Cytopathologic Detection of Circulating Tumor Cells Using the Isolation by Size of Epithelial Tumor Cell Method

Promises and Pitfalls

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Abstract

*Detection of circulating tumor cells (CTCs) morphologically may be a promising new approach in clinical oncology. We tested the reliability of a cytomorphologic approach to identify CTCs: 808 blood samples from patients with benign and malignant diseases and healthy volunteers were examined using the isolation by size of epithelial tumor cell (ISET) method. Cells having nonhematologic features (so-called circulating nonhematologic cells [CNHCs]) were classified into 3 categories: CNHCs with malignant features, CNHCs with uncertain malignant features, and CNHCs with benign features. CNHCs were found in 11.1% and 48.9% of patients with nonmalignant and malignant pathologies, respectively (*P < .001*). CNHCs with malignant features were observed in 5.3% and in 43.1% of patients with nonmalignant and malignant pathologies, respectively. Cytopathologic identification of CTCs using the ISET method represents a promising field for cytopathologists. The possibility of false-positive diagnosis stresses the need for using ancillary methods to improve this approach.*

Sensitive and specific detection of circulating tumor cells (CTCs) remains a challenge in clinical oncology.[1] Animal studies and knowledge of cell invasion processes have stimulated the development of a truly reliable method to identify CTCs. Studies performed in humans show promise for the development of clinical studies in this field.[2-9] As a consequence, the potential clinical impact of CTC identification could range from early diagnosis of invasive cancers to assessment of the risk for developing recurrence or metastasis and the early detection of response or resistance to antitumor treatments.[10] However, the use of different technologies and the differences among the populations tested make the clinical significance of CTC detection difficult to interpret. Thus, the clinical benefit of detecting CTCs in the blood of patients highly depends on the technical characteristics of the method used for detection and on its reliability in terms of sensitivity and specificity.[11-14] In this regard, a critical challenge in the field of CTC detection, recently highlighted by basic and clinical studies, relates to the fact that most malignant CTCs lose their "epithelial antigens" and start to express mesenchymal antigens, a process known as *epithelial to mesenchymal transition*.[15]

Direct and indirect methods have been proposed to detect CTCs, but their results show large variability in specificity, sensitivity, and cost.[10,16-26] Among the direct methods, cytopathologic detection of CTCs, after substantial enrichment according to their size (isolation by size of epithelial tumor cells [ISET]), seems to be a very attractive procedure providing good specificity and sensitivity in addition to its simplicity, rapidity, and low cost.[26] However, although cytopathologic analysis is predictably more specific than antigen-mediated CTC capture based on antibodies

lacking specificity for tumor cells, it still has to be shown that CTCs can be recognized using the cytopathologic criteria of malignancy already used in conventional cytology (ie, in exfoliative and in fine-needle aspiration [FNA] cytopathology). Furthermore, enriching large cells from blood could also lead to the isolation of very rare hematologic (as megakaryocytes or large monocytes) or mesenchymal (as endothelial cells) cells that are undetectable by current hematologic analyses and may be difficult to distinguish from epithelial tumor cells.

For all these reasons, we planned a blinded, multicentric, cytopathologic study of blood samples obtained from patients with miscellaneous benign and malignant pathologies and from healthy subjects, processed using the ISET method. It is interesting that we found that a consensus in CTC identification can be obtained by using the same criteria as those applied to exfoliative and FNA cytology. In fact, CTCs were found neither in healthy subjects nor in the vast majority of patients with benign diseases. Strikingly, CTCs were detected in 10 (5.3%) of 190 patients with benign diseases, including thyroid and parathyroid adenomas, thus confirming that CTC identification faces the same challenges as FNA cytology in certain pathologies. Finally, these data, obtained from a national network of 10 experienced cytopathologists, confirm the interest in the ISET method and stress the need for increasing our knowledge within a potentially very important and new field in cytopathology.

Materials and Methods

Cases

A total number of 808 subjects were included in this study. They corresponded to patients with miscellaneous nonneoplastic diseases (152 cases), miscellaneous benign (38 cases) and malignant neoplasia (569 cases) diseases, and healthy volunteers (49 cases). Blood samples were obtained before surgery in 635 cases. None of the patients had undergone a biopsy or surgical excision during the month before venipuncture. The different pathologies included in this study are listed in **Table 1**. Among the patients with metastasis, 38 had breast carcinoma, 44 had colonic carcinoma, 18 had kidney carcinoma, and 5 had head and neck carcinoma. All subjects provided a signed agreement for this study, and the protocol was approved by the local ethics committee of the University of Nice, Nice, France.

Methods

For the study, 10 mL of peripheral blood was collected in buffered EDTA (before anesthesia of patients), maintained at 4°C, and processed within 1.5 hours. Surgical specimens were obtained from patients for histologic evaluation. The ISET method was carried out as previously described.[26] The filtration device with 10 wells makes it possible to load and filter each milliliter (of 10) in parallel. Blood filtration through a polycarbonate filter with a calibrated pore size of 8 µm is

Table 1
Number of Patients With Detected CNHCs, CNHC-MF, CNHC-UMF, CNHC-BF According to Malignant and Nonmalignant Associated Diseases*

