Tumor cells may circulate in medullary thyroid cancer patients independently of serum calcitonin

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Dear Editor,

Early detection of tumor relapse is a major issue in patients with medullary thyroid carcinoma. Calcitonin has been reported as a sensitive and accurate marker for recurrence of medullary thyroid carcinoma after thyroidectomy. Recent evidence nevertheless reveals pitfalls in calcitonin immunoassays due to the presence of heterophilic antibodies or macroaggregates (i.e. falsely increased values or macrocalcitonin) (Alves et al. 2016). Calcitonin can also remain undetectable despite metastasis of rare tumor cells in thyroidectomized patients. In this context, we designed a sensitive and specific technique to identify calcitonin-positive circulating tumor cells (CTC) in medullary thyroid carcinoma. We demonstrate that calcitonin-positive CTCs are present in the peripheral blood of medullary carcinoma patients following complete thyroidectomy. Unexpectedly, the presence of CTCs could be identified up to 12 years after surgery even in the absence of non-pathological levels of serum calcitonin.

Incidence of thyroid cancer significantly increased in the past several decades (Kuo et al. 2016). Tumors most frequently arise from follicular epithelial cells to generate papillary, follicular or anaplastic thyroid cancers. Medullary thyroid carcinoma (MTC) initiates in neuroendocrine C cells accounting for approximately 4-5% of all thyroid cancers (Xing 2013). Since MTC are unresponsive to radioiodine therapy, surgical resection of the tumor is the first line treatment. With 10-year survival rates of about 75%, thyroidectomy is generally the most adequate therapeutic option. The main issue in MTC management is prediction of tumor relapse. The serum concentration of calcitonin is a specific and cost-effective biomarker that adequately predicts tumor relapse or metastasis. Indeed, the doubling time of serum calcitonin is a reliable prognostic marker used in routine management of MTC (Ito et al. 2016). Recent evidence has nevertheless challenged calcitonin-based immunoassays due to the presence of heterophilic
antibodies in patient serum or cross-reactivity with procalcitonin and related peptides (Alves et al. 2016). These issues should be solved to improve post-operative prognosis. When calcitonin levels exceed 500 pg/mL, distant metastases are almost always identified by radiographic imaging. In contrast, when serum calcitonin is low, the risk of persistent or recurrent residual disease is low. A challenge in decision-making occurs in presence of intermediate calcitonin levels (150 pg/mL) or when concentrations are persistently increased but unchanged over time. In these conditions, local or distant metastases are difficult to detect by radiographic imaging and require additional and often expensive tests during follow-up. In this context, we investigated another approach based on the identification of thyroid-specific circulating tumor cells (CTCs). CTCs are cells that detach from the primary or metastatic tumor and intravasate into the blood stream (Yu et al. 2011). Since CTCs travel in the blood to develop a secondary tumor at a distant site, their frequency can be used as a prognostic marker for relapse. Currently, main issues in CTC enumeration pertain to their extreme scarcity compared to blood cells and to phenotypic changes (epithelial to mesenchymal transition, EMT). A series of tests have been developed based on cell size fractionation, microfluidics or antigen capture. In particular, the CellSearch (Veridex) is a FDA-validated system that enumerates CTCs upon capture of EpCAM-positive epithelial cells in whole blood. If exceeding a defined threshold, CTC levels have been demonstrated to worsen prognosis of the situation (Yu et al. 2011). The principle of the technique is based on automated sorting of CTCs with an epithelial marker (EpCAM) and subsequent detection of CD45-negative, cytokeratin-positive nucleated cells. To identify CTCs in thyroid cancer, a series of 15 patients was analyzed by CellSearch (study #2014/93 approved by the ethic committee of the CHU university hospital, Liege, Belgium). In the majority of cases (60%, 9 out of 15), no CTC was identified (Figure 1 A-B and Supplementary Table 1). Four
subjects out of 15 (27%) had 1 CTC in 7.5 ml of blood. The two remaining patients (2 out of 15) with medullary and papillary thyroid cancers displaying lung and lymph node metastases had 2 and 7 CTCs, respectively. As controls, CTCs were enumerated in cancers associated with high (small cell lung cancer, SCLC) and low (non small cell lung cancer, NSCLC) cell counts. In accordance with previous studies (Krebs et al. 2011; Hou et al. 2012), CTC were detected in all SCLC cases (n=6) and in most NSCLC (n=41 out of 67) (Figure 1B and Supplementary Table 1). These data thus indicate that EpCAM-positive CTCs are undetectable in most thyroid cancer patients validating and extending previous observations (Yu et al. 2016). Therefore, we set up another technique based on cell filtration (ScreenCell device) and calcitonin expression to identify CTCs in MTC. The reactivity of a FDA-approved antibody used in routine diagnosis (SP17) was first evaluated on 3 cell lines pertaining to the major thyroid cancer subtypes: TT (medullary), TPC-1 (papillary) and C643 (anaplastic). Immunofluorescence analysis showed that only TT cells expressed calcitonin, as expected (Figure 1C and Supplementary Figure 1). Immunohistochemistry of a thyroid biopsy further indicated that parafollicular C cells specifically stained positive for calcitonin (Supplementary Figure 2). These experiments thus validate the specificity of the anti-calcitonin SP17 antibody.

