Detection of Circulating Tumor Cells in Patients With Adrenocortical Carcinoma: A Monocentric Preliminary Study

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Context: Adrenocortical carcinoma (ACC) is a rare malignancy, the prognosis of which is mainly dependent on stage at diagnosis. The identification of disease-associated markers for early diagnosis and drug monitoring is mandatory. Circulating tumor cells (CTCs) are released into the bloodstream from primary tumor/metastasis. CTC detection in blood samples may have enormous potential for assisting in the diagnosis of malignancy, estimating prognosis, and monitoring the disease.

Objective: The aim of the study was to investigate the presence of CTCs in blood samples of patients with ACC or benign adrenocortical adenoma (ACA).

Setting: We conducted the study at a university hospital.

Intervention: CTC analysis was performed in blood samples from 14 ACC patients and 10 ACA patients. CTCs were isolated on the basis of cell size by filtration through ScreenCell devices, followed by identification according to validated morphometric criteria and immunocytochemistry.

Main Outcome Measure: We measured the difference in CTC detection between ACC and ACA.

Results: CTCs were detected in all ACC samples, but not in ACA samples. Immunocytochemistry confirmed the adrenocortical origin. When ACC patients were stratified according to the median value of tumor diameter and metastatic condition, a statistically significant difference was found in the number of CTCs detected after surgery. A significant correlation between the number of CTCs in postsurgical samples and clinical parameters was found for tumor diameter alone.

Conclusions: Our findings provide the first evidence for adrenocortical tumors that CTCs may represent a useful marker to support differential diagnosis between ACC and ACA. The correlation with some clinical parameters suggests a possible relevance of CTC analysis for prognosis and noninvasive monitoring of disease progression and drug response. **(*J Clin Endocrinol Metab* 98: 3731–3738, 2013)**

Adrenocortical carcinoma (ACC) is a rare and very aggressive endocrine tumor with a poor prognosis, mainly dependent on tumor stage at diagnosis. Early diagnosis followed by surgical tumor removal, possibly associated to adjuvant mitotane therapy (1), has been proven as the best option for ACC treatment. The mean

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Abbreviations: ACA, adrenocortical adenoma; ACC, adrenocortical carcinoma; CTC, circulating tumor cell; EpCAM, epithelial cell adhesion molecule; SF-1, steroidogenic factor 1; TBS, Tris-buffered saline.

5-year survival rate ranges between 16 and 38%, although it drops to less than 10% in metastatic disease (2, 3). Considering that an early diagnosis is pivotal to the prognosis, the identification of sensitive, specific, and noninvasive biomarkers is mandatory to significantly improve the clinical management along with the survival rate and quality of life of ACC patients. The best biomarkers should not only be able to discriminate between benign and malignant adrenocortical masses, but also to provide prognostic penetrance, enabling noninvasive follow-up once the tumor has been surgically removed. Detection of circulating tumor cells (CTCs) in peripheral blood is a reliable tool for prognosis and follow-up in several solid cancers (4, 5), including rare tumors of neuroendocrine origin (6). CTCs are neoplastic cells originating from either primary tumor or metastases, and they circulate freely in the peripheral blood of cancer patients (4, 7). Tumor-induced angiogenesis and invasion processes allow localized tumors with high invasive potential to release CTCs into peripheral circulation before any detectable metastasis is established. CTC detection may therefore have enormous potential in diagnosing malignancy, estimating prognosis, and monitoring disease recurrence and response to anticancer therapy (8).

No attempt has so far been made to detect and characterize CTCs in blood samples of patients affected by ACC or adrenocortical adenoma (ACA).

Isolation of CTCs from the other circulating elements can be achieved with various methods (5), either immunological or physical. Immunological techniques are based on the separation of CTCs through their expression of epithelial cell-specific markers (epithelial adhesion molecules, such as epithelial cell adhesion molecule [EpCAM]) or tumor-specific markers (5, 9). Physical methods are based on cell separation according to size or migration along a density gradient. Among them, blood filtration allows CTC isolation on the basis of their larger size over other blood cells. The latter method has the advantage of isolating intact CTCs, but needs further morphological analysis to identify CTCs, whereas immunocytochemistry is recommended for cell origin characterization. This technique shows high sensitivity, detecting even 1 single tumor cell from 1 mL of blood in a background of 10^6-10^7 normal blood cells (10).