Histologic Type	Absence of CNHCs	Presence of CNHCs	CNHC-MF	CNHC-UMF	CNHC-BF	Overall	*P*†
Malignant tumors	291 (51.1)	278 (48.9)	245	28	5	569 (100.0)	
NSCLC			119	4	0	394	
Miscellaneous carcinoma			11	8	2	25	
Metastatic carcinoma			56	7	0	105	
Malignant pleural mesothelioma			6	3	0	10	<.001‡
Melanoma			20	6	1	30	
Sarcoma			3	0	2	5	
Nonmalignant diseases	169 (88.9)	21 (11.1)	10	5	6	190 (100.0)	
Benign tumors			0	0	0	38	
Thyroid adenoma			7	0	0	25	
Parathyroid adenoma			3	0	0	7	
Lipoma			0	0	0	4	
Chondroma			0	0	0	2	
Nontumoral diseases			0	0	0	152	NS‡
Graves disease			0	1	0	15	
Thyroid hyperplasia			0	2	0	59	
Parathyroid hyperplasia			0	2	2	15	
Amygdalitis			0	0	2	40	
Pneumonitis			0	0	2	23	
No disease	47 (96)	2 (4)	0	0	2	49 (100)	

CNHCs, circulating nonhematologic cells; CNHC-BF, CNHCs with benign features; CNHC-MF, CNHCs with malignant features; CNHC-UMF, CNHCs with uncertain malignant features; NS, not significant; NSCLC, non–small cell lung carcinoma.
* Data are given as number (percentage) or number of cases.
† Significant at the .05 level.
‡ Coding of variables for presence of CNHC: malignant (1) vs nonmalignant (2) diseases or benign tumors (1) vs nontumoral diseases (2).

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performed. The membrane is then gently washed with phosphate-buffered saline (PBS), disassembled from the filtration device, and allowed to air dry.[26] The membrane was cut into 2 parts containing 6 spots to be stained and 4 spots to be stored for further studies. The spots were stained using a modified May-Grünwald-Giemsa staining method using the following steps: May-Grünwald (undiluted, 5 minutes), May-Grünwald (diluted 50% in PBS, 5 minutes), and Giemsa (diluted 10% in PBS, 40 minutes), followed by rinsing with PBS for 1 minute. Membranes were then air dried and kept in the dark at room temperature. Stained spots were examined by light microscopy using the following procedure: (1) screening at ×100 and ×200 to look for circulating nonhematologic cells (CNHCs) and (2) observation at ×630 and ×1,000 with oil immersion for detailed cytomorphologic study.

The following criteria were taken into account: presence of cytoplasmic (only CNHCs with visible cytoplasm were considered) irregularity of the nuclear membrane, size of the nucleus, anisonucleosis, high nuclear/cytoplasmic ratio, and presence of tridimensional sheets of cells. CNHCs with malignant features (CNHC-MF = CTCs) were then characterized by the presence of at least 4 of the following criteria: anisonucleosis (ratio >0.5), nuclei larger than a 3-calibrated pore size (8 μm) (ie, >24 μm), irregular nuclei, presence of tridimensional sheets, and a high nuclear/cytoplasmic ratio **Image 1**. CNHCs with uncertain malignant potential

