Flow Cytometric Detection of Small Cell Lung Cancer Cells with Aberrant CD45 Expression in Micrometastatic Bone Marrow

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A lot of hematologists are often faced with the difficulty of diagnosing bone marrow micrometastasis of carcinoma cells. We employed a new flow cytometric immunophenotyping by a combination of CD45 with three neuroendocrine markers: CD56, microtubule-associated protein-2 and synaptophysin, and successfully detected micrometastatic tumor cells in the bone marrow of a 61-year-old male patient with small cell lung cancer (SCLC), whose marrow smears never showed a distinct morphology of metastasis. It was noteworthy that these SCLC cells accompanied the aberrant expression of CD45, leukocyte common antigen known as a specific marker for hematolymphoid neoplasms, which was not detected in the tumor of primary lesion. We describe this rare case to arouse an attention that tumors of non-hematolymphoid origin can exhibit exceptional CD45-positivity in metastatic sites.

Key words: bone marrow micrometastasis — CD45 — flow cytometry — neuroendocrine — small cell lung cancer

INTRODUCTION

Small cell lung cancer (SCLC) is derived from pulmonary cells with a neuroendocrine (NE) phenotype. Bone marrow is a metastatic site frequently involved in SCLC cases. It is easy to confirm the diagnosis of metastasis when tumor cells show typical morphology as large cohesive cells form a lot of aggregates scattering in the bone marrow smear. On the contrary, the cases of micrometastasis, tumor cells presenting the lymphoblast-like appearance (1), or artifactual distortion of hematopoietic elements simulating metastatic SCLC (2) sometimes puzzle and lead us to make a misdiagnosis. Therefore, the establishment of a useful method overcoming the diagnostic uncertainty by conventional cytopathology is of primary importance. Flow cytometry (FCM) is a powerful tool for immunophenotyping tumor cells in bone marrow, which is routinely performed in the field of modern hematology. Here, we report a case of SCLC, in which a new FCM immunophenotyping by the combination of CD45 with three NE markers: CD56, microtubule-associated protein-2 (MAP-2) and synaptophysin (SYN), successfully detected the micrometastasis of bone marrow.

PATIENTS AND METHODS

PATIENT

A 61-year-old male patient was admitted to our hospital because of continuous cough with bloody sputum, anterior chest pain and lumbago. Computed tomographic scanning revealed that a bulky mass in right upper lobe of his lung extended from right thoracic wall to mediastinum compressing superior vena cava. In addition to right pleural effusion, mediastinal and right supraclavicular lymph nodes were also involved. A radionuclide bone scan suggested the involvement of bilateral ribs. He underwent transbronchial tumor biopsy. Tumor cells showed positive for several NE markers including CD56 and SY (Fig. 1A–C), as well as cytokeratin (AE1/AE3 and CAM5.2). He was finally diagnosed as SCLC with Stage IV (T4, N3, M1). To demonstrate the bone marrow involvement, bone marrow aspiration was performed for reprints and all correspondence: Mikio Danbara, Department of Hematology, Kitasato University School of Medicine, 1-15-1, Kitasato, Sagamihara, Kanagawa 228-8555, Japan.
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at bilateral posterior iliac crests. After making smear samples, remaining aspirates were collected in sterile EDTA tubes.

