Flow Cytometric Analysis of CD200 Expression by Pulmonary Small Cell Carcinoma

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Background: CD200 is a membrane bound glycoprotein that is expressed by a variety of normal tissues and hematopoietic malignancies. Flow cytometric analysis of CD200 expression has utility in the evaluation of mature B-cell neoplasms, myeloma, and acute leukemia; however, CD200 expression in nonhematopoietic malignancies has not been extensively studied.

Methods: We studied 14 cases of biopsy proven pulmonary small cell carcinoma in which a discrete CD45 negative, CD56 positive abnormal cell population was identified by flow cytometry. We retrospectively evaluated these cases for flow cytometric and immunohistochemical evidence of CD200 expression.

Results: Twelve of the 14 cases of pulmonary small cell carcinoma showed convincing expression of CD200 by both immunohistochemistry and flow cytometry.

Conclusions: Pulmonary small cell carcinoma frequently expresses CD200 at a level that can be detected by flow cytometry and immunohistochemistry. CD200 expression therefore may be used to help identify pulmonary small cell carcinoma in flow cytometry specimens and tissue sections. CD200 may also play a role in the biology of pulmonary small cell carcinoma and is a potential target of future therapies. © 2015 International Clinical Cytometry Society

Key terms: CD200; flow cytometry; immunohistochemistry; pulmonary small cell carcinoma; nonhematopoietic neoplasms

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CD200 (also known as OX2) is a membrane bound glycoprotein that is normally expressed by many cell types including neurons, endothelial cells, lymphocytes, follicular dendritic cells, and subsets of white blood cells (1). CD200 receptor (CD200R) expression is believed to be limited to myeloid cells, and the interaction between CD200 and CD200R has been shown to inhibit myeloid activation (2). CD200 is thought to play a role in many biological pathways including autoimmunity, allergic disorders, infection, transplantation, bone homeostasis, reproductive biology, and tumorigenesis (3). In certain malignancies, CD200 expression is also thought to promote tumor formation and metastasis by allowing malignant cells to evade the immune system (1).

CD200 expression has been well studied in hematopoietic malignancies, including lymphomas, plasma cell neoplasms, and acute leukemias. CD200 is consistently expressed in chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), and CD200 expression can be used to help distinguish CLL/SLL from mantle cell lymphoma (4,5). CD200 expression has also been found in most cases of mediastinal type diffuse large B-cell lymphoma (DLBCL), and is useful in differentiating mediastinal DLBCL from other forms of DLBCL (6). Hairy cell leukemia and angioimmunoblastic T-cell lymphoma have also been shown to be CD200 positive (7,8).

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CD200 expression has been demonstrated in the majority of plasma cell myelomas; however, studies evaluating CD200 expression in polytypic plasma cells report differing results (9–12). CD200 expression in myeloma is stable after treatment which suggests that CD200 may be a useful marker for minimal residual disease detection (9). Attempts to determine the prognostic significance of CD200 expression in multiple myeloma have yielded conflicting results (4,9).

CD200 expression has also been studied in acute leukemias. Normal hematogones have been shown to express CD200, and aberrant overexpression or underexpression of CD200 has been demonstrated in approximately 95% of cases of B-cell precursor acute lymphocytic leukemia B-lymphoblastic leukemia/lymphoma (B-ALL) (4). As a result, CD200 analysis has potential value in the detection of minimal residual disease in B-ALL (13). Strong CD200 overexpression has also been reported in association with hyperdiploid or TEL-AML1 subtypes of B-ALL, but this relationship needs further study (13). In addition, CD200 is abnormally expressed in a subset of acute myeloid leukemias and may be a marker of poor outcome (14).

In contrast, CD200 expression in nonhematopoietic malignancies has not been as extensively studied. In an analysis of tumor cell lines, CD200 expression was found in renal carcinoma, ovarian carcinomas, neuroblastoma, and melanoma with no evidence of expression in prostatic carcinoma, lung carcinoma, breast carcinoma, astrocytoma, or glioblastoma (15). A subset of cells in basal cell carcinoma express CD200, and these cells appear to initiate tumor growth (16). CD200 expression has also been demonstrated in cutaneous squamous cell carcinoma where it may promote metastasis (17). To our knowledge, CD200 expression has not been previously described in pulmonary small cell carcinoma (PSCC).