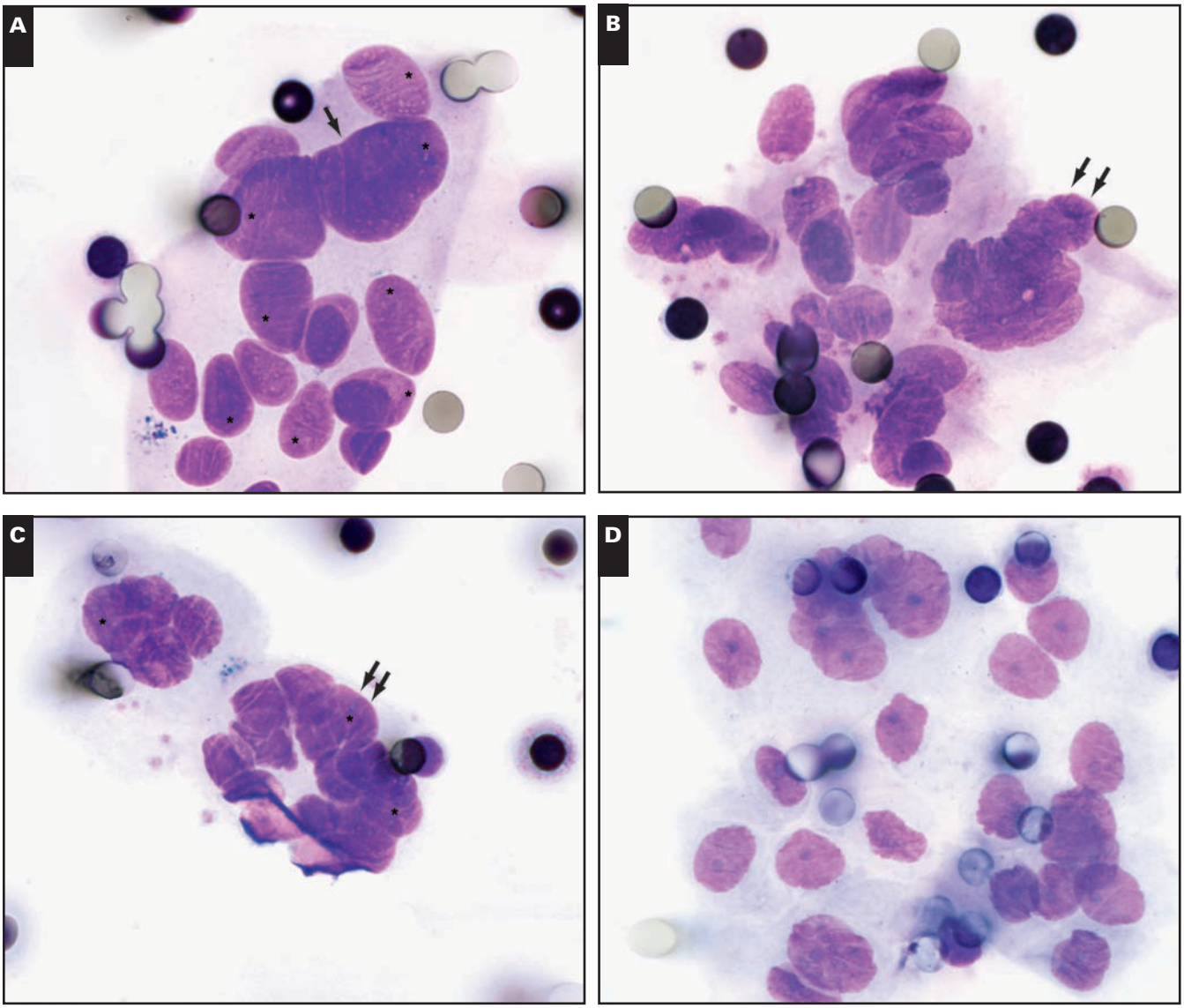

Image 1 Cytomorphologic criteria for circulating nonhematologic cells with malignant features (CNHC-MF) obtained by the isolation by size of epithelial tumor cell method. **A**, Esophageal adenocarcinoma. **B**, Head and neck carcinoma. **C**, Malignant mesothelioma. **D**, Lung adenocarcinoma.

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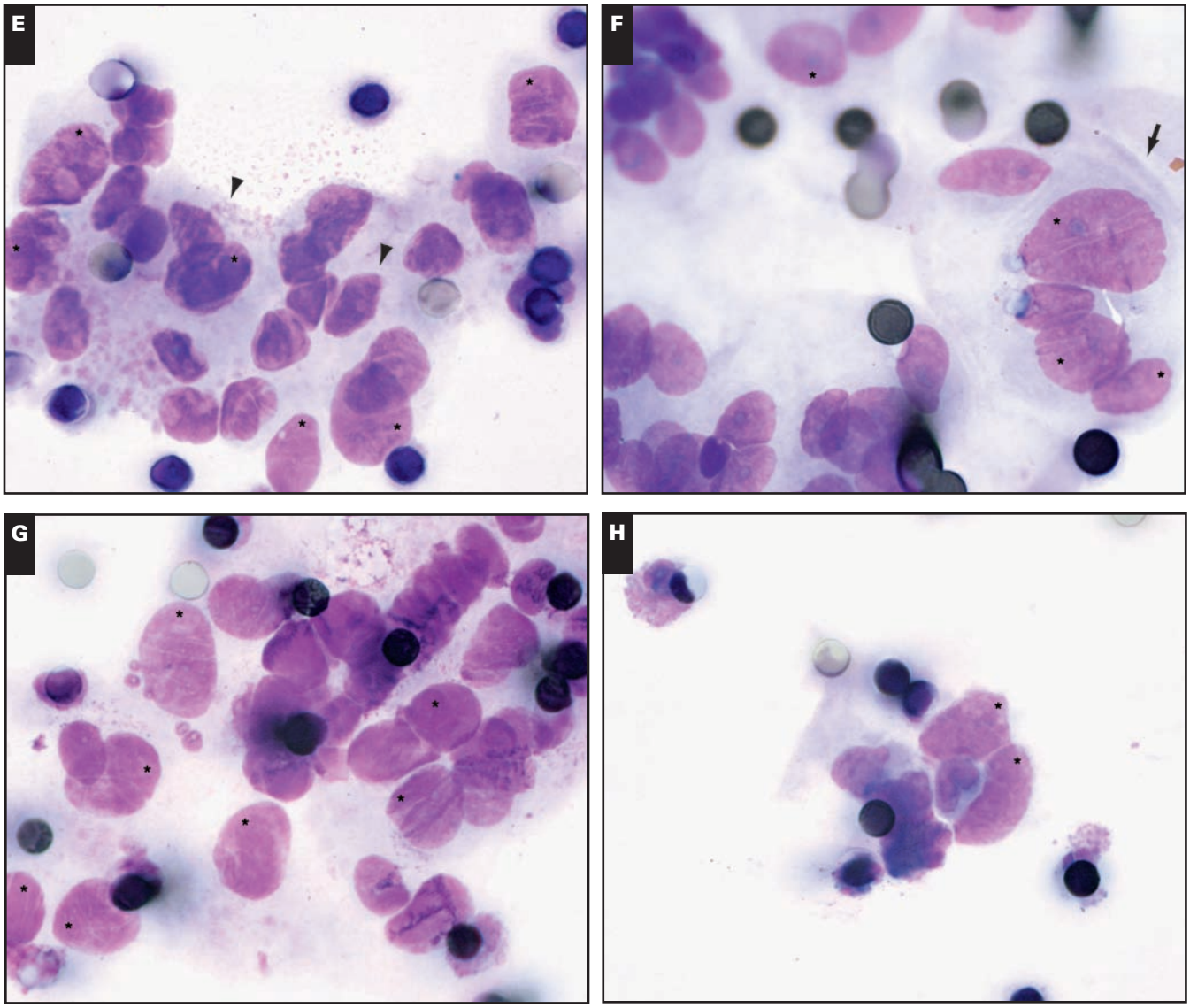
(CNHC-UMF) were defined when fewer than 2 criteria were present **Image 2**. CNHCs with benign features (CNHC-BF) were characterized by the absence of these criteria **Image 3**. A semiquantitative analysis was performed on each filter, and cases were categorized into 3 groups according to the number of CNHCs: group 1, fewer than 10 CNHCs; group 2, between 10 and 100 CNHCs; and group 3, more than 100 CNHCs. In all, 1,025 pictures (average, 5 pictures per filter; range, 1-21) were recorded, and images were digitized and collected by 3 observers (V.J.H., C.B., and P.M.H.). All images were reviewed independently by the members of the panel (V.J.H., C.B., T.M., J.M.V., J.F.F., S.L., E.P., N.M., P.V., and P.M.H.) without knowledge of the patients' clinical status and pathologic diagnosis.