Flow Cytometry

After lyses of erythrocytes, a total of $1 \times 10^6$ cells were stained for 15 min at room temperature in the dark with 10 µL of phycoerythrin-cyanin 5.1-conjugated mouse anti-human CD45 monoclonal antibody (Immunotech, France) and R-phycoerythrin (PE)-conjugated mouse anti-human CD56 monoclonal antibody (Immunotech). As a negative control, PE-conjugated irrelevant mouse isotype IgG (Immunotech) was used instead of anti-CD56 antibody. Stained cells were fixed and permeabilized using a commercial kit, IntraPrep™ Permeabilization Reagent (Beckman–Coulter, CA, USA), following the manufacturer’s instructions, subsequently stained with the primary antibody-cocktail containing 1:1 goat anti-human MAP-2 polyclonal antibody (Santa Cruz, CA, USA) and goat anti-human synaptophysin polyclonal antibody (Santa Cruz, CA), a final concentration of 2 µg/mL, for 15 min at room temperature. As a control antibody, an equal concentration of goat IgG (Sigma) was used. After one wash, cells were stained with fluorescein isothiocyanate-conjugated rabbit anti-goat monoclonal antibody (Sigma), a final concentration of 10 µg/mL, for 15 min at room temperature. After final washes, the stained cells were analyzed using an EPICS® XL-MCL Flow Cytometer. SYSTEM II™ software (Beckman–Coulter) was used for subsequent data analysis.

Results and Discussion

Bone marrow smears demonstrated slight hypocellularity. The close examination of smears from right iliac crest (RIC) made it possible to detect a few cohesive cell aggregates containing small vacuoles suggesting SCLC cells (Fig. 2). The smears from the left iliac crest (LIC) showed almost normal bone marrow, except for the finding of very few naked nuclear cells of unknown origins (data not shown). These equivocal findings made us confused. To clear diagnostic dilemma, we performed immunocytochemistry using smears from RIC. Only showed atypical cell aggregates (Fig. 3A and C) as seen in Fig. 2 positive for cytokeratin (CAM5.2) (Fig. 3B and D), indicating that they were tumor cells of non-hematolymphoid origin. We employ an FCM immunophenotyping with CD45 and three NE markers for searching the definite evidence as SCLC metastasis in bone marrow. CD56 has been widely employed as a marker of this phenotype of tumors (3). It
is, however, expressed on a variety of cell types including certain T cell subsets and neoplastic cells other than NE tumors, e.g. natural killer cell lymphomas, tumors from marrow cell origin (4,5). To reinforce the specificity of NE immunophenotyping with CD56 alone, we added two cytoplasmic proteins, MAP-2 and SYN, both of which showed high specificity for the NE phenotype (6,7). A set of antibodies, two primary antibodies from the identical species of the immunized animals and a fluorescence-conjugated secondary antibody, were used to detect cells expressing either of the two proteins or both, resulting in the gain of sensitivity for the NE phenotype.

The results from preliminary experiments using 4 established SCLC cell lines (Lu135, Lu165, N231 and HL69) and 16 normal bone marrow aspirates were promising. Neither CD56 nor MAP-2/SYN expression was detected in normal marrow cells as seen in negative control cells from a B-cell leukemia cell line (BALL-1) (Fig. 4A and B). Contrastively, both of them were highly positive in all SCLC cell line cells (Fig. 4C–F). We confirmed that MAP-2/SYN and CD56 were stained correctly in cytoplasm and on the surfaces of the cells, respectively, by analyzing the localization of fluorescence using confocal laser-scanning microscopy (data not shown). Dilution study in which SCLC cell line cells were mixed with normal marrow cells to make dilution samples with a serial tumor cell concentration (50%, 5% and 1%) showed that this immunophenotyping clearly separated the tumor cell population from normal marrow cells in 1% dilution samples simulating tumor micrometastasis (data not shown). Besides, cells showing NE immunophenotype were clearly detected in the massively metastatic bone marrow from a 51-year-old male patients with SCLC Stage IV (T3, N1, M1), indicating that our method was useful in the clinical setting (Fig. 4G).

