Original Article

Analysis of clonality in cutaneous B-cell lymphoma and B-cell pseudolymphoma using skin flow cytometry: Comparison of immunophenotyping and gene rearrangement studies

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Abstract [243 words]

To identify clonal neoplastic cells in skin affected by B-cell lymphoma using skin flow cytometry (FCM) techniques, we investigated light-chain restriction using skin FCM with clonality assessed by polymerase chain reaction and light-chain restriction by *in situ* hybridization (ISH). We retrospectively analyzed 16 cases of B-cell lymphoma with cutaneous involvement: primary cutaneous diffuse large B-cell lymphoma, leg type (pcDLBCL-LT) (n=7), DLBCL–not otherwise specified (DLBCL-NOS) (n=6), primary cutaneous follicle center lymphoma (pcFCL) (n=1), and follicular lymphoma (n=2), as well as cutaneous B-cell pseudolymphoma (n=9). Results of skin FCM light-chain restriction analyses were compared with immunoglobulin H (*IgH*) gene rearrangement and κ/λ ISH findings. Skin FCM detected light-chain restriction in 11 of 14 B-cell lymphoma patients but none of the B-cell pseudolymphoma patients. The sensitivity of skin FCM for distinguishing B-cell lymphoma and B-cell pseudolymphoma was 79%, and the specificity was 100%. Eleven of 13 B-cell lymphoma patients exhibited gene rearrangement (sensitivity: 85%), whereas 6 of 7 pseudolymphoma patients were negative (specificity: 86%). ISH was positive in 3 of 16 B-cell lymphoma cases (sensitivity: 19%) but none of the B-cell pseudolymphoma cases (specificity: 100%). ISH sensitivity was 29% for pcDLBCL-LT, 17% for DLBCL-NOS, and 0% for pcFCL and follicular lymphoma. Skin FCM therefore appears to be more sensitive than ISH in detecting light-chain restriction in DLBCL and follicular lymphoma and as sensitive as *IgH* gene rearrangement analysis in detecting clonality. Skin FCM is thus a promising diagnostic tool for identifying monoclonal neoplastic B-cell populations.

Key words: B-cell lymphoma, gene rearrangement, light-chain restriction, pseudolymphoma, skin FCM

Introduction

Identifying clonal B-cell populations can provide powerful evidence of B-cell neoplasms. Distinguishing between cutaneous B-cell lymphoma and pseudolymphoma remains challenging, however, particularly if no clonal B-cell population is detected using established diagnostic approaches.[1-5]

Flow cytometry (FCM) analyses of lymph node or peripheral blood mononuclear cells can reveal significant clues that may aid the diagnosis of lymphoma and leukemia and determine the specific type of these diseases.[6, 7] The presence of light-chain restriction in a B-cell lineage is highly suggestive of a clonal neoplastic process, although there are some exceptions.[5]

To determine whether skin FCM is a useful diagnostic tool for identifying clonal B-cell populations, we compared the results of light-chain restriction analyses obtained using skin FCM with clonality by polymerase chain reaction (PCR) and light-chain restriction by *in situ* hybridization (ISH).

Patients and Methods

Definition of B-cell lymphoma and B-cell pseudolymphoma

We focused on idiopathic nodular B-cell pseudolymphoma based on Mitteldorf's criteria.[8] Patients were selected based on the following criteria: (1) clinical manifestations compatible with nodular B-cell pseudolymphoma; (2) idiopathic disease (i.e., no obvious cause such as drugs, insect bites, etc.); (3) spontaneous involution without invasive treatment (only skin-directed therapy); and (4) histopathologically compatible with pseudolymphoma.