Criteria for Evaluation

The presence of CNHCs was evaluated and compared in patients with nonmalignant and malignant diseases and healthy volunteers using the χ^2 statistical test. A P value of .05 or less was considered significant. Interobserver agreement was assessed for the diagnosis of CNHC-MF, CNHC-UMF, and CNHC-BF for CNHCs detected in patients with nonmalignant diseases and with malignant diseases using κ as the measure of agreement.

Results

Interobserver agreement for the 3 cytopathologists working in the same institution (V.J.H., C.B., and P.M.H.;



E and **F**, Thyroid adenoma. **G** and **H**, Parathyroid adenoma. Arrows, anisonucleosis; arrowhead, irregular nuclear borders and large nuclei; double arrows, 3-dimensional sheets; asterisks, cells satisfying the criteria for CNHC-MF (**A**-**H**, May-Grünwald-Giemsa, ×1,000).

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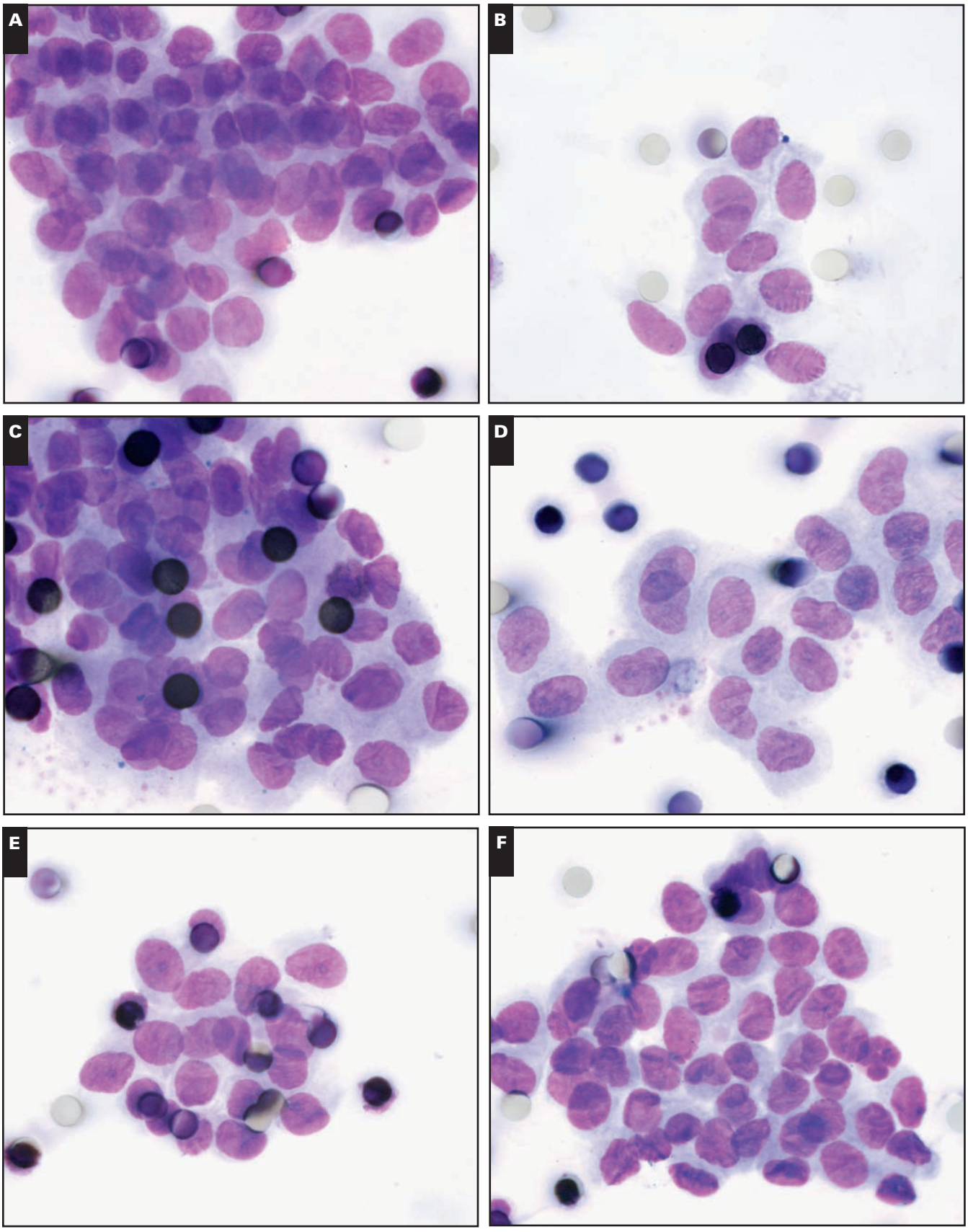

Image 2 Cytomorphologic criteria for circulating nonhematologic cells with uncertain malignant features obtained by the isolation by size of epithelial tumor cell method. **A**, Lung epidermoid carcinoma. **B**, Malignant mesothelioma. **C**, Metastatic large bowel carcinoma. **D**, Head and neck carcinoma. **E** and **F**, Thyroid adenoma.

