Digital Pathology Slides-based Measurement of Tumor Cells and Lymphocytes Within Cytology Samples Supports the Relevance of the Separation by Size of Nonhematological Tumor and Hematological Nontumor Cells in Liquid Biopsies

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Abstract: Filtration by size is one method used to study circulating tumor cells in blood samples. Filtration-migration ability is highly dependent of the size of cell nucleus. This implies to search for the appropriate nucleus size able to separate between hematological nucleated and nonhematological nucleated blood cells to maximize circulating tumor cell isolation. Digitalized cytology slides [May-Grünwald Giemsa (MGG) stained and immunocytochemistry (ICC) slides] from various cancer metastases served for manual measurements of nuclei about tumor cells and adjacent lymphocytes to determine the diameters the more able to separate between tumor cells and lymphocytes. Among 2022 cells analyzed (1067 tumor cells and 955 lymphocytes) on MGG stained slides, the mean diameter of tumor cells nuclei was 14.77 μm whereas the mean diameter of lymphocytic nuclei was 6.47 μm ($P < 0.001$). In ICC slides, about 6583 cells (4753 tumor cells and 1830 lymphocytes), the mean diameter of tumor cells nuclei was 9.28 μm whereas the mean diameter of lymphocytic nuclei was 4.95 μm ($P < 0.001$). Areas under the receiver operating characteristic curves analyses concluded that diameters of 9.37 μm and 6 μm separated the best between tumor cells and lymphocytes in MGG and ICC slides, respectively. Measuring manually the diameters of the smallest tumor cells in ICC slides, we established more than 99% of tumor cells had diameters superior to 8 μm. The sizes differences between tumor cells and lymphocytes support the relevance of the filtration by size to isolate blood circulating nonhematological tumor cells. The existence of small tumor cells with sizes overlapping with those of lymphocytes is worth to optimize the threshold to separate between tumor cells and hematological cells.

Key Words: circulating tumor cells, isolation by size, lymphocytes, tumor cells, digital pathology

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At present time, metastatic cancers remain a major cause of mortality and morbidity worldwide. The progression of cancer from tumor initiation and growth to its metastatic spread, responsible for the large majority or cancer-related deaths, implies close relationships between the tumors (primitive ones and metastases) and the circulatory system.[1] In this manner, various amounts of tumor-derived materials of different natures as circulating tumor cells (CTCs), cell-free DNA or RNAs (cfDNA-cfRNAs) or exosomes are inevitably present in the blood of patients with cancer. The detection of these different tumor-derived materials in the blood of patients is the growing field of "liquid biopsy." The relative advantages and limitations of searching for CTCs, cfDNA-cfRNAs or exosomes are the subject of several global, technical or organs/tumor subtype-dedicated review articles dealing with the different questions to solve for the management of patients, for diagnostic, prognostic and theranostic purposes.[2–7] The detection of CTCs appears promising for these different purposes, allowing a molecular-status independent detection of cancer-derived circulating elements. Nevertheless, because of their extreme rarity (even in patients with advanced cancers as metastatic ones) among billions of nontumor hematological cells contained in a blood sample, their detection consists in a challenge and requires specific methods for the detection, enrichment, and characterization of CTCs.

Two main methods can be used for the detection of CTCs in blood samples. First, immunophenotype-based ones that use a selection based on the presence of surface markers to differentiate between the tumor cells and the nontumor ones (ie, cytokeratines, vimentine, CD45). An intrinsic limitation of these methods is to be highly dependent of the markers expressed by the CTCs: the absence or loss of expression of markers used for the selection by some CTCs (eg, as epithelial ones which can be lost during the epitheliomesenchymal transition of carcinomatous CTCs) may impair the success of the detection. Secondly, immunophenotype-independent methods used nonimmunologic ways to detect and isolate the CTCs. The size of cancer cells, admitted to be higher than the size of nontumor cells, is notably used in different widespread CTCs detection filtration-based methods.[8–11]

A crucial question to maximize the nonimmunologic size-based detection and isolation of cancer cells for different purposes is in this manner as simple as which cell size better differentiate between tumor cells and nontumor hematological cells. Highly sensitive filters will have the advantage to retain more numerous CTCs but the drawbacks of retaining also large amounts of nontumor hematological cells that can impair the individualization of CTCs among them. Inversely, a more specific filter will only retain the biggest CTCs but some small of them can be missed by the test increasing the risk of a false-negative test.

