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Original Research

Using flow cytometry for the diagnosis of lung neuroendocrine carcinoma with samples from fine-needle aspiration and pleural fluid

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ABSTRACT

Objective: Diagnosis of primary and metastatic lung neuroendocrine carcinoma (LNC) is challenging with fine-needle aspiration (FNA) since the morphologic features of LNC and lymphoid lesions overlap. Flow cytometry (FC) is commonly utilized for lymphoid lesions; however, FC with cluster differentiation (CD) 56 and cytokeratin antibodies can also be used for the diagnosis of neuroendocrine neoplasia. Our studies compared the role of FC to immunohistochemistry (IHC) for the diagnosis of LNC from cytological specimens. **Materials and Methods:** Cases of LNC with both cytology and FC were identified in our pathology databases from 2008 until June 2012. IHC and FC results were compared in these cases. **Results:** Fifteen of 17 cases had immunohistochemical studies from cell block including cytokeratin (11/11, 100%), thyroid transcription factor-1 (6/8, 75%), synaptophysin (12/14, 86%), chromogranin (4/13, 31%) and CD 56 (10/11, 91%). Of all 17 cases, 13 cases had FC performed with antibodies for CD 56, cytokeratin (Cam5.2) and CD 45. Twelve of the 13 cases were positive for CD 56 (12/13, 92%), which was similar to the IHC results (10/11, 91%). Eleven cases were positive for cytokeratin by IHC and 10 were positive (10/12, 83%) for cytokeratin by FC. One case in our study which was CD 56+/Cam5.2+/CD 45− was diagnosed as carcinoid tumor by final morphology. **Conclusions:** Our results suggest that FC utilizing antibodies for CD 56/cytokeratin/CD 45 is a reliable alternative method for detecting LNC from FNA specimens when IHC fails or becomes unavailable. However, the morphology is still necessary for the diagnosis since the FC panel is not wholly specific for the subtypes of LNC.

KEY WORDS: Cluster differentiation 56, cytokeratin, fine needle aspiration, flow cytometry, immunohistochemistry, lung small cell carcinoma

INTRODUCTION

Lung neuroendocrine carcinoma (LNC) accounts for <15% of all lung cancers, but is an aggressive tumor with neuroendocrine differentiation, a high mitotic rate, and often presents with metastasis at the time of diagnosis. Surgical intervention is rarely helpful, but chemotherapy can be useful, however, survival rates remain dismal [1,2].

The workup of primary and metastatic lung carcinoma has increasingly been performed using endobronchial ultrasound fine needle aspiration (EBUS-FNA) and FNA on lymph nodes [1]. The sample obtained at time of the procedure can be triaged for appropriate workup of either small cell carcinoma or non-small cell carcinoma. The diagnosis of LNC is based on review of morphology and is supported by immunohistochemistry (IHC) performed on a cell block or biopsy for markers such as cluster differentiation (CD) 56, pancytokeratin, thyroid transcription factor-1 (TTF-1), chromogranin (CH) and synaptophysin [1]. However, if there is no good cell block from FNA, the differentiation of LNC from reactive lymphocytes or lymphoma sometimes is very challenging. In addition, preparation of a cell block for immunostaining often requires more than 48 h from the time of the procedure until a finalized report is ready. Alternative methods to supplement this diagnostic process would be desirable.

Flow cytometry (FC) is a rapid method for analyzing cells in suspension for both physical properties such as cell size and complexity, as well as staining the cell surface for various markers of differentiation. While this method is commonly used for lymphoma and leukemia, FC has also been used as an alternative method for the diagnosis of neuroendocrine tumors including small cell carcinoma [3-8], merkel cell carcinoma [3,6,9,10], primitive neuroectodermal tumor (PNET)/Ewing sarcoma and neuroblastoma [6,8,11-17]. Small cell carcinoma, for example, is very amenable to FC due to its small cell size, lack of cell to cell cohesion, and expression of cell surface markers such as CD 56 and cytokeratin. Lymphoma is almost always considered in

the differential along with small cell carcinoma when working up cases of possible LNC. An expanded analysis to include cytokeratin (Cam5.2) and CD 56 helps to identify a CD 45 negative population of cells representing a neuroendocrine neoplasm, especially when there is no cell block available. The use of FC for the diagnosis of lung neuroendocrine carcinoma has been reported in several papers [4-6,8].