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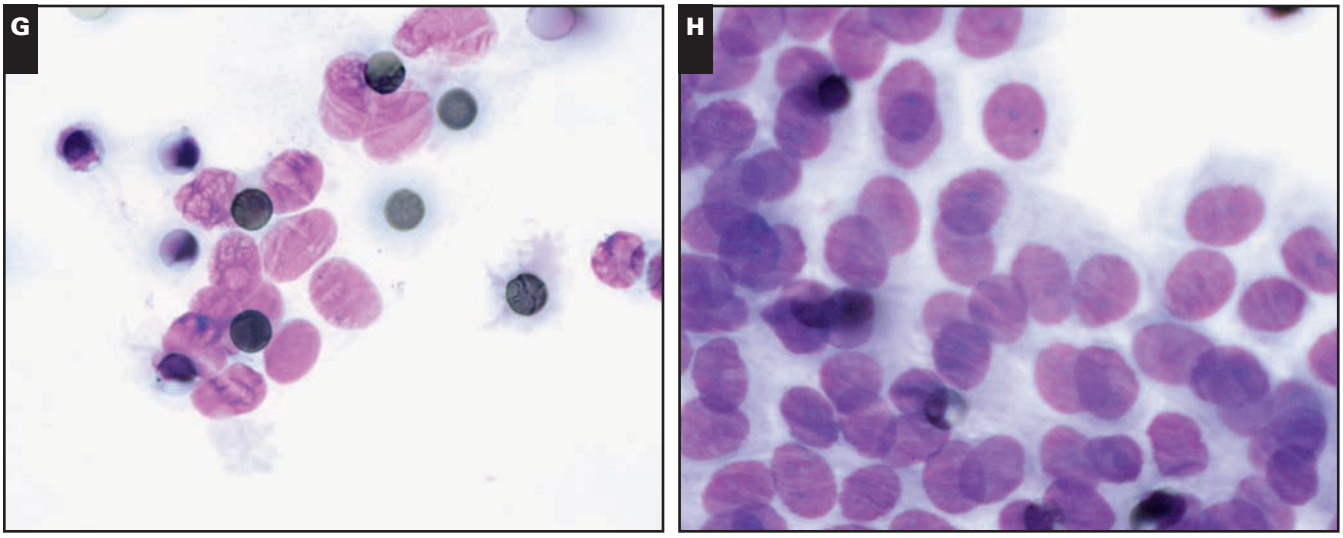

Image 2 (cont) **G** and **H**, Parathyroid adenoma (**A**-**H**, May-Grünwald-Giemsa, ×1,000).

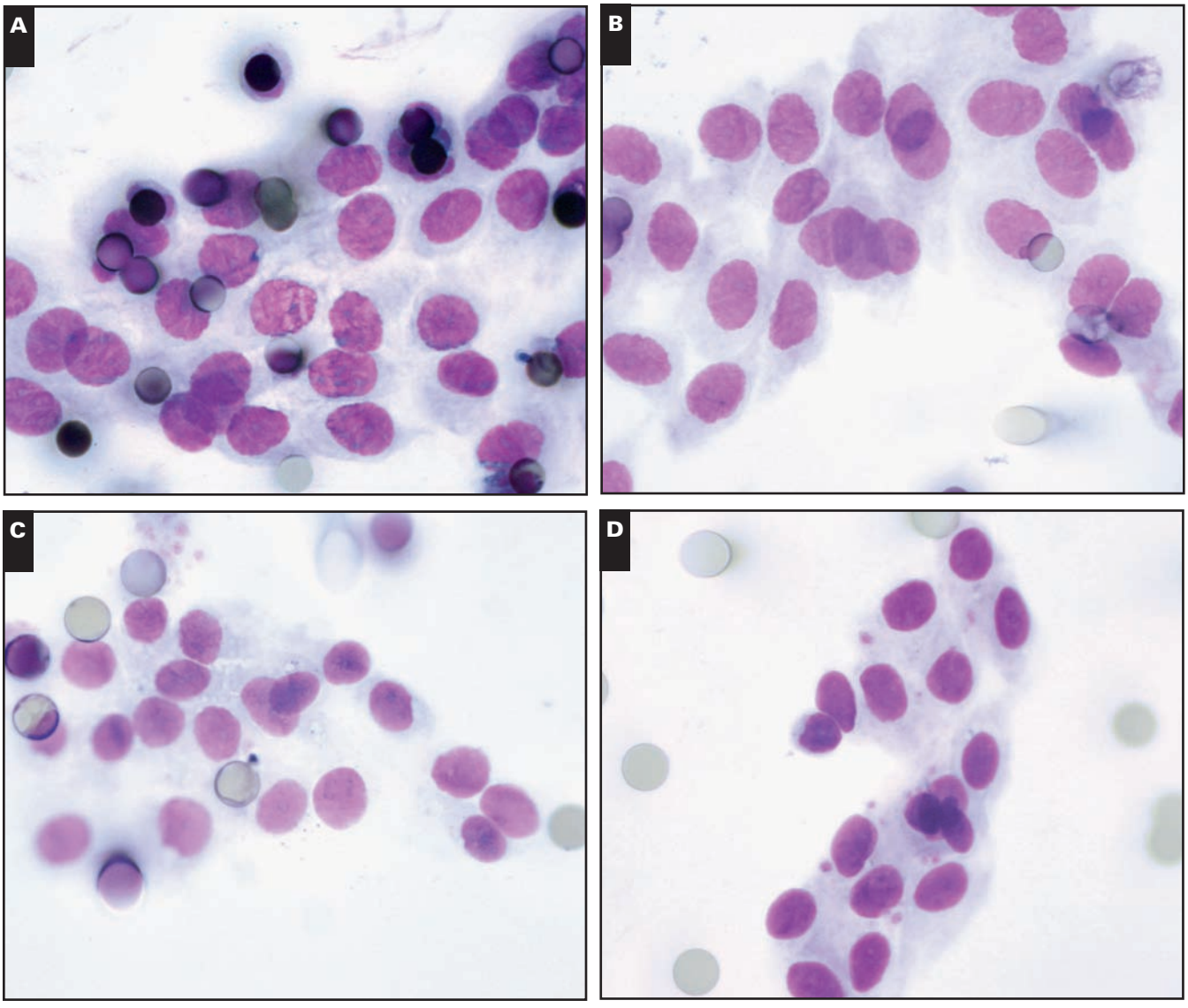

Image 3 Cytomorphologic criteria for circulating nonhematologic cells with benign features obtained by the isolation by size of epithelial tumor cell method. **A**, Lung epidermoid carcinoma. **B**, Sarcoidosis. **C**, Nodular hyperplasia of the thyroid. **D**, Thyroid adenoma.