Beyond large amounts of surgical pathology and cytopathology textbooks describing the morphological features of tumor cells of different organs including information about their mean sizes, the question of the minimal size of cancer cells able to separate between nucleated blood cells and the most common cancer subtypes remains paradoxically poorly studied, despite its crucial interest for the design of size-based CTCs detection methods. This question of the minimal size to differentiate between tumor cells and nontumor hematological cells among different cancers is the subject of the present study.

MATERIALS AND METHODS

Cases Selection

The cases included in this study were patients with various metastatic cancers diagnosed at the Brest University Hospital (France). May-Grünwald Giemsa (MGG) stained cytologic slides of patients with various cancers were collected for novel digital analyses, as well as immunocytochemistry (ICC) slides having permitted the diagnosis of various cancers on the basis of nuclear markers of tumor cells. This retrospective noninterventional study was conducted according to our national and institutional guidelines in compliance with the Declaration of Helsinki and after approval by our Institutional Review Board with all tumor material included in a registered tumor tissue collection (CHRU Brest, CPP no. DC-2008-214).

Digitalization and Analysis of Cytology and Histology Slides

The slides were digitalized with a 3DHistech Pannoramic Midi scanner (3DHistech, Budapest, Hungary) at ×400 magnification. The CaseViewer software (3DHistech) was used for the analyses. Tumor and nontumor cells were individualized for each slide by a pathologist (A.U.) using the built-in annotation tool of the CaseViewer software. Assuming that the size of the nucleus is the main parameter conditioning the capacity of a size-based CTCs detection method to retain a given cell, we only focused on the size of nuclei and not of whole cells as a primary measurement.[12] For each slide, nuclei of tumor cells and, when feasible, of tumor-adjacent hematological cells (lymphocytes) were manually identified, circled and measured (perimeter and area) using the built-in measurement tool of the CaseViewer software. Mean diameter was then deducted from measured perimeter and area for each nucleus. On MGG slides, the distinction between tumor cells and nontumor cells was based on morphological criteria whereas, on ICC slides, a tumor cell was diagnosed in case of any clear nuclear staining using the tumor-specific nuclear markers. For the 10% of the smallest tumor nuclei measured on ICC slides, a second measurement was performed about the whole cell each time the cytoplasm was clearly distinguishable.

Statistical Analyses

The diameters of tumor and nontumor nuclei were compared using analysis of variance. Comparisons of areas under the receiver operating characteristic curves (AUROC) were used searching for the nucleus diameter the more able to separate between tumor and nontumor cells on the basis of MGG and ICC slides on the one hand and, in the other hand, to calculate the sensitivity and specificity of a separation between tumor and nontumor nuclei using different thresholds of nuclei diameters. Statistical analyses were performed using MedCalc Statistical Software version 13.2.2 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2014). The level of significance was set at $P < 0.05$.

RESULTS

Cases Included

Twelve MGG cytology slides including 9 lymph node metastases and 3 samples without any identified tumor cell were analyzed for a total of 2022 cells (1067 tumor cells and 955 lymphocytes) measured on digital slides. Twenty-four ICC slides were also used to measure 4753 tumor nuclei and 1830 lymphocytes (ie, a total of 6583 cells). More details about cases are listed in Tables 1 and 2.

TABLE 1. Summary of Cases and Nuclei Measurements of the 9 Nodal Metastases in May Grünwald Giemsa Stains

Cases	Sex (M/F)	Age (y)	Diagnosis	Tumor Nuclei Analyzed	Mean Tumor Nuclei Diameters (SD) (μm)
#1	M	74	Nonsmall cell lung carcinoma (nodal metastasis)	119	15.17 (2.32)
#2	M	71	Small cell lung carcinoma (nodal metastasis)	111	11.91 (1.57)
#3	M	63	Nonsmall cell lung carcinoma (nodal metastasis)	108	13.49 (2.5)
#4	F	65	Melanoma (nodal metastasis)	112	13.06 (1.41)
#5	M	78	Melanoma (nodal metastasis)	100	13.17 (2.13)
#6	F	65	Melanoma (nodal metastasis)	113	16.99 (2.31)
#7	M	49	Esophageal adenocarcinoma (nodal metastasis)	122	15.3 (2.62)
#8	F	51	Breast adenocarcinoma (nodal metastasis)	140	17.43 (2.69)
#9	F	63	Urothelial carcinoma (nodal metastasis)	142	15.26 (2.38)

F indicates female; M, male.