Using the ScreenCell device, CTCs were isolated from peripheral blood of a patient with MTC and stained with SP17. Figure 1D illustrates a typical fluorescence scan of CTCs expressing calcitonin in the cytoplasm and containing a DAPI-stained nucleus (blue arrows). A major criteria to distinguish CTCs from leucocytes is their larger diameter (at least 3 fold) that does not allow filter pore bypass. Although the filter pores yielded some background autofluorescence, the specificity of labeling was demonstrated by the presence of another cell lacking calcitonin expression (see * on Figure 1D) and by spike-in experiments (Supplementary Figure 3). In
contrast, no calcitonin-positive CTCs could be detected in peripheral blood from 4 healthy volunteers (Supplementary Figure 4).

Using these optimized experimental conditions, the presence of calcitonin-positive CTCs was investigated in a series of subjects with medullary thyroid carcinoma. We selected 9 patients at different TNM stages having undergone complete thyroidectomy and, except for #9, lacking any evidence of other cancer (see Table 1 for clinical and pathological characteristics). The calcitonin concentrations in the serum were above the basal level of the assay (i.e. 10 pg/ml) and ranged from 48 to 10,600 pg/ml, further validating the diagnosis. Calcitonin-positive CTCs were identified in all patients (from 1 to 7), except one (#1). Interestingly, the CTC counts did not correlate with TNM classification as illustrated for example by patient #1 at pT3N1bM1 lacking any detectable calcitonin-positive circulating cell while 5 CTCs were scored in subject #7 at pT1N1bMx. Finally, the serum calcitonin concentrations did not correlate with CTC counts ($R^2=0.11272$, Figure 1E). In particular, the calcitonin levels were low in patients #5, #7 and #8 (70, 48 and 82 pg/ml) while CTC counts were high (n=4, 5 and 7), respectively.

We have thus set up a technique that combines the ScreenCell device and calcitonin labeling that very specifically identifies CTCs in MTC patients even after thyroidectomy. Compared to the FDA-cleared CellSearch, our experimental protocol has a series of advantages. First, the CellSearch enumerates CTCs by cell sorting using an epithelial marker (EpCAM) that is frequently lost due to EMT. Our data indicate that this approach is indeed inadequate for thyroid carcinoma due to lack of sensitivity. This conclusion is in agreement with a recent report showing that, even at metastatic stages, only 7 out of 18 MTC have more than 2 CTCs (Yu et al. 2016). In contrast, our method is able to identify calcitonin-positive CTCs in thyroidectomized patients considered to be in persistent disease. Secondly, calcitonin labeling specifically
identifies CTCs derived from MTC excluding any other neoplasm such as breast cancer (patient #9 in this study). Third, gentle cell filtration by ScreenCell is also compatible with microemboli that cannot be scored by CellSearch. Fourth, the protocol does not require any expensive equipment in addition to filtration columns.

The ScreenCell device and calcitonin labeling approach also has some disadvantages. Although visual inspection very specifically reveals fluorescent cells expressing calcitonin, validation of CTC phenotype requires the intervention of a trained pathologist. The technique also requires further automation to be compatible with routine diagnosis. Another limitation of the technique is that blood samples should be analyzed fresh or at least within a maximum of 92 hours. Despite these drawbacks, the ScreenCell device and calcitonin labeling methodology can, as described, readily be set up in standard conditions of routine diagnosis.