An early study by Bryson *et al.* showed that by screening solid tissue samples with FC for a CD 56+/CD 45− population, they were able to identify 12 specimens from 2000 studies that were neuroendocrine. Five of these turned out to be small cell carcinoma, whereas another three were merkel cell carcinoma [3]. Farinola *et al.* reviewed their previous experience with 89 cases of FNA biopsy that had concurrent FC performed. Ten of those cases were non-lymphoid tumors and the FC analysis revealed all ten of them to be neuroendocrine tumors based on CD 56+ staining by FC. Eight of those cases had a final diagnosis of small cell carcinoma, one merkel cell carcinoma and one Ewing Sarcoma/PNET [6]. Cornfield *et al*. had one of the largest studies to date that retrospectively reviewed 27 cases that had been submitted for FC (three needle aspiration and 24 tissue biopsy), which had CD 56+/CD 45− populations and also submitted those cases for analysis by IHC. In 25 of 27 cases, they had complete agreement with the FC and IHC results. Their overall results led them to conclude that "the combination of the CD 56 +CD 45− phenotype, as determined by FCM, and typical small cell neuroendocrine-type morphology, in the proper clinical setting, is highly suggestive of small cell carcinoma"[4]. However, there is no study to comparing the FC and IHC tests in cytopathological specimen.

In the current study, we compared the role of FC to immunohistochemical studies from pure cytological samples including FNA and pleural fluid. The extended markers, CD 56 and cytokeratin (Cam5.2), were used for FC. Immunohistochemical studies were performed on cell blocks from cytological samples. We wanted to examine if FC could play reliable role in the diagnosis of small cell lung carcinoma (SCLC) or other neuroendocrine neoplasia.

MATERIALS AND METHODS

Case Material

The pathology database at the University of Rochester Medical Center (Rochester, New York) was searched for cases of LNC that had both cytology and FC performed from 2008 until June 2012. In most of these cases, rapid assessment was performed at the time of EBUS-FNA to asses for adequacy of diagnostic material. A dedicated pass for FC was requested when a diagnosis of lymphoma was suspected. In addition, needle rinses and dedicated passes for preparation of a cell block were routinely collected. Patient information, including age, sex, and location of the lesion that was sampled by EBUS-FNA were recorded. This project was approved by the University of Rochester Medical Center RSRB officer.

FC

The aspirated specimens were submitted in RPMI-1640 for flow cytometric analysis. The cell pellets were washed (PBS) and suspended in stain buffer (BD Pharmingen, BD Biosciences) following centrifugation to a final concentration of approximately 1×10^6 to 1×10^7 cells/ml. Sample tubes were then prepared using 100 microliter aliquots of cell suspension and these were then subjected to cell surface staining. The fluorescently conjugated antibodies were added in concentrations recommended by the manufacturer and incubated for 20-45 min while protected from light. The samples were washed twice with 1-2 ml of stain buffer and the resulting pellets resuspended in stain buffer for a final volume of 0.5 ml. Cell surface staining for neuroendocrine carcinoma was performed using a single tube containing anti-CD 45 PerCP, anti-CD 56 PE and anti-cytokeratin (Cam5.2) FITC (BD Biosciences, San Jose, CA). A total of at least 5,000 events per sample tube were collected. Data acquisition was performed on BD FACS Canto and Canto II flow cytometers (BD Biosciences) and data analysis was performed with BD FacsDiva software (BD Biosciences).

IHC

Tissue sections from the cell blocks were deparaffinized, rehydrated through graded alcohols, and washed with phosphate buffered saline. Antigen retrieval was performed by heating sections in 99°C water bath for 40 min. After endogenous peroxidase activity was quenched and nonspecific binding was blocked, antibodies [Table 1] were incubated at room temperature for 30 min. The secondary antibody (Flex HRP) was allowed to incubate for 30 min. After washing, sections were incubated with Flex DAB Chromogen for 10 min and counterstained with Flex Hematoxylin for 5 min. A previously diagnosed SCLC served as positive control. Negative control was performed by replacing antibodies with normal serum.