TABLE 2. Summary of Cases and Nuclei Measurements in the 24 Metastases Immunocytochemistry Stains

Cases	Sex (M/F)	Age (y)	ICC Slide (Marker)	Diagnosis	Tumor Nuclei Analyzed	Mean Tumor Nuclei Diameters (SD) (μm)
#10	F	64	CDX-2	Colorectal adenocarcinoma (pleural liquid)	202	7.24 (1.05)
#11	F	56	CDX-2	Colorectal adenocarcinoma (bone metastasis)	212	9.62 (1.92)
#12	M	67	CDX-2	Colorectal adenocarcinoma (peritoneal liquid)	230	8.91 (1.76)
#13	M	42	CDX-2	Colorectal adenocarcinoma (peritoneal liquid)	214	10.45 (1.59)
#14	F	64	TTF1	Lung adenocarcinoma (pleural liquid)	313	6.46 (0.78)
#15	M	69	TTF1	Lung adenocarcinoma (nodal metastasis)	119	11.18 (2.29)
#16	M	72	TTF1	Lung adenocarcinoma (pleural liquid)	247	10.33 (2.25)
#17	F	51	TTF1	Lung adenocarcinoma (pleural liquid)	257	7.38 (1.07)
#18	F	78	TTF1	Lung adenocarcinoma (pleural liquid)	139	9.36 (1.74)
#19	F	50	Estrogens receptor	Breast adenocarcinoma (pericardic liquid)	215	8.86 (1.32)
#20	F	79	Estrogens receptor	Breast adenocarcinoma (pleural liquid)	219	6.42 (1.04)
#21	F	57	Progesteron receptor	Breast adenocarcinoma (pericardic liquid)	300	9.67 (1.35)
#22	H	78	SOX10	Melanoma (nodal metastasis)	30	15.64 (3.77)
#23	H	76	SOX10	Melanoma (nodal metastasis)	238	8.32 (1.68)
#24	F	81	SOX10	Melanoma (nodal metastasis)	211	11.12 (2.53)
#25	F	60	p53	Ovarian adenocarcinoma (peritoneal liquid)	313	13.76 (2.84)
#26	F	60	p53	Ovarian adenocarcinoma (peritoneal liquid)	204	10.62 (1.94)
#27	F	69	PAX-8	Ovarian adenocarcinoma (pleural liquid)	248	8.08 (1.57)
#28	F	84	PAX-8	Ovarian adenocarcinoma (peritoneal liquid)	25	6.99 (0.88)
#29	F	59	PAX-8	Ovarian adenocarcinoma (pleural liquid)	64	7.78 (1.68)
#30	H	51	p40	Squamous cell carcinoma (nodal metastasis)	227	7.8 (1.50)
#31	M	64	p40	Squamous cell carcinoma (nodal metastasis)	221	12.1 (2.36)
#32	M	83	p40	Squamous cell carcinoma (nodal metastasis)	202	9.16 (1.33)
#33	M	63	p40	Squamous cell carcinoma (bone metastasis)	103	7.53 (1.64)

CDX-2: clone EPR2764Y, Cell Marque, 1:50 dilution; TTF1: clone 8G7G3/1, Dako, 1:50 dilution; estrogens receptor: clone SP1, Roche, prediluted; progesteron receptor: clone 1E2, Roche, prediluted; SOX10: clone SP267, Roche, prediluted; p53: clone DO-7, Dako, 1:50 dilution; PAX-8: clone MRQ-50, Cell Marque, prediluted; p40: polyclonal, Clinisciences, 1:100 dilution.
F indicates female; ICC, immunocytochemistry; M, male.

Measurements and Comparisons of Nuclei of Tumor Cells and Lymphocytes in MGG Slides

Among the 12 cytologic samples, the mean diameter of lymphocytic nuclei (955 measurements) was 6.47 μm [95% confidence interval (CI): 6.40-6.55] and the mean diameter of tumor nuclei (1067 measurements) was 14.77 μm (95% CI: 14.6-14.9) with significant variations across tumor subtypes from 11.91 to 17.4 μm mean diameters in a small cell lung carcinoma sample and in a breast carcinoma sample respectively ($P < 0.001$). The tumor nuclei were significantly larger than lymphocytic ones ($P < 0.0001$) and AUROC analysis (AUROC = 1.0; 95% CI: 0.997-1.0) concluded in an optimal cut-off value separating tumor and lymphocytic nuclei of 9.37 μm diameter with a sensitivity of 99.63% (95% CI: 99-99.9) and a specificity of 99.16% (95% CI: 98.4-99.6) (Fig. 1A). Among the tumor nuclei, the smallest had a diameter of 8.8 μm and 4/1067 nuclei (0.37%) were smaller than 9.37 μm.