The diagnosis of B-cell lymphoma was based on the classification of the World

Health Organization – European Organization for Research and Treatment of Cancer.[9]

Patient samples

We retrospectively analyzed the cases of patients diagnosed with primary cutaneous B-cell lymphoma, systemic non-Hodgkin B-cell lymphoma with cutaneous involvement, or cutaneous B-cell pseudolymphoma who were seen at our dermatology department and underwent FCM examination of skin lesions over a 7-year period (2014-2020). Skin biopsy specimens were obtained from patients with primary cutaneous diffuse large B-cell lymphoma, leg type (pcDLBCL-LT) (n=7), DLBCL–not otherwise specified (DLBCL-NOS) (n=6), primary cutaneous follicle center lymphoma (pcFCL) (n=1), follicular lymphoma (n=2), as well as patients with B-cell pseudolymphoma (n=9). Clinical data for the study subjects are summarized in Table 1.

The study was approved by the Institutional Review Board of Okayama University Hospital. Informed consent was obtained from the selected patients when any additional examination was performed, in accordance with the Helsinki Declaration.

Skin FCM

Skin-infiltrating mononuclear cells (SIMCs) were obtained as follows from skin lesion biopsies of each patient. Tissue samples were gently pricked with a 21-gauge needle in saline to isolate tissue-infiltrating lymphoid cells. The isolated infiltrating cells were then analyzed on a Navios EX flow cytometer (Beckman Coulter, Indianapolis, IN, USA) using the following panel: CD3, CD4, CD5, CD8, CD10, CD16, CD19, CD20, CD45, CD56, Igκ, and Igλ (Beckman Coulter). Debris was excluded based on forward scatter and side scatter as described elsewhere.[10] CD45-positive cells were gated and

analyzed as lymphoid cells, whereas CD19- and CD20-positive cells were gated as B cells.

The κ to λ ratio was evaluated to determine B-cell clonality. Although no established criteria are available, the following definition of clonality was used: κ/λ >5 or λ/κ >3 with a monoclonal population of properly collected cells.[5, 11, 12] Threshold lines were set based on isotype controls. For cases in which cells were not collected properly, results were not included in the statistical analyses.

Analysis of clonality

Clonality of malignant B cells was evaluated using PCR-based analysis of immunoglobulin genes (LSI Medience, Tokyo, Japan) using DNA extracted from SIMCs or formalin-fixed, paraffin-embedded (FFPE) sections of skin biopsy tissue. Samples for which insufficient material was available were not included in the statistical analyses.

ISH of Igκ and Igλ

ISH was carried out using FFPE tissue sections. Staining was performed in accordance with the manufacturer's instructions using a ZytoFast™ Plus CISH implementation kit HRP-DAB, ZytoFast® human Ig-kappa probe, and ZytoFast® human Ig-lambda probe (ZytoVision, Bremerhaven, Germany). Clonality was defined based on κ/λ >5 or λ/κ >3. Positive controls were well stained for all specimens.

Statistical analyses

Statistical analyses were carried out using GraphPad PRISM software, ver. 4.03 (GraphPad, La Jolla, CA). Differences in data were evaluated for significance using the

Mann-Whitney *U*-test; *p*-values <0.05 were considered significant.

Results

Table 1 summarizes the characteristics of B-cell lymphoma patients (n=16) and pseudolymphoma patients (n=9) regarding age, sex, diagnosis, type and site of skin lesions, treatment, outcome, gene rearrangement, and presence/absence of light-chain restriction as shown by skin FCM and ISH.

In skin FCM analyses, light-chain restriction was detected in all 6 pcDLBCL-LT patients (Table 1, Fig. 1A) and 4 of 5 DLBCL-NOS patients (Table 1, Fig. 1B). Light-chain restriction was positive in the single pcFCL patient (Table 1, Fig. 1C) and negative in the follicular lymphoma patients (Table 1, Fig. 1D) by skin FCM. Light-chain restriction was examined by skin FCM in all patients in the B-cell pseudolymphoma group, but the cells were not collected properly for patients P1, P2, and P3. Patients P4, P5, P6, P7, P8, and P9 did not exhibit light-chain restriction by skin FCM (Table 1, Fig. 1E, F).