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Pasteur Hospital, Nice, France) was total (κ = 1) for detecting CNHCs on filters. According to the number of observed images among each category of CNHCs, interobserver variation was low for the diagnosis of CNHC-MF (1.9%) and relatively high for the diagnosis of CNHC-UMF (7.5%) and CNHC-BF (8.9%). Consequently, agreement of cytopathologists for the diagnosis of the different categories of CNHCs was high (κ = 0.93) for the diagnosis of CNHC-MF, moderate (κ = 0.64) for the diagnosis of CNHC-UMF, and relatively low (κ = 0.35) for the diagnosis of CNHC-BF **Table 2**. Among patients with malignant pathologies, 278 (48.9%) of 569 (Table 1) showed the presence of CNHCs that were diagnosed by all cytopathologists as CNHC-MF (Image 1), CNHC-UMF (Image 2), and CNHC-BF (Image 3) in 245 cases, 28 cases, and 5 cases, respectively. Among the patients with benign pathologies, 21 (11.1%) of 190 had CNHCs (Table 1) that were classified by all cytopathologists as CNHC-MF (Image 1), CNHC-UMF (Image 2), and CNHC-BF (Image 3) in 10 patients (thyroid, 7 cases; parathyroid adenomas, 3 cases), 5 patients, and 6 patients, respectively. CNHCs were detected in 2 (4%) of 49 healthy volunteers, all corresponding to CNHC-BF (Table 1). Cumulatively different categories of CNHCs were observed in most patients with malignant disease. Cytopathologic features of CNHCs were similar in patients with the different pathologies (not shown). Of note, the numbers of CNHCs detected on filters were usually higher in patients with malignant diseases than the numbers observed in patients with nonmalignant diseases ($P < .001$) **Table 3** **Image 4**.

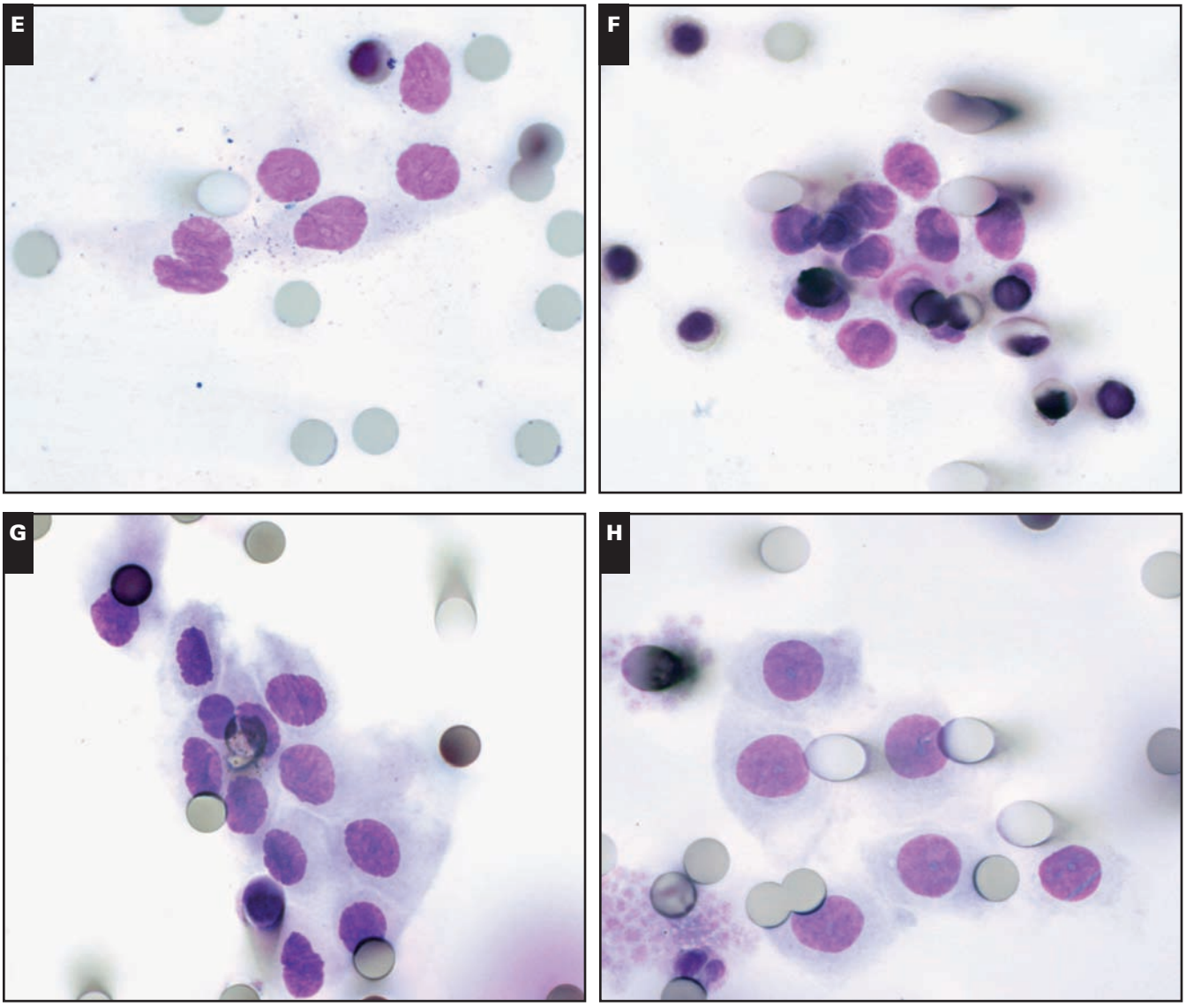

Image 3 (cont) **E**, Parathyroid hyperplasia. **F**, Amygdalitis. **G**, Pneumonitis. **H**, Parathyroid adenoma (**A**-**H**, May-Grünwald-Giemsa, ×1,000).