In this study, we evaluated CTC presence in blood samples of 14 patients with ACC and 10 patients with ACA using a cytomorphological technique based on filtration, specifically the ScreenCell device system (ScreenCell), followed by immunocytochemical characterization with the same markers employed in tumor tissue for ACC diagnosis. Moreover, we tried to correlate the number of CTCs detected in postsurgical blood samples with some clinical parameters of ACC.

Patients and Methods

Patients

All patients gave their written informed consent to the study, which was approved by the Local Ethical Committee. The study includes 24 patients evaluated at our university hospital for adrenocortical tumors (14 ACC and 10 ACA).

Blood sample collection

In each patient, 6 mL of blood were collected in EDTA tubes. Sampling was performed before surgery (n = 3 ACC and 10 ACA patients) or at different time points during postsurgical follow-up (n = 14 ACC and 2 ACA). All blood samples were processed within 3 hours after collection and then evaluated for CTC presence.

CTC analysis

CTC analysis was performed through 3 sequential steps consisting of isolation from blood by filtration on ScreenCell Cyto filtration devices, followed by CTC identification through validated morphometric criteria (10, 11), and finally identification of cell origin by immunocytochemistry using antibodies against adrenocortical markers.

1. Isolation

Blood was filtered by the ScreenCell Cyto filtration devices according to the procedure previously described (12). Briefly, before filtration and in order to lyse red blood cells, 3-mL blood samples were diluted in 4 mL of a specific dilution buffer for fixed cells (ScreenCell FC dilution buffer; ScreenCell). After filtration, an additional 1 mL of PBS was filtered to remove red blood cell debris. Filtration was usually completed within approximately 50 seconds. The filter was then disassembled from the filtration module and allowed to air dry. For each patient's blood sample, filtration was performed in duplicate.

2. Identification

Cytological studies, including staining and immunocytochemistry, were conducted directly on the filter. The track-etched filters were stained with hematoxylin solution S (Merck KGaA), applied to the membrane for 1 minute, and Shandon eosin Y aqueous (Thermo Electron Corporation, Thermo Fisher Scientific Inc) for 45 seconds. For microscopic observation, the ScreenCell Cyto filter was placed on a standard microscopy glass slide, and a 7-mm circular cover slip (Menzel-Glaser) was laid on the filter with the appropriate mounting medium.

CTCs were identified according to the following morphological criteria: cell size $\geq$ 16 μm, nucleocytoplasmic ratio $\geq$ 50%, irregular nuclear shape, hyperchromatic nucleus, and basophilic cytoplasm. Under these criteria, red cells and platelets were not entrapped in the filters, and leukocytes could be excluded (10, 11).

3. Cytological characterization

For immunostaining, the ScreenCell Cyto filters were hydrated with Tris-buffered saline (TBS; pH 7.4). The excess TBS was removed with absorbent paper, and the filters were put on the paraffin film in a humid chamber. Each spot was incubated for 5 minutes at room temperature with 70 μL of permeabilizing buffer. All antibodies required heat-induced epitope retrieval, so the Metafilter spots were treated in a bath containing the Target Retrieval Solution (S2367; Dako) (pH 9.0) at 99°C for 20 minutes.

After being washed quickly in a bath containing distilled water, each filter was incubated overnight with 70 μL monoclonal mouse antihuman MART-1/Melan A (clone A103; Ventana Medical Systems), monoclonal mouse antihuman synaptophysin (clone MRQ-40; Ventana) and polyclonal anti-steroidogenic factor 1 (anti-SF-1; catalog no. 07–618; Upstate, Millipore) antibodies ready to use. The filters were then washed once with TBS for 1 minute and immersed in a bath containing distilled water. Staining was achieved by treating each spot with 70 μL EnVision Detection System Peroxidase/DAB, Rabbit/Mouse (K5007; Dako) for 40 minutes at room temperature, followed by the chromogen 3.3′ diaminobenzidine (Dako) for 10 minutes at room temperature. Each filter was then placed on paraffin film, and the nuclei were slightly counterstained with Mayer's hematoxylin for 6 minutes. Finally, the filters were rinsed with running water and dried for 10 minutes at room temperature.

Histological analysis and immunohistochemistry of the primary tumor

Histological diagnosis was performed by the reference pathologist on tumor tissue removed at surgery (n = 14 ACC and 3 ACA). In 7 patients affected by nonhypersecreting adrenal incidentaloma, the diagnosis of ACA was established by computed tomography/magnetic resonance imaging tumor characteristics and unchanged imaging characteristics at least 1 year after diagnosis.