RESULTS

Clinicopathological Findings

From January 2008 to June 2012, there were 17 cases with the diagnosis of SCLC and neuroendocrine tumor of lung that had both cytology and FC performed during the study period. These patients consisted of 11 males and 6 females with a

Table 1: Antibodies used for immunohistochemistry of SCLC

Antibody	Clone	Dilution	Producer
Pan-cytokeratin	AE1+3: AE1/AE3	1:300	Dako
	Cam5.2: cam5.2	1:30	Becton dickinson
Synaptophysin	MRQ-40	1:100	Cell marque
CH	LK2H10	1:100	Cell marque
CD 56	1B6	1:200	Nova castra
TTF-1	8g7G3/1	1:100	Dako

TTF-1: Thyroid transcription factor-1, CD: Cluster differentiation, SCLC: Small cell lung carcinoma, CH: Chromogranin

mean age of 65 years (39-85 years). Clinical symptoms were variable including chest pain, cough, headache, dysphagia and shortness of breath. These 17 specimens were from mediastinal or supraclavicular lymph nodes (11/17), lung masses (4/17) or pleural fluid (2/17). Table 2 provides a breakdown of the individual results for each case. Only 9 cases were available that had CD 56 performed by both FC and IHC.

The cytological smear with Diff-Quick® stain showed small blue cells. These small blue cells formed small loose clusters surrounded by single naked-like cells with focal necrosis and crushed artifact [Figure 1]. The cytological morphology with both Diff-Quick® and Papanicolaou stains showed small cells with very high nuclear/cytoplasmic ratio (scant cytoplasm), round to irregular nuclei, hyperchromatic fine chromatin, and internuclear molding [Figure 1].

Table 2: Results from both FC and IHC tests in total 17 cases

Case no	Age	Sex	LF	IHC					FC			
				CK	SY	CH	I56	TF	FC	FC56	FC5.2	FC45
1	39	M	MA		+	+	+	+	Y	+	+	
2	64	M	LN						Y	+	+	-
3	79	M	LN	F+	+	-	+	F+	Y	+	-	-
4	60	M	LN	+	-	-	-	-	Y	-	+	-
5	79	M	LN		F+	F+	+	+	Y	+	+	-
6	53	M	LN						Y	+	+	-
7	69	F	LN	+	-	-	+		Y	+	+	-
8	85	F	LN	+	F+	F+			Y	+	+	-
9	74	M	MA	+	F+	-	+	+	Y	+		-
10	48	M	FL		+	-	+		Y	+	+	-
11	67	M	FL	+	+	-	+		Y	+	-	-
12	73	M	LN	+	+	-	+	+	Y	+	+	-
13	58	F	LN	+	+	-	+	+	Y			
14	77	F	MA	F+				-	Y			
15	82	F	LN		F+	F+	+		Y			
16	53	M	MA	+	+				Y	+	+	-
17	52	F	LN	+	F+	-			Y			

LF: Location of fine needle aspiration (MA-mass, LN-lymph node, FL-pleural fluid), CK: IHC pan-cytokeratin, SY: Synaptophysin, CH: Chromogranin, I56: IHC CD 56, TF: TTF-1, FC: flow cytometry performed, FC 56: CD 56 by flow cytometry, FC5.2: Cytokeratin by flow cytometry, FC 45: CD 45 by flow cytometry, FC: flow cytometry, CD: Cluster differentiation, IHC: Immunohistochemistry

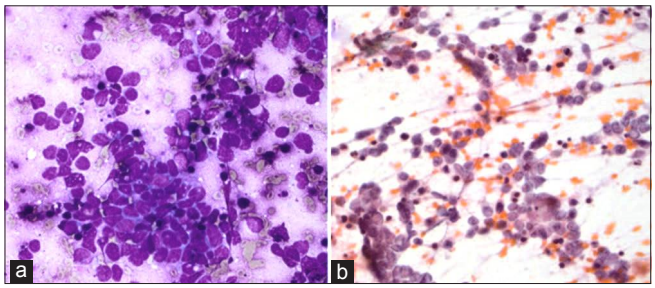

Figure 1: Representative cytological findings in a case of small cell carcinoma obtained by endobronchial ultrasound fine needle aspiration. Small blue cells showed clusters and single cells with high nuclear/cytoplasm ratio, fine granular hyper-chromatin, nuclear molding, and pleomorphic nuclei. (a) Diff-quick stained smear and (b) papanicolaou stained smear