PSCC frequently metastasizes to lymph nodes, and it may mimic lymphoma in touch preparations and frozen sections. As a result, fresh PSCC samples may be submitted to the laboratory for flow cytometric evaluation. In a previous report, Chang et al. demonstrated flow cytometric identification of possible small cell carcinoma cases using expression of CD56 and lack of CD45 (18). Similarly, we have also identified CD45-negative/CD56-positive cell populations in clinical flow cytometry samples that are suspicious for involvement by PSCC. Interestingly, we have noted multiple cases in which the neoplastic cells were CD200 positive, as CD200 analysis has been part of our initial flow cytometry screening tubes since 2012. To further investigate this incidental finding, we retrospectively evaluated the flow cytometric and immunohistochemical expression of CD200 in 14 cases of PSCC.

### MATERIALS AND METHODS

#### Samples

After obtaining institutional review, we retrospectively searched the Tacoma General Hospital flow cytometry archives from 2012 to 2014 to find tissue biopsy samples sent for flow cytometry which contained discrete CD56-positive, CD45-negative cell populations that were suspicious for involvement by a nonhematopoietic malignancy. We identified 14 such cases that were diagnosed as pulmonary small cell carcinoma. We reviewed the concurrent H&E and immunohistochemically stained slides from these cases to confirm the diagnosis of PSCC and to exclude alternative diagnoses such as carcinoid, large cell neuroendocrine carcinoma, adenocarcinoma, or squamous cell carcinoma. Cases included both primary and metastatic PSCC (see Table 1).

#### Flow Cytometry

The tissue samples included in this study were evaluated in the flow cytometry laboratory at MultiCare Health Systems Tacoma General Hospital. At the time of initial testing, the specimens were collected fresh in RPMI and analyzed within 24 h of collection. Following tissue disaggregation, 100 μL of sample was incubated for 15 min in the dark, with titrated amounts of the following antibodies (manufactured by Beckman Coulter):...
unless stated otherwise): CD2 FITC, Kappa FITC, CD7 PE, Lambda PE, CD34 ECD, CD19 ECD, CD56 PC5.5, CD22 PC5.5, CD3 PC7, CD38 PC7, CD4 APC, CD200 APC (eBioscience, clone OX104), CD16 APCF700, CD10 APCAF700, CD5 APCAF750, CD8 PB, CD20 PB, and CD45 KO. Specimens were then subjected to fixation and erythrocyte lysis for 15 min in the dark, using an ammonium chloride lyse solution with 10% methanol-free formaldehyde (Polysciences). Finally, specimens were washed with a PBS/BSA solution, and evaluated using a 3-laser, 10-color Beckman Coulter Gallio flow cytometer. Wherever possible, 150,000 events were collected per sample. Data were analyzed using Kaluza Flow Cytometry Analysis software from Beckman Coulter (version 1.3). Specimens were initially gated using forward scatter properties to exclude doublets, followed by forward scatter/side scatter gating to exclude nonviable cells.

Each sample contained a discrete CD45-negative, CD56-positive tumor cell population which was subsequently evaluated for CD200 expression by flow cytometry. The criteria used to define CD200 positivity by flow cytometry were determined empirically based upon our experience with evaluating CD200 expression in B-cell neoplasms and involved determination of the CD200 median fluorescence intensity (MFI) in comparison with the CD200 negative T cells present in each sample. Cells were considered CD200-positive if the CD200 MFI was \( \geq 10 \), and was at least 0.5 log greater than the MFI of the CD200-negative T cells.