Tumor specimens were evaluated according to the Weiss System, which combines 9 morphological parameters: 3 related to tumor structure (description of cytoplasm, diffuse architecture, and necrosis), 3 related to cytology (atypia, atypical mitotic figures, and mitotic count), and 3 related to invasion (veins, sinusoids, and tumor capsule). The presence of 3 or more criteria highly correlates with malignant behavior (13).

Immunohistochemistry was performed on formalin-fixed and paraffin-embedded tissues using antibodies directed against adrenocortical markers such as MART-1, inhibin-α, and synaptophysin to define the adrenocortical origin of the tumor. Ki67 index was evaluated as a proliferation marker to assess ACC prognosis (14, 15). Immunohistochemistry analysis with mouse antihuman Ki67 monoclonal MIB1 antibody (Dako) was performed with the Ventana Benchmark XT system (Ventana Medical Systems). Nuclei were hematoxylin-counterstained. Ki67-positive nuclei were counted on 1000 tumor cells, and Ki67 was expressed as the percentage of proliferating cells. Negative controls were achieved by omitting the primary antibody.

Tumor stage was assessed according to the revised TNM classification of ACC proposed by the European Network for the Study of Adrenal Tumors (16).

Statistical analysis

All data were expressed as mean ± SD and median [interquartile range]. Statistical analysis was performed by SPSS ver-

Table 1. Characteristics of ACC Patients

	Mean ± SD, Median [Interquartile Range]	No. of Patients	%
Age at surgery, y	44 ± 18, 47 [25–59]	14	100
Sex			
Male		5	36
Female		9	64
Adrenal			
Left		5	36
Right		9	64
Secretion		9	64
Cortisol		6	67
Androgens		6	67
DHEAS		1	11
Progestins		1	11
Tumor diameter, cm	10.1 ± 5.7, 8.8 [5.7–14.6]	14	100
Ki67, %	27.4 ± 20.7, 20.0 [12.5–40.0]	14	100
WEISS	6.6 ± 1.6, 7 [6–8]	12	86
Stage			
1		2	14.3
2		5	35.7
3		3	21.4
4		4	28.6
Metastases	Lung, liver, bone, pancreas	4	28.6
Surgery		14	100
MTT therapy		13	93
Other chemotherapies (EDP)		5	35.7
Radiotherapy		0	0
Follow-up from surgery, mo	32.6 ± 20.7, 22.5 [16.2–54.0]	14	100
Survival		11	79

Abbreviations: EDP, etoposide-doxorubicin-cisplatin combined chemotherapy; MTT, mitotane; DHEAS, dehydroepiandrosterone sulfate. Mean ± SD and median [interquartile range] values for the indicated parameters are reported, along with the number of patients and their percentage.

sion 18.0 for Windows (Statistical Package for the Social Sciences). *P* values of less than .05 were considered statistically significant. Univariate correlation was carried out using Pearson's test. Groups of data were compared using the nonparametric Mann-Whitney *U* test or Student's *t* test for independent values, when appropriate.

Results

Patient characteristics

The enrolled cohort of 24 adrenal tumor patients consisted of 14 patients with ACC and 10 with ACA whose main characteristics are detailed in Tables 1 and 2, respectively.

Of the 14 ACC patients, 5 (36%) were male, and 9 (64%) presented a secreting ACC. Mean ± SD age at diagnosis was 44 ± 18 years. Stages at diagnosis were as follows: stage 1, 2 patients (14.3%); stage 2, 5 patients (35.7%); stage 3, 3 patients (21.4%); and stage 4, 4 patients (28.6%). All patients underwent adrenalectomy. After surgery, 13 (93%) were administered adjuvant mitotane therapy. Among these, five also received etoposide-doxorubicin-cisplatin combined chemotherapy. None underwent radiotherapy. Survival rate was 79% with a mean ± SD follow-up of 32.6 ± 20.7 months from surgery.

Of the 10 ACA patients, 5 (50%) were males and 3 (30%) had a cortisol-secreting tumor. Mean ± SD age at diagnosis was 59 ± 14 years. Adrenalectomy was performed in the 3 patients with cortisol-secreting tumors. The mean ± SD duration of follow-up was 29.3 ± 16.4 months after diagnosis.

