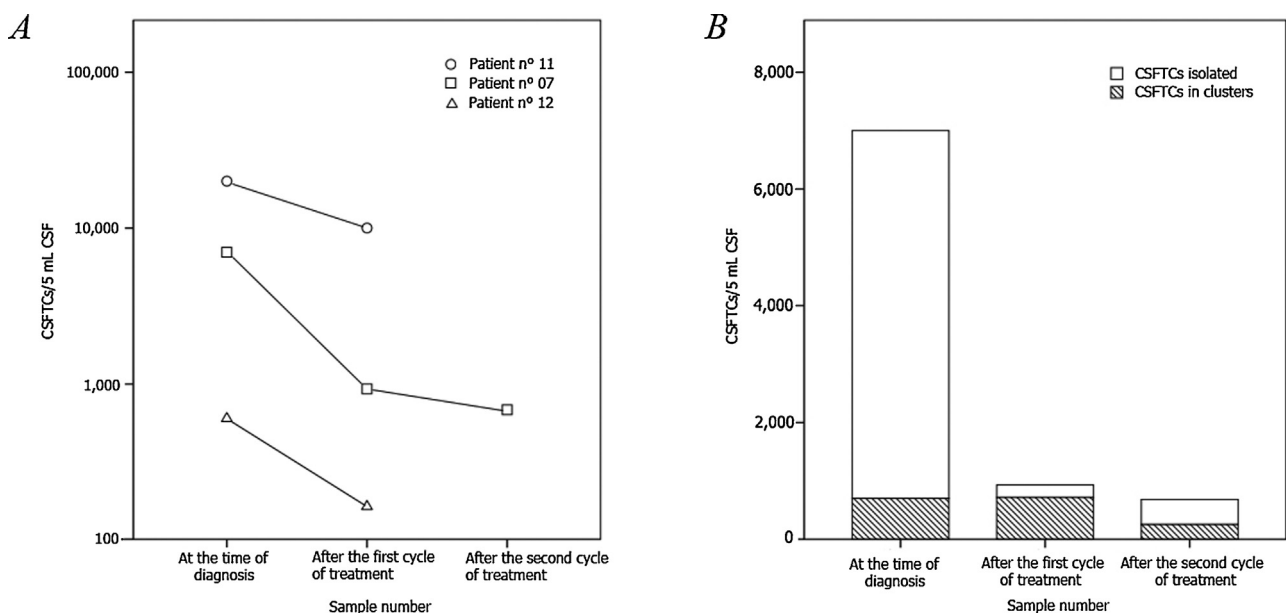

Fig. 3. Sequential CSFTCs detections in LM from LC patients. (A) Individual numbers of CSFTCs detected using the CellSearch® technology from 3 patients during follow-up. (B) Change of the numbers of total CSFTCs and aggregated CSFTCs in one patient. CSFTC: CSF tumor cell, LM: leptomeningeal metastasis, LC: lung cancer.

racy in CTC detection [10]. It depends on the magnetic selection with anti-EpCAM coated nanoparticles which could enrich EpCAM$^+$ tumor cells with a high sensitivity. Among the different antigens expressed by epithelial tumor cells, EpCAM plays a central role in helping identify the underlying tumor and EpCAM expression in malignant effusions depicts the situation in the corresponding primary carcinoma [26]. EpCAM expression has been shown to be present in CSF tumor cells. Fourthly, with a semi-automatic method and a standard criterion (CK$^+$DAPI$^+$CD45$^-$), interpretation of the CellSearch® technology results is more objective and much easier than conventional cytology which was highly dependent on experienced operators and cytologists. Additionally, with conventional cytology there is no quantification or characterization of tumor cells and clinicians must make judgments on the binary presence or absence of malignant cells as determined by cytology. In our adapted CellSearch® technology, the results were not only qualitative, but also quantitative and also recorded for possible revision at any time. Finally and most importantly, the presence of an additional free channel for other reagents in the CellSearch® also makes it possible to potentially investigate for other relevant markers expressed by CSFTCs. After enrichment by CellSearch®, the possibility of performing analyses of CSFTCs, through genotyping or molecular characterization methods could open new insights for LM patients by tailoring therapeutic strategies and monitoring treatment efficacy. Recent studies have demonstrated great potential for using circulating cell-free DNA (cfDNA) in blood for cancer diagnosis, prognosis, and directed treatment [27]. In a recent study, Pan et al. [28] have demonstrated that tumor mutations were detectable in the CSF of patients with different types of brain tumors by using high-throughput sequencing-based methods. The development of such genotyping methods could be complement of CellSearch®, the latter however providing results more rapidly.

In this study, CSF samples from LC patients with brain metastases but without LM who were included as negative control, displayed no CSFTCs, and there was no difference for CSFTC detection whether metastases were present or not.

CTMs are present in the blood of cancer patients, but their contribution to metastasis is not well defined. In some studies [29,30], peripheral CTMs are recognized as a "signature" of malignant metastases. In a recent study [31], using mouse models with tagged mammary tumors, it was demonstrated that CTMs arise from oligoclonal tumor cell groupings and not from intravascular aggregation events. Although rare in the circulation compared with single CTCs, CTMs have 23- to 50- fold increased metastatic potential. Here, in most of the patients, CSFTCs were presented as clusters, but the significance of this feature needs further research to be confirmed.

Moving forward, we evaluated the dynamical change of CSFTC counts with treatment response of three patients and found that a sequential quantitative monitoring of therapy can be obtained from CSF of LM, using the CellSearch® technology. Although decreased numbers of CSFTCs in consecutive samples can be observed, they were not necessarily correlated with clinical treatment response. However, it must be noted that LM is usually a terminal stage of cancer and that despite vigorous therapy, many patients do poorly and the median survival is only about several months. In this context, decreased numbers of CSFTCs could probably indicate the efficacy of the treatment, but their obvious presence in the CSF can always be a negative prognostic marker in these patients.

The limitation of study is its small number of studied patients, so the findings require validation. Also, we only enrolled LM patients with positive MRI. All patients with strong suspicion of LM, whatever the MRI result, should be included to fully evaluate the sensitivity of CellSearch. In addition, the therapeutic strategy here was still based on the binary result of conventional cytology. In the future, a stratification based on the quantitative results from

CellSearch® should be used to help in the management of LM patients. Finally, from this pilot study, we might observe false-negative results for CellSearch® which may be related to the low expression of EpCAM in lung cancer. This is the most important reason which hampers the validation of CellSearch® for the detection of CTCs in lung cancer after breast cancer, prostate cancer and colorectal cancer.

5. Conclusion

In conclusion, to the best of our knowledge, this is the first study focused on detecting and enumerating CSFTCs from LM patients with LC, according to an adapted CellSearch® technology. This method is relatively simple, examiner-independent, with a high sensitivity, specificity and reproducibility. The preliminary evidence from our data suggests that this direct detection and quantification of CSFTCs using the CellSearch® technology may be a promising procedure for diagnosis, and therapy-monitoring of LM in LC patients. Further studies are needed to validate the detection of CSFTCs in large cohorts, to explore their use as a diagnostic and prognostic marker in the management of LM.

Conflict of interest

None declared.

Acknowledgements

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