**Immunohistochemistry**

Formalin fixed, paraffin embedded tissue sections were deparaffinized, rehydrated, and blocked for endogenous peroxidase. Rehydrated specimens were steamed in a Tris-EDTA buffer, pH 9, for 20 min and allowed to cool for another 20 min. Ultra Vision Block (Thermo #TA-125-UB) was applied to the slides via a DAKO autostainer, incubated for 5 min, and washed off the slides. Prediluted (1:500) CD200 antibody (R&D Systems, goat polyclonal) was applied and specimens were incubated for 30 min on the DAKO stainer. Anti-goat IgG was then applied for 10 min, followed by a wash step. Ultra Vision HRP polymer (Thermo #TL-999-PH) was then applied, incubated for 10 min, and washed off. DAKO DAB (L3468110-2) was applied for 10 min and then washed. The immunostains were reviewed by a single pathologist (J.L.) and evaluated for the percentage of stained tumor cells and the intensity of staining. Intensity was graded as weak, intermediate, or strong. Cases showing immunoreactivity for CD200 were further classified as “focally,” “variably,” or “uniformly” positive based upon the percentage of stained tumor cells as follows: “focally positive” = 1-25% reactivity, “variably positive” = 25-75% “reactivity, and uniformly positive” = >75% reactivity. Cases that were at least variably CD200-positive (reactivity on >25% of tumor) with at least intermediate intensity were considered immunohistochemically positive for CD200.

**RESULTS**

Twelve of the 14 PSCC cases were positive for CD200 by flow cytometry, and included both primary and metastatic disease. These 12 cases showing CD200 expression by flow cytometry were also positive for CD200 expression by immunohistochemistry. The two cases without demonstrable CD200 expression by flow cytometry were also negative for CD200 by immunohistochemistry (see Table 1). Representative flow cytometry and immunohistochemical results for CD200 expression are depicted in Figures 1 and 2. The fourteen cases examined in our study were selected based upon the presence of a CD45-negative/CD56-positive population as identified by flow cytometry. CD56 expression was confirmed by immunohistochemistry in 13 of 14 cases (data not shown). In one case there was insufficient tissue available for CD56 assessment by immunohistochemistry.
FIG. 2. a. Pulmonary small cell carcinoma specimen, forward scatter height versus forward scatter area gating to exclude doublets and debris. b. Forward scatter versus side scatter gating, to exclude nonviable cells. c. CD45 versus CD56 gating to identify the CD45-negative/CD56-positive tumor cells (colored red). d. The tumor cells lack both CD19 and CD5 expression. e. Pulmonary small cell carcinoma shows a CD200 positive, CD45-negative immunophenotype.
DISCUSSION

We used flow cytometry and immunohistochemistry to demonstrate CD200 expression in 12 of 14 cases of PSCC, including both primary tumors and metastatic disease. PSCC frequently metastasizes to lymph nodes, and may lead to initial diagnostic confusion as it can mimic lymphoma in touch preparations and frozen sections. As a result, fresh PSCC samples are often submitted to the laboratory for flow cytometric evaluation as part of an initial diagnostic work-up. Recognition that CD200 is expressed on a significant subset of PSCCs is important to ensure that flow cytometry studies are not misinterpreted when both PSCC and CLL/SLL (or other B-cell lymphomas) are diagnostic considerations. CD200 therefore joins CD56, EPCAM, CD99, CD90, and cytokeratin as antigens that can potentially identify nonhematopoietic malignancies by flow cytometry (18). Since CD200 also has utility in evaluating B-cell neoplasms, it is arguably one of the more useful of the above antigens, akin to CD56, as it is included in the diagnostic B-cell panels in many flow cytometry laboratories. As an immunohistochemical marker, CD200 joins synaptophysin, chromogranin, and CD56 in the battery of markers that can be used to identify PSCC in tissue sections. While the identification of a CD45-negative/CD56-positive/CD200-positive population by flow cytometry can raise the possibility of PSCC and help guide initial immunohistochemical panels, it should be noted that this immunophenotype is not pathognomonic and flow cytometry is not a recommended modality for the diagnosis of PSCC. Current treatment strategies for lung carcinomas require histologic examination in order to definitively diagnose PSCC and to distinguish it from squamous cell carcinoma, adenocarcinoma, large cell variants, and other variants. Histologic and/or immunohistochemical subtyping of lung carcinoma is further supplemented by molecular testing to determine the most appropriate chemotherapeutic regimen for a patient.