Detection of circulating ACC cells

CTCs were isolated and detected in all patients affected by ACC after hematoxylin/eosin staining of filters (Figure 1, A–F). Tumor cells were observed mostly as isolated units (Figure 1). On the other hand, CTCs were not found in the blood of ACA patients.

CTCs were detected in all patients tested before surgery (mean CTCs/3 mL = 14.5 ± 14.6; n = 3 patients and 3 samples) and in all patients tested in the postsurgical period (mean CTCs/3 mL = 3.9 ± 7.1; median CTCs/3 mL = 1.9 [interquartile range, 0.8–4.5]; n = 14 patients and 21 samples). The presurgery blood samples were collected at hospital recovery (12–24 h before surgery). The postsurgery blood samples were collected 17 ± 15 months (mean ± SD) after surgery. In 2 patients affected by stage 2 ACC and positive for CTCs shortly after surgery, CTCs were not detected in blood samples drawn 12 and 24 months after surgery, respectively.

No CTCs were detected in presurgical blood samples from any of the ACA patients analyzed (n = 10). The presurgery blood samples were collected at hospital recovery (12–24 h before surgery) in patients who were going to be operated (n = 3 patients; Table 2) or in nonoperated patients during a control visit. In 2 ACA patients, we also obtained blood samples 2 months after surgery. These blood samples remained CTC negative.

Immunocytochemical analysis of the filters performed using antibodies against MART-1 (Figure 1G), synaptophysin (Figure 1H), and SF-1 (Figure 1I) demonstrated a marked positivity of CTCs, confirming their adrenocortical nature (n = 14 patients).

Surgery affects the number of CTCs

In 3 of the 14 patients analyzed, we obtained presurgical as well as postsurgical blood samples at different follow-up times (0, 2, 6, and 12 mo). When presurgical and postsurgical samples from the same patient were com-

Table 2. Characteristics of ACA Patients

	Mean ± SD, Median [Interquartile Range]	No. of Patients	%
Age at diagnosis, y	59 ± 14, 65 [52–68]	10	100
Sex			
Male		5	50
Female		5	50
Adrenal			
Bilateral		1	10
Left		2	20
Right		7	70
Secretion		3	30
Cortisol		3	100
Tumor diameter, cm[a]	2.9 ± 0.9, 3.0 [2.3–3.5]	10	100
Follow-up surgery/diagnosis, mo	29.3 ± 16.4, 27.5 [14.0–42.7]	10	100
Surgery		3	30
Survival		10	100

Mean ± SD and median [interquartile range] values for the indicated parameters are reported, along with the number of patients and their percentage.

[a] Evaluated by computed tomography/magnetic resonance imaging scan.

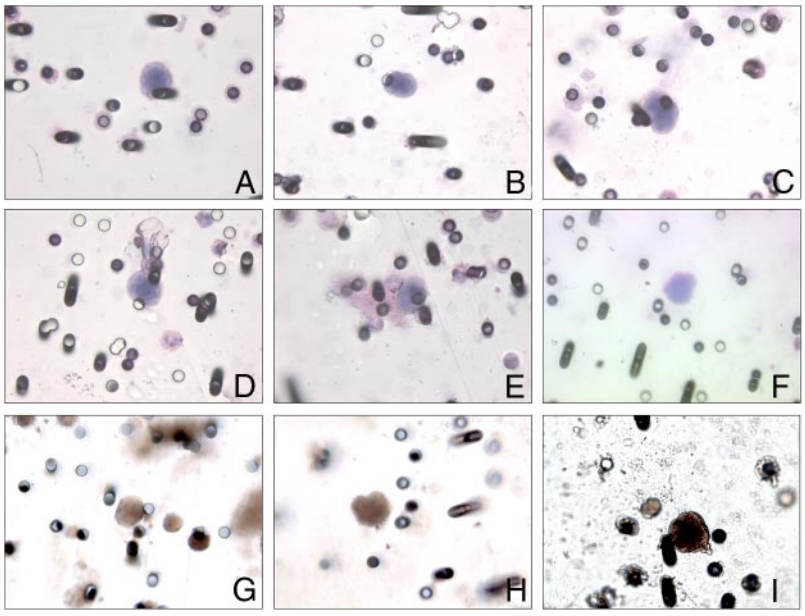

Figure 1. Circulating adrenocortical cancer cells are present in blood samples from ACC patients. A–F, Hematoxylin and eosin staining of representative track-etched filters obtained after filtration of blood samples from different ACC patients. Neoplastic cells fulfilled criteria for CTCs, including: 1) cell size ≥ 16 μm; 2) nucleocytoplasmic ratio ≥ 50%; 3) irregular nuclear shape; 4) hyperchromatic nucleus; and 5) basophilic cytoplasm (original magnification, ×63). G–I, Immunocytochemistry with anti-MART-1 (G), synaptophysin (H), and SF-1 (I) antibodies displayed a strong positivity of CTC, confirming their adrenocortical origin (original magnification, ×63).