IgH gene rearrangement could not be determined in three patients (B4, B9, and B15) in the B-cell lymphoma group due to insufficient material, as gene rearrangement in these patients was examined using FFPE sections. Among the patients for whom reliable results were obtained, *IgH* gene rearrangement was positive in 5 of 6 pcDLBCL-LT patients, 4 of 5 DLBCL-NOS patients, 1 pcFCL patient, and 1 follicular lymphoma patient. Of the 9 B-cell pseudolymphoma patients, only patient P5 exhibited clonality; the amount of material was insufficient to determine clonality in patients P7 and P9. Results were negative for the remaining patients (Table 1).

Light-chain restriction was detected by ISH in 3 of the 16 B-cell lymphoma

patients, and the results demonstrating light-chain dominance were compatible with those obtained by skin FCM (Table 1). ISH identified Ig κ or λ on the proliferating cells in patients P1, P6, P7, P8, and P9.

The median ratios of dominant light-chain ratios (κ/λ or λ/κ) were as follows: pcDLBCL-LT patients, 54.25 (range 8.7-174.6); DLBCL-NOS patients, 88.7 (range 2.1-107.8); and B-cell pseudolymphoma patients, 1.22 (range 1.09-1.42). There were too few patients in the follicular lymphoma (n=2) and pcFCL (n=1) groups for statistical analysis. None of the pseudolymphoma patients exceeded the criteria. The light-chain ratio differed significantly between the pseudolymphoma group and the pcDLBCL-LT (p=0.002) and DLBCL-NOS (p=0.004) groups.

Table 2 summarizes the results of *IgH* gene rearrangement, light-chain restriction by skin FCM, and light-chain restriction by ISH analyses. The sensitivity of skin FCM for detecting light-chain restriction was 100% in pcDLBCL-LT patients and 80% in DLBCL-NOS patients. The lone pcFCL patient exhibited light-chain restriction. Ig was expressed in the 2 patients with follicular lymphoma, but no light-chain restriction was detected. Overall, light-chain restriction was identified by skin FCM in 11 of 14 B-cell lymphoma patients (i.e., those patients in which cells were collected properly), and the sensitivity of skin FCM in B-cell lymphoma patients was 79%.

In contrast to the above results, none of the B-cell pseudolymphoma patients exhibited light-chain restriction by skin FCM (specificity: 100%). Therefore, the positive predictive value of skin FCM was 100%, and the negative predictive value was 67%. With regard to gene rearrangement, 11 of 13 B-cell lymphoma patients exhibited gene rearrangement (sensitivity: 85%), but 6 of 7 pseudolymphoma patients were negative for gene rearrangement (specificity: 86%). The sensitivity of ISH in B-cell lymphoma

patients was therefore 19%. Light-chain restriction was not detected by ISH in any patients in the pseudolymphoma group (specificity: 100%) (Table 2).

Discussion

Among patients with B-cell lymphoid proliferation, skin FCM is reportedly more sensitive than either immunohistochemistry (IHC) or ISH and *IgH* gene rearrangement examinations for identifying clonal B-cell populations.[5, 12, 13] In those studies, the sensitivity of IHC/ISH was 37-55%, and that of *IgH* gene rearrangement analysis was 39-43%; FCM exhibited a sensitivity of 68-100%.[5, 12, 13] The sensitivity of ISH is reportedly low because ISH only stains tumor cells that have differentiated into plasma cells, and the diagnostic value of ISH for DLBCL or follicular lymphoma in general is low,[5] consistent with the findings in our present patients.

PCR-based analysis of *IgH* gene rearrangement sometimes shows a high rate of false-negative results (up to 30%) depending on the PCR strategy used and lymphoma subtype analyzed. DNA can be irreversibly damaged during extraction from FFPE tissue sections, which negatively impacts the reliability of results.[14-16] Despite having sufficient material for PCR, two of the present DLBCL cases (patients B1 and B11) showed negative results. In contrast, the material was insufficient for analysis in all cases involving FFPE sections (patients B4, B9, and B15), although light-chain restriction was detected by FCM in some of those cases.