Although the series of samples are limited, this report highlighted an unexpected and interesting observation: relatively high CTC counts can be detected despite low calcitonin levels in the serum of 3 patients (#5, #7 and #8). Since CTC numbers correlate with the probability of tumor relapse, there is an intrinsic risk that prognosis of these subjects will worsen in a near future. Careful follow up of these patients and further validation in larger cohorts will confirm this observation. This is particularly important because follow-up periods in our series are longer in the group of low CTC count (especially in cases 1, 2 and 4) compared to the group of high CTC count (cases 5-9). Therefore, overall survival or disease- free survival may show poorer in high CTC count group than in low CTC count group. Nevertheless, we believe that the presence of calcitonin-positive CTCs in the absence of significant levels of serum calcitonin should be considered to initiate additional tests during follow-up. There is indeed growing evidence that the presence of CTC correlates with poorer prognosis (Yu et al. 2016). Further follow-up
experiments are required to assess whether prognosis depends on calcitonin-positive CTC
counts. Since neither radiotherapy nor chemotherapy has demonstrated durable objective
responses in patients with advanced MTC, early identification of tumor relapse is predicted to
increase overall survival.
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AUTHORS CONTRIBUTION

SNS performed the lab experiments and analyzed raw data. AB, EH, MC and RL organized trial and analyzed clinical data. MC contributed to patient recruitment and ethics. AB, EH and
LW conceived and designed the experiments. All co-authors contributed to writing and critical reading of the manuscript.

DECLARATION OF INTEREST

The authors fully declare any financial or other potential conflict of interest.
REFERENCES


FIGURES

FIGURE 1

(A) Representative results of CTCs identified using the CellSearch system (Veridex). CTC were isolated with an EpCam antibody and labeled for cytokeratin (CK), nuclei (DAPI) and leukocyte common antigen (CD45) as described in the Supplementary methods section. (B) Summary of CTC counts in thyroid cancer, small cell lung cancer (SCLC) and non small cell lung cancer (NSCLC). The numbers and percentages of patients without CTC (0), with 1 or with more than 1 are indicated. (C) The TT cell line derived from medullary thyroid carcinoma was labeled with SP17 anti-calcitonin antibody and visualized by fluorescence microscopy. (D) CTCs were isolated by size of epithelial tumor cells (ScreenCell device) from peripheral blood of a patient with medullary thyroid cancer. Cells were labeled and visualized as described in panel B. Arrows indicate calcitonin-positive CTCs (green) and their nuclei (DAPI in blue). (E) The number of calcitonin-positive CTCs was plotted against the concentration of serum calcitonin (pg/ml). $R^2$ is the correlation coefficient.

TABLE 1

Clinical characteristics of MTC patients, CTC numbers, calcitonin concentrations (pg/ml) and carcinoembryonic antigen (CEA in ng/ml) in serum. DT means doubling time. RET gene mutations were screened by routine sequencing according to standard guidelines of the American Thyroid Association. (m): multiple tumors, N1a: regional lymph node metastases, cervical homolateral, N1b: cervical lymph node metastasis, bilateral, medians, contralateral, metastasis mediastinal lymph node, Mx and M1: undetermined and distant metastasis, respectively. Patient #9 was also diagnosed for breast cancer. Mx and M1 mean undetermined and distant metastasis,
respectively. TT means total thyroidectomy.

Supplementary Figure 1: Only medullary TT thyroid cancer cell line stained positive for calcitonin.

A series of cell lines from different thyroid cancer sub-types (medullary TT, papillary TPC1, anaplastic Uhth74 and C643) were analyzed by confocal microscopy using the anti-calcitonin SP17 antibody. Nuclei were labeled with DAPI. The SP17 antibody was omitted in mock.

Supplementary Figure 2: Only parafollicular C cells from medullary thyroid cancer biopsies are labeled with anti-calcitonin SP17 antibody

Formalin fixed, paraffin embedded biopsy sections were heat-treated during 20 minutes and incubated overnight with rabbit monoclonal anti-calcitonin (SP17) primary antibody in a humid chamber. The primary antibody was omitted in mock. Sections were revealed with the DAB+ kit (Dako). After counterstaining with hematoxylin, slides were visualized with an Eclipse 90i microscope (Nikon).

Supplementary Figure 3: Only TT cells but not peripheral blood mononuclear cells are labeled by SP17

TT cells were spiked into peripheral blood mononuclear cells from a healthy subject and analyzed by ScreenCell filtration - SP17 labeling. Nuclei were labeled with DAPI. In panel A, the SP17 was omitted (mock). In panel B, no TT cells were added to peripheral blood mononuclear cells. In panel C, spiked-in TT cells stained positive for anti-calcitonin antibody.

Supplementary Figure 4: Blood from healthy subjects does not contain SP17-positive cells

Blood from healthy subjects (#1, #2, #3 and #4) was analyzed by confocal microscopy after ScreenCell filtration and SP17 labeling.
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<th>Type of surgery</th>
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