pared, a statistically significant decrease in the number of CTCs was noted (Figure 2A, Student's *t* test for unpaired samples; $P = .02$). In 2 of the 3 patients, the CTC number considerably decreased after surgery and remained stable, whereas in the third patient, surgery did not seem to affect CTCs (Figure 2B). No significant correlation between the CTC number and the length of follow-up was evident. The characteristics of these patients are described in Supplemental Table 1 (published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org).

Correlation of CTC values with clinicopathological prognostic parameters

To ascertain any association between the CTC number in postsurgical blood samples and the main clinicopathological characteristics of ACC patients, we performed univariate regression analysis between CTC number/3 mL and available parameters—namely, patient age, tumor diameter, Ki67, stage, and Weiss score, using the first sample available at follow-up (mean ± SD = 15 ± 11 mo of follow-up). A statistically significant linear correlation was found only with the tumor diameter ($R^2 = 0.362$; R = 0.602; $P = .023$; n = 14), but not with the other parameters analyzed such as Ki67 ($R^2 = 0.147$; R = 0.384; $P = .196$; n = 13), age, stage, and Weiss score (data not shown).

When patients were stratified into 2 classes according to the tumor diameter median value in the ACC cohort and to metastatic condition (stage 4), a statistically significant difference was found in the number of postsurgical CTCs. CTC mean number ± SD per 3 mL was 8.3 ± 11.2 vs 1.8 ± 2.0 ($P = .006$) for tumor diameter ≥ 8.8 cm and < 8.8 cm, respectively, and was 11.7 ± 14.5 vs 2.1 ± 2.1 ($P = .031$)

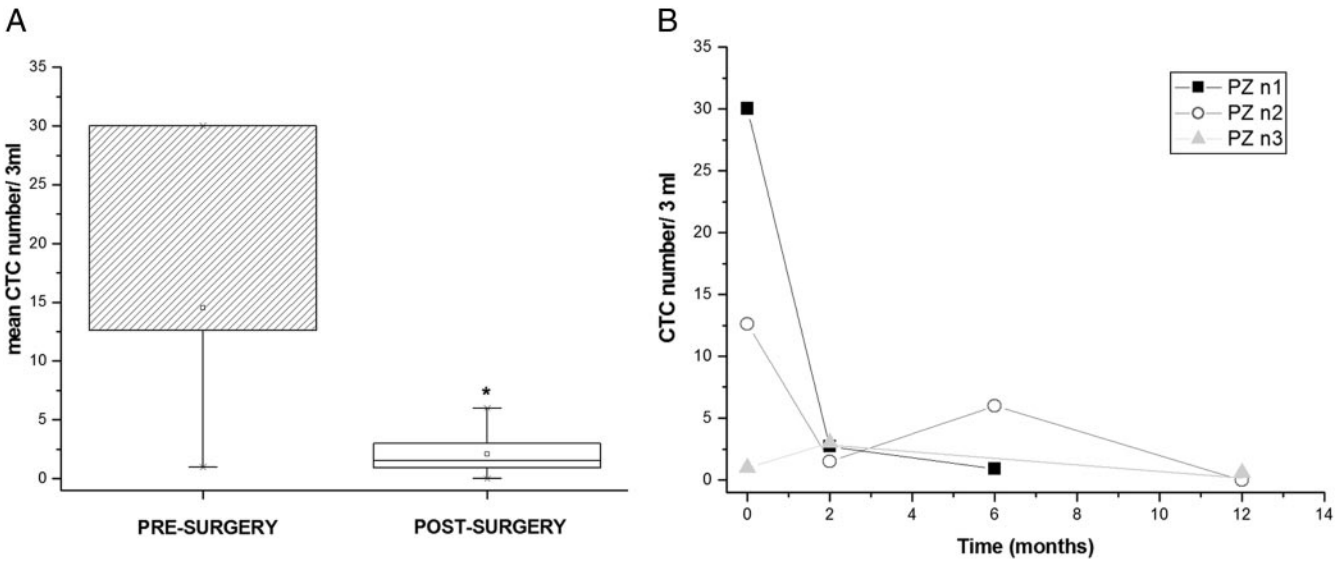

Figure 2. Time course analysis of CTC levels in ACC. CTC levels were evaluated in 3 ACC patients before surgery and at different time intervals during follow-up. Numbers of CTCs/3 ml are expressed as mean or median values in presurgical and postsurgical samples from the 3 patients as box charts (A) or as absolute values in each sample from each patient during follow-up (B). *, $P < .05$, Student's *t* test.