As for the present case, FCM failed to detect cells co-expressing CD56 and MAP-2/SYN in the samples from

**Figure 2.** Bone marrow aspirate from right iliac crest (RIC). Low-magnification view.

**Figure 3.** Positive immunoreactivity for cytokeratin in metastatic tumor cells of bone marrow. Smear from RIC was once bleached by ethanol–HCl, followed by stained with the identical anti-CAM5.2 antibody used in staining tumor of primary site by conventional method. Only atypical cell aggregates as seen in Fig. 2 showed weakly positive for CAM5.2. (A and C) May–Giemza stain. (B and D) Immunocytochemistry.
Figure 4. Two-color analysis with CD56 and microtubule-associated protein-2/SYN. Following materials were prepared: 4 established SCLC cell lines (Lu135, Lu165, N231 and HL69, kindly gifted by Dr T. Terasaki), a B-cell leukemia cell line (BALL-1), 16 normal bone marrow aspirates examined by both morphology and cytogenetics from patients with no malignancy, and clinical specimen from a 51-year-old male patient with SCLC, Stage IV (T3, N1, M1), whose bone marrow smear showed massive infiltration of tumor cells. Tumor cells (red dots) were clearly defined in dot plots. (A) Normal bone marrow, (B) BALL-1, (C) Lu135, (D) Lu165, (E) N231, (F) HL69 and (G) a clinical case described above. Black dots depict control antibody stains.

Figure 5. Detection of SCLC cells in bone marrow from the present case. Black dots in two-color analysis depict control antibody stains. LIC, left iliac crest; SS, side scatter.
both sides of the iliac crest when setting the gate on the whole-cell population except lymphocytes and cell debris on the side scatter (SS) versus CD45 dot plots (data not shown). Of note, by resetting the gate on the faint population contiguous to lymphocyte population, which showed CD45\textsubscript{high} and SS slightly higher than that shown in lymphocytes (Fig. 5, upper panels), a small number of cells with NE phenotype (CD56\textsuperscript{high} and MAP-2/SYN\textsuperscript{high}) as shown in the tumor of primary lesion (Fig. 1B and C) were clearly defined in the sample from the LIC (0.026% of non-gated all analyzed cells) (Fig. 5, right column), as well as that from the RIC (0.27% of non-gated all analyzed cells) (Fig. 5, left column). Additional immunohistochemistry revealed that tumor cells of primary lesion showed no immunoreactivity for CD45 (Fig. 1D). The gated region shown in Fig. 5 overlapped with that of monocytes/macrophages population. To exclude the possibility that the sticky monocytes/macrophages induced by the staining procedure would absorb antibodies for CD56 and MAP-2/SYN non-specifically, we reviewed the results of dilution study described above. In the region of monocytes/macrophages population, none of cells showed immunoreactivity for both CD56 and MAP-2/SYN in any of dilution samples (data not shown). Considering these results together with the reproducible results from two separate aspirates (LIC and RIC), we concluded that CD45\textsuperscript{high} immunoreactivity in our case was not a mere artifact but a rare event which would be occurred with bone marrow metastasis of SCLC as described in very few cases (8,9). Although the significance of this aberrant expression of CD45 remains elucidated, recent works give us the clue to answer the question. CD45 is a transmembrane protein tyrosine phosphatase and functions as a modulator of signals from diverse receptor tyrosine kinases (10). It is of great interest that SCLC cells express favorable molecules as the target of CD45; c-Kit, the receptor of stem cell factor, and its downstream signaling molecules including SFKs (11), or hepatocyte growth factor receptor tyrosine kinase (c-MET) and focal adhesion kinase governed by c-MET (12). These molecules are closely involved in SCLC cell motility, adhesion and proliferation (11,12). Accordingly, it sounds very rational to speculate that CD45 expressed in SCLC cells modulates growth factor receptors and/or their downstream signaling molecules to gain advantage of cell adhesion, survival and proliferation in the metastatic sites.

This new immunophenotyping not only endorsed the morphological diagnosis (bone marrow from RIC), but also successfully detected micrometastasis overlooked by morphology (bone marrow from LIC). Especially, CD45, besides discriminating lymphocyte population, played an important role to disclose the unexpected pitfall hiding in the common diagnostic knowledge about SCLC.

In conclusion, we report here a rare case of SCLC with bone marrow micrometastasis, successfully diagnosed on a new FCM immunophenotyping by the combination of CD45 with three NE markers, in which tumor cells showed aberrant expression of CD45.

**Conflict of interest statement**

None declared.

**References**