Our present analyses demonstrated that skin FCM enables determination of clonality at a rate comparable to examination of gene rearrangement and better than that possible with ISH, suggesting that skin FCM is useful for identifying monoclonal B-cell populations in cutaneous B-cell neoplasms.

The detection of light-chain restriction in plasma cells or B cells is suggestive of a clonal population and neoplastic process rather than an atypical reactive process.[5] However, some lymphomas do not show clonality when examined using IHC, gene rearrangement, or ISH analyses.[2-4, 8, 11, 17-19] In our present study as well, some patients did not exhibit clonality by ISH or gene rearrangement analysis but did with skin FCM (e.g., patient B1). IHC and ISH provide better detection of light-chain restriction in plasma cells and cells undergoing plasmacytic differentiation; cells not undergoing plasmacytic differentiation are not usually stained.[5] Skin FCM can be used to detect light chains on the surface of B cells even if the cells have not differentiated into plasma cells and even when the number of cells is small.[5]

Some patients did not show clonality by skin FCM, as only reactive lymphocytes are detected using this technique when no large, fragile neoplastic cells survive processing.[5, 12, 20] Technical errors introduced during the collection of cells from the skin or crushing of the skin during biopsy can also lead to unreliable results.

In conclusion, the results of our study suggest that skin FCM can be a promising diagnostic tool for identifying monoclonal neoplastic B-cell populations in cutaneous B-cell lymphomas.

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Table Captions and Figure Legends

Table 1. Characteristics of patients with B-cell lymphoma (n=16) and B-cell pseudolymphoma (n=9).

*1: Patient B10 was not fully investigated by computed tomography (CT) or fluorodeoxyglucose–positron emission tomography/CT scans.

*2: Clonality was identified in patient P5 by gene rearrangement analysis of *IgH*, but the patient was diagnosed with B-cell pseudolymphoma (he met the criteria for B-cell pseudolymphoma).

[footnote]

Pt: Patients

Diagnoses
pcDLBCL-LT: primary cutaneous diffuse large B-cell lymphoma, leg type
DLBCL-NOS: diffuse large B-cell lymphoma, not otherwise specified
pcFCL: primary cutaneous follicle center lymphoma
FL: follicular lymphoma

Outcomes
CR: complete response
PR: partial response

Treatments
BSC: best supportive care
RT: radiotherapy
R: rituximab
CHOP: cyclophosphamide, doxorubicin hydrochloride, vincristine, and prednisone
THP-COP: pirarubicin, cyclophosphamide, vincristine, and prednisolone
VP16: etoposide
HD-MTX: high-dose methotrexate
ITA: intralesional triamcinolone acetonide
NBUVB: narrow-band UVB

Examinations
FCM: flow cytometry
ISH: in situ hybridization
U.V. (light-chain restriction by skin FCM): unable to obtain value (B7: crushed material, B11: too few cells, P1: too few cells, P2: mainly T cells, P3: debris)
I.M.: insufficient material due to use of FFPE samples
N.S.: not stained

U.V. (ISH): only small numbers of tumor cells were stained; therefore, unable to obtain value

※1 Achieved CR with RT.
※2 Treated with R.
※3 Achieved CR with the same treatment.
※4 Achieved CR with topical corticosteroids.

Table 2. Summary of skin FCM, gene rearrangement, and ISH results for each group.

Fig. 1. A-F: Light-chain examination by skin FCM of patients with pcDLBCL-LT **(A)**, DLBCL-NOS **(B)**, pcFCL **(C)**, follicular lymphoma **(D)**, and pseudolymphoma **(E, F)**. *Red font* indicates the dominant light chain ($\kappa/\lambda > 5$ or $\lambda/\kappa < 3$). Gate and results of *IgH* gene rearrangement and ISH analyses are shown in the boxes.

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