for stage 4 and stages < 4, respectively (Table 3). Finally, there was no statistically significant difference in the mean number of CTCs in postsurgical samples between alive and deceased patients (data not shown).

Discussion

In this study, we demonstrated the ability of the ScreenCell method to detect CTCs of adrenocortical origin dependent on cell size in blood samples from ACC patients after surgical removal of the tumor, with no positivity in ACA samples. Our analysis revealed that CTC positivity was found in all presurgical blood samples, as well as in all postsurgical blood samples in metastatic patients. Moreover, the false-positive outcome among the benign adrenocortical tumors was zero, thereby suggesting the high specificity and sensitivity of the method. Interestingly, CTCs were not found in ACA patients after surgery (2-mo follow-up), thus excluding that intraoperative manipulation of the adrenal mass may cause tumor cell dissemination, as has been suggested for other solid tumors (11, 17, 18). However, in the absence of long-term follow-up, these studies failed to demonstrate any cause-and-effect relationship between surgical indirect cell dissemination and the development of metastasis. Further longitudinal studies on larger cohorts of ACC patients operated in various surgical centers are needed to evaluate the clinical impact of different surgical procedures (open vs video-assisted) in shedding adrenocortical cancer cells into the circulation.

Our findings indicated that CTC retrieval from peripheral blood by minimally invasive procedures could be a valid and sensitive marker to support the differential diagnosis between malignant and benign adrenocortical tumors. The importance of this diagnostic biomarker is even more relevant in adrenocortical tumors because the prognosis is strictly dependent on early diagnosis. Indeed, up to now, ACC diagnosis was only possible after surgical removal of the mass and histological confirmation.

The ScreenCell method allows separation of CTCs from blood based on cell size and morphological criteria, with subsequent specific characterization to identify the adrenocortical origin. We chose this method of separation to avoid CTC selection on the basis of the expression of specific markers, thus allowing the capture of all CTCs present in blood samples, irrespective of surface markers. In fact, other separation methodologies based on cell surface expression of epithelial markers, such as EpCAM (4, 5, 19), might underestimate CTCs derived from adrenal carcinomas, which have been demonstrated to be negative for EpCAM (20).

Immunocytochemistry performed on enriched CTCs confirmed the ACC origin because they were positive for markers routinely used for characterization of primary adrenocortical tumors (MART-1 and synaptophysin), and in particular displayed nuclear positivity for SF-1, which is strongly expressed in ACC (21) and H295R (22), with a positive correlation with tumor aggressiveness.

Metastatic cells from various tumors have often been demonstrated to express phenotypic and genotypic characteristics at variance with the primary tumor (9). Thus, continuous monitoring and characterization of such differences on isolated CTCs from blood samples during patient follow-up may be relevant for modulating personalized anticancer therapies specific for metastatic rather than for the primary tumor cells (19, 23).

In metastatic patients, CTCs isolated in postsurgical blood samples are likely to derive from metastases or tumor recurrence. Conversely, the origin of CTCs still detectable in 90% of disease-free patients even after extended follow-up is unclear. In breast cancer patients, tumor cell detection has been described in both blood (CTCs) and bone marrow samples even at longer follow-up (median, 40 mo) from primary operation (24), suggesting a long-lasting reminiscence of the bulk of cells spilled out from the primary tumor before its removal. Due to this persistent presence of CTCs in the bloodstream, it would probably be more important to evaluate over time the change in the number of CTCs, rather than the absolute number. In the 3 patients studied at different time points, the number of CTCs after surgery either remained stable, as in the case of stage 1 and 2 patients, or signifi-