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Table 2
Agreement Among Cytopathologists on the Diagnosis of the Different Categories of CNHCs*

	Cytopathologist Agreement					
	10/10	**9/10**	**8/10**	**7/10**	**6/10**	**5/10**
Malignant tumors						
CNHCs	100% (555)	—	—	—	—	—
CNHC-MF	94% (182)	92% (134)	83% (68)	25% (31)	11% (12)	3% (3)
CNHC-UMF	52% (97)	34% (69)	22% (31)	11% (13)	5% (9)	2% (5)
CNHC-BF	29% (19)	11% (15)	9% (12)	7% (6)	4% (4)	2% (3)
Nonmalignant diseases						
CNHCs	100% (197)	0%	0%	0%	0%	0%
CNHC-MF	100% (7)	92% (2)	75% (1)	61% (1)	9% (1)	3% (1)
CNHC-UMF	61% (42)	34% (10)	26% (7)	12% (5)	8% (4)	5% (3)
CNHC-BF	61% (55)	42% (19)	14% (15)	5% (10)	2% (9)	1% (5)

CNHCs, circulating nonhematologic cells; CNHC-BF, CNHCs with benign features; CNHC-MF, CNHCs with malignant features; CNHC-UMF, CNHCs with uncertain malignant features.
* The percentages represent the positive responses made by the panel of cytopathologists for the diagnosis of the different categories of CNHC. The numbers in parentheses are the number of examined images.

Discussion

CTC detection is a highly relevant issue in clinical oncology. It is supposed to help clinicians in identifying patients with cancer with a high risk of recurrence or metastases of their solid tumors and patients with invasive tumors at a very early stage. Therefore, solving the technological challenges of sensitive and specific identification of CTCs by a noninvasive approach may represent a crucial step in modern clinical care.

An average number of 10 million leukocytes and 5 billion erythrocytes are present in 1 mL of blood. Detection of 1 single CTC per mL is expected to be clinically important, meaning that 5,000 CTCs are present in the blood circulation. Hematologic Coulter (Beckman Coulter, Fullerton, CA) automated instruments analyze blood volumes of 50 μL or less. Thus, "rare" cells, defined in this setting, must account for at least 20 of them per milliliter. The CTC field of investigation requires a very high sensitivity combined with an "absolute" specificity of malignant cell diagnosis. Taking all these requirements into account, indirect detection of CTCs based on reverse transcriptase–polymerase chain reaction amplification of RNA markers and cell capture based on antigens lacking "absolute" specificity for malignant tumor cell identification are not expected to reach all of the mentioned clinical goals.[10]

Among the different reported methods for CTC detection, the ISET method allows enrichment of CNHCs in a powerful manner.[26] Because these isolated cells are then available for cytopathologic study, the method could also provide the specificity expected to bring advantage to patients.[26] However, it has never been assessed whether application of classic cytopathologic criteria, currently used in "conventional" cytopathology, to the CTC field allows pathologists to reach a consensus in patients with solid cancers and to pinpoint a cell type absent in healthy subjects.

Table 3
Number of Cases With Detected CNHCs According to Pathology

Pathology	<10 CNHCs	10-100 CNHCs	>100 CNHCs	Total
Nontumoral	5	6	0	11
Benign	5	4	1	10
Malignant	58	99	121	278
Total	68	109	122	299

CNHCs, circulating nonhematologic cells.

The present work was designed to investigate whether a national panel of 10 experienced cytopathologists, working independently and without knowledge of clinicopathologic data, could consistently and reliably identify CTCs in patients with malignant solid tumors vs in patients with benign pathologies and vs in healthy subjects. Our results clearly show the following: (1) Different morphologic subtypes of CNHCs circulate in the blood of patients, but only one, defined as CNHC-MF, represents "true" CTCs because they were never found in healthy subjects. (2) CNHC-MF were found in 10 (5.3%) of the patients with benign pathologies consisting of thyroid (7 cases) and parathyroid (3 cases) adenomas, which are known to be diagnostically challenging in FNA cytopathology. This clearly means that (1) experienced cytopathologists can reliably identify CTCs by applying classic cytopathologic criteria; (2) caution is highly recommended in the case of benign pathologies such as thyroid and parathyroid adenomas, which may give false-positive results; and (3) because CNHCs without malignant features that are easily recognized by cytopathologic analysis are indeed present in healthy subjects and in patients with nonmalignant diseases, they represent a challenge for other methods aimed at detecting CTCs using nonspecific markers for malignant cells.