The nucleoli in the nuclei are inconspicuous or absent. Based on the morphology alone, it is very difficult to differentiate the small tumor cells from benign or malignant lymphocytes.

IHC

15 of 17 cases had immunohistochemical studies on the cell block including cytokeratin (11/15), TTF-1 (8/15), synaptophysin (14/15), CH (13/15) and CD 56 (12/15) [Table 2]. The cytokeratin immunostain showed strong granular cytoplasmic staining in 9 of 11 cases and faint staining in 2 of 11 cases (Table 2, 100%). TTF-1 immunostaining had a strong nuclear stain except for one faint nuclear stain (6/8; 75%). The tumor cells also showed strong or faint granular cytoplasmic staining for synaptophysin (12/14, 86%), CH (4/13, 31%) and CD 56 (10/11, 91%) [Figure 2].

FC

13 of the 17 cases had FC performed using a dedicated tube with antibodies for CD 56 and 12 for cytokeratin (Cam5.2) and CD 45 [Table 2; Figure 3]. 12 of the 13 cases surface stained for CD 56 were positive (12/13, 92%), which is similar to IHC results (10/11, 91%). Only one case (1/13) that was diagnosed as SCLC by morphology was negative for CD 56 by both FC and IHC; however, the case was positive for cytokeratin by both FC and IHC. Ten of the 12 cases were positive (10/12, 83%) for cytokeratin (Cam5.2) by FC. Two cases (2/12) were negative for Cam5.2 by FC, but positive for pancytokeratin by IHC. The concordance rate of cytokeratin (Cam5.2) by FC and cytokeratin by IHC was 71%. All cases were negative for CD 45 by FC.

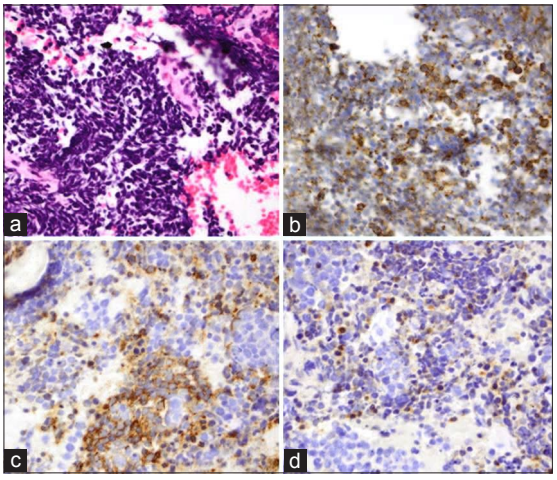

Figure 2: Representative stains performed on the cell block include H&E stain, (a) small blue cells with nuclear molding, high nuclear/cytoplasm ratio and crushed artifact, cluster differentiation 56 positive cells with granular cytoplasmic stain adjacent to nuclei (b), synaptophysin positive cells with granular cytoplasmic stain adjacent to nuclei, (c) and chromogranin positive cells with weak granular cytoplasmic stain (d).