Measurements and Comparisons of Nuclei of Tumor Cells and Lymphocytes in ICC Slides

Among the 24 ICC slides, the mean diameter of lymphocytic nuclei (1830 measurements) was 4.95 μm (95% CI: 4.91-5) with variations from 2.5 μm to 11.03 μm. The mean diameter of tumor nuclei (4753 measurements) was 9.28 μm (95% CI: 9.21-9.38) with significant variations from 4.08 to 30.4 μm mean diameters in a colorectal carcinoma sample and an ovarian carcinoma sample, respectively ($P < 0.001$). The tumor nuclei were larger than lymphocytic ones ($P < 0.001$) and AUROC analysis (AUROC = 0.972; 95% CI: 0.968-0.976) concluded in an optimal cut-off value separating tumor and lymphocytic nuclei of 6 μm diameter with a sensitivity of 93.84% (95% CI: 93.1-94.5) and a specificity of 90.27% (95% CI: 88.8-91.6) (Fig. 1B). The diameters of lymphocytes and tumor cells in MGG and ICC slides are summarized in Figure 2.

Among the tumor nuclei, the 10th percentile corresponded to a diameter of 6.31 μm. Measuring individually the cell diameters of cells with nuclei smaller than 6.31 μm, the smallest tumor cell was measured at 6 μm. Three hundred and ninety two tumors cells (8.3% of the 4753 tumor cells in our study) had a size between 6 μm (ie, the minimal size of tumor cell) and 11.03 μm (ie, the maximal

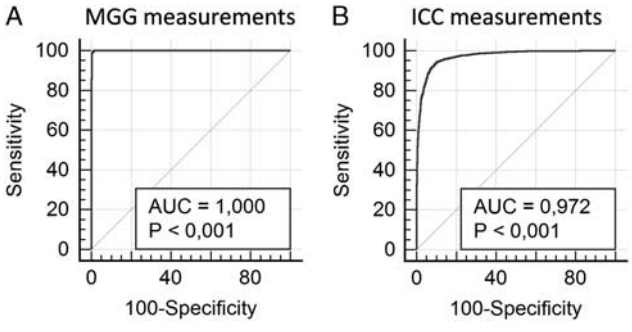

FIGURE 1. Receiver operating characteristic curves in differentiating tumor cells and lymphocytes in May-Grünwald Giemsa (MGG) (A) and immunocytochemistry (ICC) (B) slides. AUC indicates area under the curve.

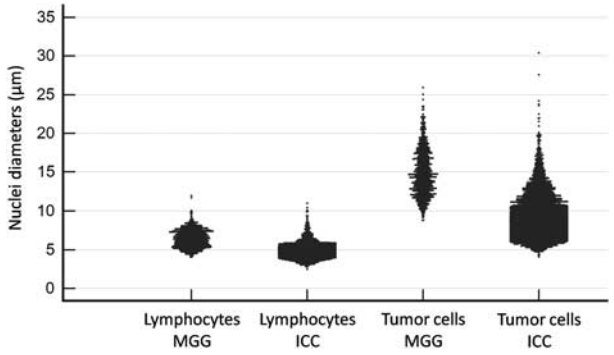

FIGURE 2. Summary of the nuclei measurements of lymphocytes and tumor cells in May-Grünwald Giemsa (MGG) and immunocytochemistry (ICC) slides.

size of a lymphocyte). Five (0.1%) and 55 (0.9%) of tumor cells had cells diameters inferior to 7 µm and 8 µm, respectively (Fig. 3 for examples).