As mentioned above, the identification of a CD45-negative/CD56-positive/CD200-positive population by flow cytometry is not unique to PSCCs as this immunophenotype can be seen in cases of plasma cell myeloma, ALL, or AML. Therefore, utilization of carefully designed flow cytometry panels is extremely important for appropriate interpretation of CD200 expression. While PSCC and lymphoma can be difficult to distinguish based upon initial touch preparation or frozen section findings, discriminating between mature B-cell lymphoma expressing CD200 and small cell carcinoma in flow cytometry samples should not be difficult in the vast majority of cases. Mature B-cell lymphomas express CD45, CD19, CD22, CD20, and surface light chains, while small cell carcinomas are negative for these antigens. Distinguishing between an abnormal plasma cell population and small cell carcinoma by flow cytometry based purely upon the immunophenotypic findings is a little more problematic as both may exhibit a CD200 positive, CD45 negative, CD19 negative, CD56 positive immunophenotype; however, the expression of CD38, CD138, and cytoplasmic light chains should allow myeloma to be differentiated from small cell carcinoma in almost all instances. Evaluation of broad panels of myeloid, monocytic, T cell, and B-cell antigens should be adequate to distinguish between small cell carcinoma and acute leukemias in flow cytometry samples. In all cases, correlation of flow cytometric findings with morphologic features and the clinical context is required to prevent error.

In H&E stained slides, small cell carcinoma and lymphoma can have similar morphologic features and it is possible that a CD200 positive PSCC could be mistaken for CLL or another lymphoma in immunohistochemically stained tissue sections if only limited IHC antibodies are employed. This is especially concerning given that PSCC may express PAX-5, a commonly used immunohistochemical marker of B-cell lineage (19). However, immunohistochemistry for CD45 should allow differentiation between small cell carcinoma and mature B-cell lymphoma in this situation.

In our study, 85% of all evaluated PSCC cases were CD200 positive. Flow cytometric CD200 expression was uniformly associated with the classical markers of small cell carcinoma and CD200 positivity could be demonstrated by immunohistochemistry in the tumor samples. However, the number of cases evaluated is low and limits our ability to extract statistically significant information from our findings. Larger studies with more extensive assessment of CD200 expression in a variety of neuroendocrine neoplasms, as well as a comparison between CD200-positive and CD200-negative PSCC cases, are needed. Note that our evaluation of CD200 expression in other neuroendocrine neoplasms is ongoing, in order to determine if CD200 expression is unique to PSCC or is a common feature of neuroendocrine malignancies and to determine if CD200 expression correlates with grade or outcome in neuroendocrine malignancies.

The significance of CD200 expression in the biology of PSCC is currently not well understood. It is postulated that CD200 expression may allow tumors to evade the immune system or play a role in the invasiveness and/or metastatic potential of PSCC, but further studies to elucidate the function of CD200 in human health and disease are needed. Despite the low number of cases, our study showed CD200 expression in pulmonary small cell carcinoma using two techniques and two different antibody clones. This suggests that the CD200 expression we demonstrated is genuine; however, additional mRNA or protein studies to confirm our findings would also be useful. Given the retrospective nature of our study and the small amount of tissue submitted for the clinical samples tested, adequate fresh tissue was not available for such an investigation as part of this study.

In summary, our findings demonstrate convincing CD200 expression in PSCC by flow cytometry and immunohistochemistry. Although flow cytometry will never replace conventional morphology and immunohistochemistry in

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the diagnosis of nonhematopoietic neoplasms, the value of flow cytometry in identifying nonhematopoietic neoplasms as a way to guide subsequent studies, or evaluate potential therapeutic targets, should not be ignored. With our expanding flow cytometry panels and antibody libraries, attention should be given to nonhematopoietic flow cytometric immunophenotyping as an adjunct diagnostic tool and an opportunity to discover novel antigen expression. Our identification of CD200 expression by PSCC represents an exciting development as studies suggest that anti-CD200 antibody treatment might be therapeutically beneficial for treating CD200 positive malignancies (20). If anti-CD200 antibody therapies are found to be viable, similar trials in PSCC patients, a disease with a dismal clinical prognosis, need to be explored.

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