Table 3. Evaluation of CTCs in Postsurgical Blood Samples in ACC Patients

Tumor	Stage < 4	Stage 4	*P*	Diameter < 8.8 cm	Diameter ≥ 8.8 cm	*P*
Postsurgical CTCs, n/3 mL						
Mean (SD)	2.1 (2.1)	11.7 (14.5)	.031	1.8 (2.0)	8.3 (11.2)	.006
Median [interquartile range]	1.1 [0.7–3.0]	5.8 [2.4–27.0]		1.0 [0.5–2.3]	3.0 [2.2–9.0]	
Patients, n (%)	10 (71)	4 (29)		7 (50)	7 (50)	

Patients (n = 14) were stratified in 2 classes for stage and diameter using stage-4 or diameter median value as cutoff. Mean (SD) and median [interquartile range] values for postsurgical CTC are reported, along with the number of patients and their percentage. The range of follow-up was 2–36 months from surgery. Statistical difference between mean values in the 2 classes was evaluated using the nonparametric Mann-Whitney *U* test for independent values.

cantly decreased compared to presurgical samples, as in the case of the stage 4 patient. Although based on a limited number of patients, this may confirm the absence of surgical dissemination as well as the fact that mass removal may reduce the number of CTCs entering the bloodstream. In some patients, CTCs became undetectable during follow-up, although a significant correlation between the number of CTCs and follow-up duration could not be found.

When correlating CTC detection in postsurgical blood samples with clinical parameters of the tumor, a significant correlation was found with tumor diameter, but not with Ki67. Indeed, cell metastatic potential may be independent from the proliferative characteristics of the tumor, of which Ki67 can be considered a valid marker. Conversely, tumor diameter has been demonstrated as one of the best predictors of malignancy (25, 26) and an independent parameter of survival. Indeed, large tumors with diameter $>$ 12 cm have been associated with lower survival after complete resection (27). The tumor diameter consequently represents a good independent parameter to be correlated with the number of CTCs detaching from the primary mass. A significant correlation between tumor diameter and the number of CTCs has been observed in liver (28) and gastric tumors (29).

The other interesting finding is the statistically significant difference found in the mean number of CTCs in metastatic vs nonmetastatic patients. The prognostic value of CTCs has already been recognized in non-small-cell lung cancer because metastatic and nonmetastatic patients significantly differed in the CTC mean number (30). A cutoff higher than 5 CTCs per 7.5 mL was the strongest predictor of overall survival on multivariate analysis in non-small-cell lung (28), breast (4), and prostate (31, 32) cancer and metastatic melanoma (33). A recent meta-analysis, conducted on articles published between January 1990 and January 2012, has pointed out the clinical prognostic power of CTCs for overall, disease-free, and progression-free survival in early and metastatic stages of breast cancer, irrespective of the CTC detection method and time point of blood withdrawal (34). However, some warnings on the prognostic potential of this new biomarker have to be considered, due to the heterogeneity of the studies performed, characterized by intra- and inter-study variability, at least in melanoma meta-analysis (35). The clinical meaning of CTCs found in stage 1 and 2 patients is at present unclear. Further studies with larger cohorts of patients at different stages of ACC are required to define a potentially prognostic threshold for ACC.

The main limitation of our study is the small number of patients enrolled and scanty presurgical data. Such limitations are mainly due to the rarity of ACC and to the fact that most patients had already undergone surgery when enrolled in the study. Another limiting point is the variability of the CTC number found in different blood samples collected during follow-up. Indeed, the number of CTCs may also be affected by the discontinuous shedding of CTCs from primary and metastatic lesions, as already described for tumors at other sites (35). Multiple sampling is therefore required to limit such variability and improve the reliability of CTC detection. Finally, we here report the results obtained by blood filtering after cell fixation, which prevented us from evaluating CTC viability. Cell viability is crucial to better analyze cell biological characteristics, metastatic potential, and sensitivity to chemotherapy.

In conclusion, our findings provide the first evidence that CTCs may be a valid and useful presurgical marker to support differential diagnosis between benign and malignant adrenocortical tumors. These cells seem to correlate with some clinical parameters of ACC, such as stage and tumor diameter, suggesting that this so-called "liquid biopsy" might be a useful mini-invasive tool for prognosis and for monitoring progression and response to treatments. Moreover, in the near future, evaluation of the molecular expression profile of CTCs may help to develop tailored antimetastatic therapies in ACC. Further studies, performed on larger cohorts of patients and on blood samples taken before surgery and at different follow-up intervals, are required to definitively validate the prognostic value of this novel biomarker in ACC.

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