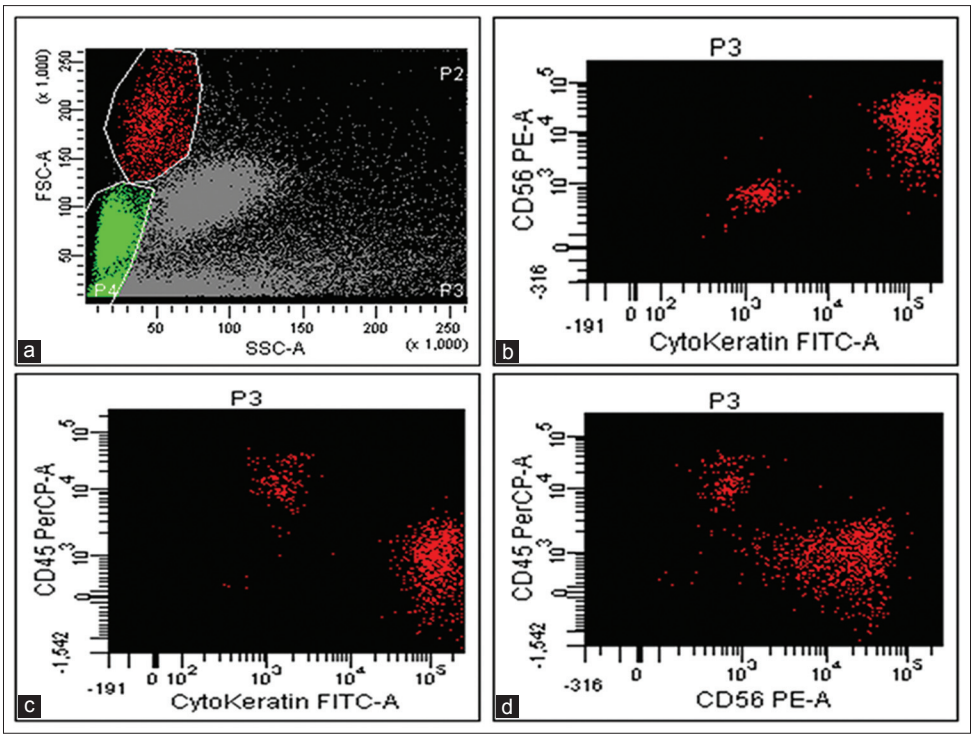

Figure 3: Representative flow dot plots showing gating strategy for lung neuroendocrine carcinoma. Forward scatter versus side scatter (upper left) showed a population (red) of larger cells based on forward scatter corresponding to the neuroendocrine carcinoma; lymphocytes were gated out (green). Analysis of the gated cells (red) showed the cells co-express cluster differentiation 56 and cytokeratin (upper right); the population was cluster differentiation 45 negative while expressing cytokeratin (lower left) and cluster differentiation 56 (lower right).

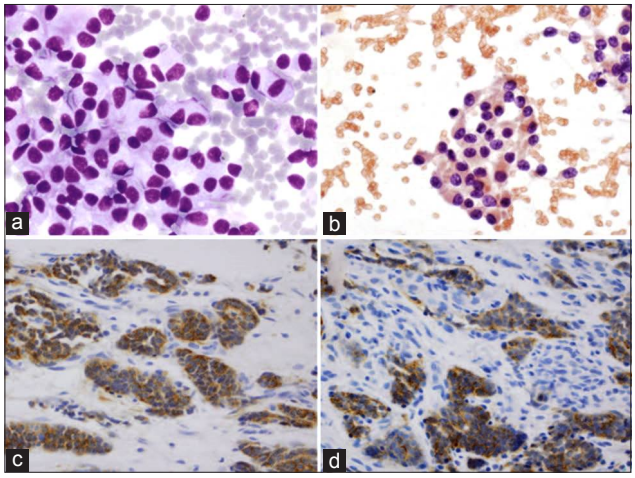

Figure 4: A unique case of a carcinoid tumor in the lung that was cluster differentiation 56+/cytokeratin+ by flow cytometry and immunohistochemistry, but was evident only by morphology. (a) Diff-Quick stained smear, (b) Papanicolaou stained smear, small cells with fine nuclear chromatin, plenty of cytoplasm and sheets of distribution. Synaptophysin positive cells with strong cytoplasmic staining (c) and (d) cluster differentiation 56 positive cells with strong cytoplasmic staining

Eight cases were positive for CD 56 by both FC and IHC; one was negative by both FC and IHC, making a concordance rate of 100%. One of the eight cases that were positive for both CD 56 and cytokeratin (Cam5.2) had the final diagnosis of typical carcinoid tumor [Figure 4].