These data are in favor of the use of cytopathologic methods to reliably identify CTCs and strongly encourage further

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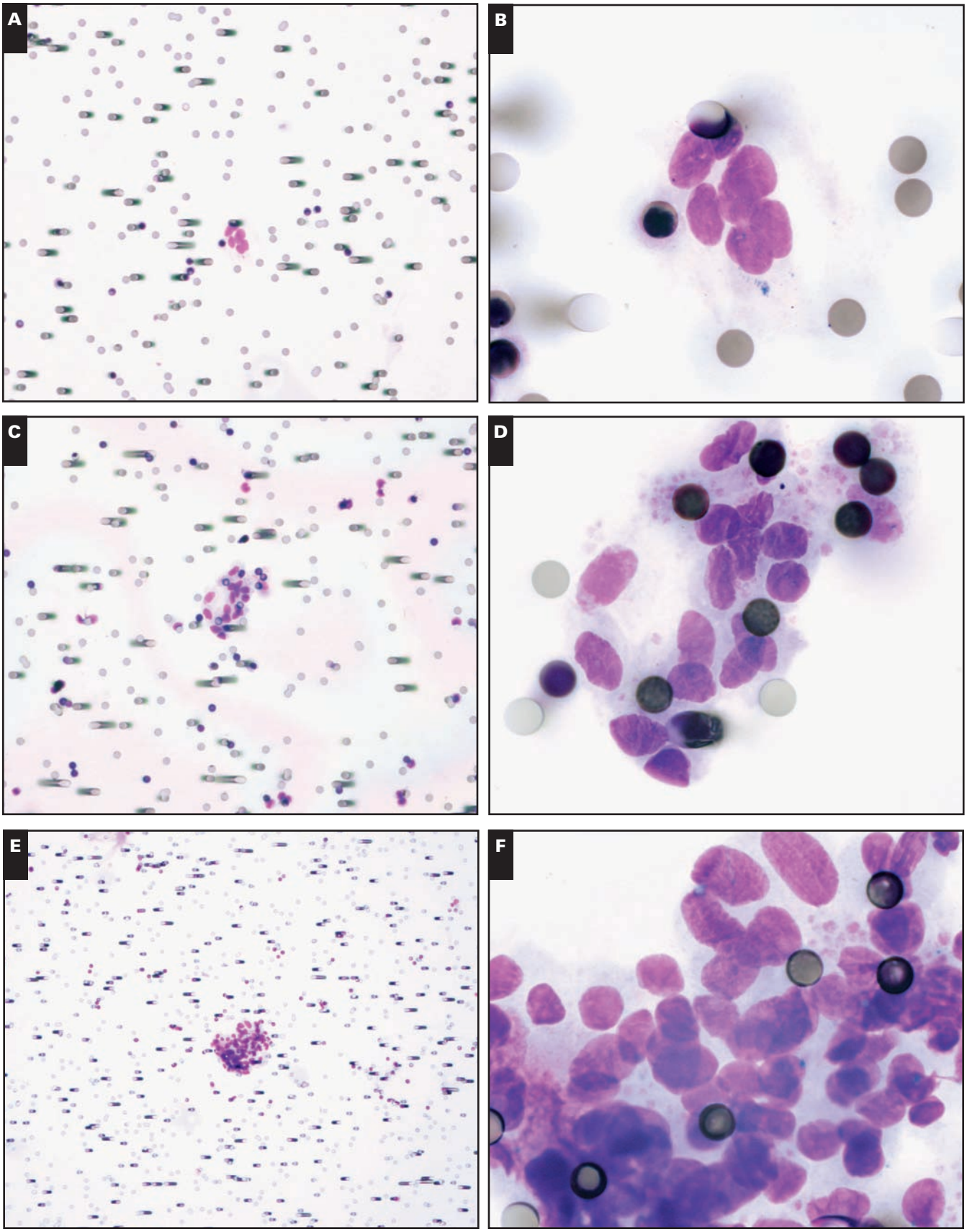

Image 4 Different numbers of circulating nonhematologic cells (CNHCs) observed in nonmalignant and malignant diseases. **A** and **B**, Isolated CNHCs or fewer than 10 CNHCs. Thyroid hyperplasia. **C** and **D**, Between 10 and 100 CNHCs. Thyroid adenoma. **E** and **F**, More than 100 CNHCs. Malignant mesothelioma (**A**, **C**, and **E**, May-Grünwald-Giemsa, ×200; **B**, **D**, and **F**, May-Grünwald-Giemsa, ×1,000).

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studies in this field. It is interesting that in a series of 199 subjects with nonmalignant diseases, the only patient with more than 2 CTCs detected by the CellSearch system (Veridex, San Diego, CA) had a hyperthyroid disorder.[27] Thus, further comparison of the respective sensitivity and specificity of the ISET and CellSearch methods for CTC detection will be of great interest. Furthermore, new molecular markers should certainly be developed in the future and might help better identify and characterize CTCs. Immunocytochemical studies performed on cells obtained by the ISET method could be used to phenotype CNHCs with challenging cytopathologic features and distinguish epithelial from endothelial cells and megakaryocytes. Immunolabeling can also be of value to identify invasive CTCs and cells with an ongoing epithelial to mesenchymal transition. Studies performed on samples from patients with solid cancers at different stages and correlating immunocytochemical results with clinicopathologic data and patient follow-up need also to be planned. Most of the published studies have demonstrated that the presence of CTCs correlates with tumors of high stage and/or metastases.[6,25,28,29] However, the different results were obtained using different methods for CTC detection.[6,25,28,29] Further studies are needed to determine in our cohort of carcinoma patients if the presence and number of CTCs detected by ISET correlate with tumors of higher stages.

Our data show that cytomorphologic examination, the reference diagnostic method in clinical oncology, is also relevant in the field of CTC detection. However, we still have to investigate, in larger clinical studies, the potential pitfalls of this approach and its potential impact on cancer staging and follow-up. This seems to be worthwhile because combining cytomorphologic diagnosis with immunologic and molecular characterization should improve CTC detection and bring new hope to patients with solid cancers.

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