DISCUSSION

The isolation of CTCs still consists in a technical challenge and both immunological and nonimmunological methods have limitations as changes in immunophenotypic features of tumor cells and cell size variations, respectively. In the field of the isolation by size of tumor cells, how tumor cells are retained by filtration methods that allow the elimination of nontumor cells is determined mainly by 2 parameters: on the one hand, the size of the tumor cells when compared with nontumor ones and, in the other hand, the deformability of these cells allowing then to cross or not the pores of the filters designed for their filtration. The deformability of tumor cells that conditions their migration through small diameters spaces as filters pores is mainly limited by the size of their nucleus that has contributed to the choice of first measuring cells nuclei in the present study.[12]

The size of tumor cells, at least for nonhematological solid tumors, even those classed of small size subtypes as small cell neuroendocrine carcinomas, is admitted to be superior to the size of small mature lymphocytes and, among other morphological features, the size is one parameter analyzed by pathologists to differentiate between malignant and nonmalignant tumor and nontumor cells in cytopathologic and histopathologic samples. Some authors have proposed that very small tumor cells, of diameters even smaller than lymphocytes exist and can be isolated only using immunologic methods but not using current available size-based isolation methods.[13–16] Nevertheless, the cut-off value of size to differentiate between malignant tumor cells and mature lymphocytes, that conditions greatly the success of size-based isolation of CTCs in liquid biopsy appears to the best of our knowledge having not been reported in cytologic samples and the present study could contribute to solve this question.

Differentiating between tumor cells and lymphocytes in metastases of different solid cancers subtypes and measuring their nuclei size has permitted in the present study to conclude that the proportion of tumor cells of solid cancers with nuclei diameters overlapping with the sizes of lymphocytes is about 8% and better revealed by ICC than on MGG sections given the unusual small size of these small tumor cells. In this manner, using a cut-off value of cell size above the mean size of lymphocytes (eg, 7 or 8 µm according our data) appears relevant to eliminate most of nucleated blood cells and to permit the isolation of most (ie, > 99%) CTCs (at least of nonhematological subtype) in blood samples.

CONCLUSION

To conclude, systematic ICC analyses reveal the presence of small size tumor cells which can be underdiagnosed using classic cytopathology criteria. These small size tumor cells could be missed by the separation by size of isolation of CTCs from hematological ones. Nevertheless, more than 99% of tumor cells would be retained using filters with 7 to 8 µm pores diameters. The present

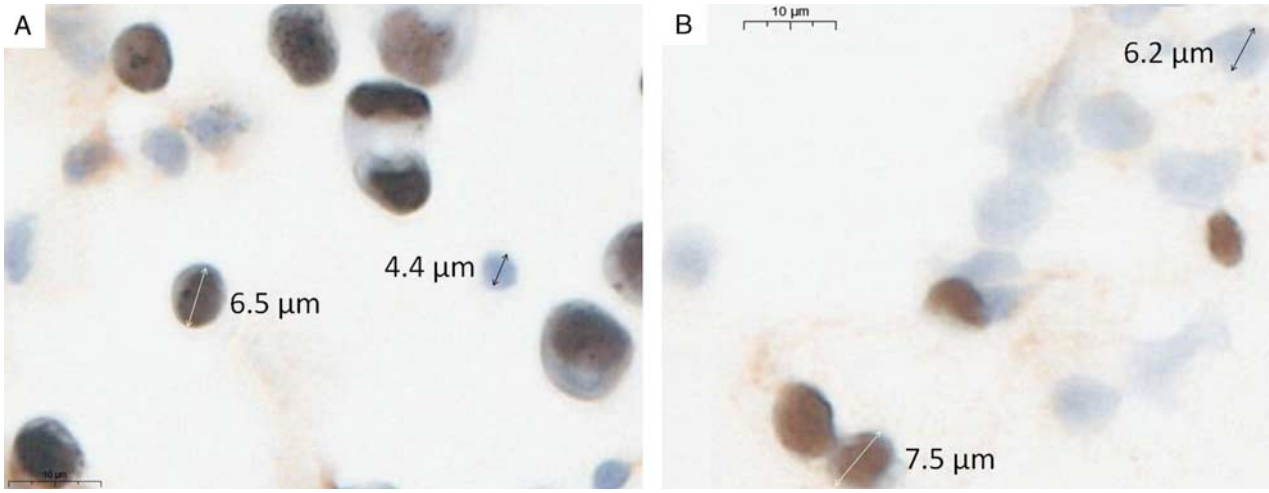

FIGURE 3. Examples of small size tumor cells in a nonsmall cell lung cancer metastasis (A, TTF1 immunocytochemistry) and in a breast cancer metastasis (B, estrogen receptor immunocytochemistry) (stained tumor cells and unstained lymphocytes).

work is limited by the small sample size and the limited number of tumor types analyzed. Another limitation is that cells measures were not performed in liquid biopsy specimens. For these reasons, the present finding must be validated on the one hand on real liquid biopsy samples and in the other hand about more numerous cells and tumor subtypes.

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