DISCUSSION

In our institution, FNA and other cytopathological samples are commonly used for the diagnosis of lung tumor and/or metastatic tumors in thoracic lymph nodes. The diagnosis of neuroendocrine carcinoma from lung and metastatic lymph nodes has increased dramatically with cytopathological methods nationwide. If there is no good cell block, the differentiation of neuroendocrine lung carcinoma from reactive lymphocytes or lymphoma sometimes is very challenging since their morphological features overlap.

FC is frequently used for the diagnosis of lymphoproliferative disorders. Several studies have found that FC was a useful alternative method for the diagnosis of neuroendocrine tumors including small cell carcinoma [3-8], merkel cell carcinoma [3,6,9,10], PNET/Ewing sarcoma and neuroblastoma [6,8,11-17].

In the current study, FC, IHC and morphology were used to diagnose LNC. We found that the neuroendocrine marker, CD 56, in both immunohistochemical studies and FC showed a 100% concordance rate; cytokeratin (Cam5.2) by FC and pancytokeratin by IHC showed an 83% concordance rate.

CD 56 is the CD assignment for the neural cell adhesion molecule, which is frequently used as a marker for neuroendocrine differentiation in immunohistochemical studies and also for non-Hodgkin's lymphoma in FC (natural

killer (NK) cells, malignant plasma cells, etc.). Cornfield and colleagues found CD 56 positivity by FC in 27 cases and 26 of 27 cases showed CD 56 positivity by IHC [4]. However, only three cases were from aspiration; the others from tissue biopsy. Eight cases of SCLC with positive CD 56 were reported from FNA samples [6]. IHC for CD 56 was only performed in two cases. We found 17 cytopathological cases with both LNC and FC in our database. Eight cases were positive for CD 56 by both FC and IHC; one case was negative by both FC and IHC [Table 2]. CD 56 by FC and IHC showed a 100% concordance rate. Cytokeratin markers such as pancytokeratin, Cam5.2 and AE1/AE3 were used to differentiate lymphoid lesions and carcinoma by FC and IHC. Cornfield and colleagues found that AE1/AE3 and Cam5.2 with IHC showed 100% concordance rate comparing to those with FC in a total of 12 cases. They only selected three FNA cases for their study. In our study, Cam5.2 by FC was positive in 10 of 12 cytopathological cases (83%), but pan-cytokeratin by IHC was 11/11 (100%) positive. This difference may be caused by different antibodies. The IHC used pancytokeratin that covered both AE1/AE3 and Cam5.2, but the FC used only Cam5.2.

From our study, CD 56+/Cam5.2+/CD 45− with FC is a reliable panel to aid in the diagnosis of small cell carcinoma. However, one of the 17 cases with CD 56+/Cam5.2+/CD 45− had a final diagnosis of typical carcinoid tumor based on the morphology. In addition, CD 56 is not a specific neuroendocrine marker, which can be expressed in various tumors including non-small cell lung cancer (squamous cell carcinoma and adenocarcinoma) [18], ovarian sex cord stromal tumors [19], NK cell lymphoma, plasma cell leukemia, leiomyoma [20], leiomyosarcoma [20], gastrointestinal stromal tumor [20], solid pseudopapillary tumor [21], synovial sarcoma [22] and nephroblastoma [23]. In lung non-small cell carcinoma, 86 of 575 (15%) squamous cell carcinoma cases and 30 of 262 (11%) lung adenocarcinoma cases were positive for CD 56 [18]. Cam5.2 immunostain is also positive in most of lung and metastatic carcinomas. If only using CD 56+/Cam5.2+/CD 45− as a diagnostic criteria for lung neuroendocrine tumors, it will cause some false diagnoses such as lung squamous cell carcinoma. Therefore, the diagnosis has to be a combined effort utilizing FC and/or IHC with cytomorphological features.

CONCLUSION

FC with CD 56+/cytokeratin (Cam5.2)+/CD 45− phenotype is a reliable alternative test for lung neuroendocrine carcinoma in cytopathological samples when IHC fails or becomes unavailable (cell block material exhausted). However, cytomorphological features still play a major role in the final diagnosis since CD 56 is not very specific for neuroendocrine tumor subtypes.

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