Immunohistochemical Characterization of Nasal-Type Extranodal NK/T-Cell Lymphoma Using a Tissue Microarray

An Analysis of 84 Cases

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Abstract

Nasal-type extranodal natural killer (NK)/T-cell lymphoma is an uncommon malignancy. By using a tissue microarray, we characterized 84 cases of extranodal NK/T-cell lymphoma with regard to expression of 18 immunohistochemical markers and the presence of Epstein-Barr virus (EBV) RNA. In our series, CD2 was positive in 69 (93%) of 74 cases, CD3 in 68 (84%) of 81, CD5 in 22 (27%) of 81, CD20 in 0 (0%) of 82, CD29 in 75 (91%) of 82, CD30 in 29 (35%) of 84, CD43 in 81 (96%) of 84, CD54 in 58 (72%) of 81, CD56 in 46 (58%) of 79, CD62L in 23 (28%) of 83, CD183 in 66 (80%) of 83, BCL2 in 33 (39%) of 84, cutaneous lymphocyte antigen in 21 (25%) of 84, granzyme B in 70 (83%) of 84, Ki-67 in 59 (71%) of 83, linker for activation of T cells in 60 (71%) of 84, perforin in 66 (86%) of 77, TIA1 in 76 (90%) of 84, and EBV in 73 (87%) of 84. Hierarchical cluster analysis separated primary cutaneous cases from cases manifesting in other sites based on lower expression of the cell adhesion molecule CD54.

Neoplasms of natural killer (NK) cells and NK-like T cells are uncommon malignancies,[1-3] which occur with increased frequency in Asian[4-8] and Central[9,10] and South American[11,12] populations. In the current World Health Organization (WHO) classification of hematolymphoid tumors, 4 entities of probable NK/T derivation are recognized.[13] These include NK-cell large granular lymphocytic leukemia, aggressive NK-cell leukemia, nasal-type extranodal NK/T-cell lymphoma, and blastic NK-cell lymphoma. Controversy exists regarding whether malignancies placed in the last of these categories truly represent NK neoplasms or are instead derived from plasmacytoid dendritic cells.[14,15] In addition to the recognized WHO categories, malignancies showing phenotypic overlap between immature NK cells and myeloid leukemia have been described.[16-18]

Of the aforementioned entities, nasal-type NK/T-cell lymphoma is the most common and best characterized.[19] As implied by the nomenclature, this tumor shows a predilection for the upper aerodigestive tract, accounting for the historic terms *lethal midline granuloma* and *midline reticulosis.* Other sites that are commonly involved include the skin, testis, soft tissue, gastrointestinal tract, and spleen.[5,7] Tumor behavior is aggressive with a reported median overall survival ranging from 13 to 38 months.[20-22]

The neoplastic lymphocytes range from small cytologically bland cells to large cells that are frankly malignant. Most cases show a morphologic spectrum with intermediate-sized and large cells predominating. Neoplastic cells are usually admixed with an inflammatory infiltrate containing small lymphocytes, histiocytes, neutrophils, and eosinophils. An angiocentric or even angiodestructive arrangement of tumor cells is often seen. Areas of necrosis are

frequently present, complicating diagnosis on small biopsy specimens.

The classically described immunophenotype is CD56+, surface CD3–, CD3ε (epsilon chain)+, and Epstein-Barr virus (EBV)+.[23-27] As CD3ε is expressed in T cells and NK cells, immunoperoxidase staining for CD3 using a polyclonal antibody on paraffin-embedded tissue samples does not distinguish lineage.[28] Instead, molecular studies to determine if T-cell receptor genes show a germline configuration are required to assign an NK-cell lineage.[29]

Although numerous case reports and small series have been published showing the expression of various immunohistochemical markers by these malignancies, there is a paucity of studies in the literature documenting the immunologic profile of large numbers of tumors with a broad range of antibodies that are in common diagnostic use. Furthermore, as the number of commercially available reagents of diagnostic or prognostic usefulness increases, a facile method of screening new markers becomes increasingly important. For example, expression of many of the homing receptors impacts clinical outcome in various lymphoma subtypes.[30] In the present study, we used a tissue microarray (TMA) to assess expression of 19 markers in 84 cases of nasal-type NK/T-cell lymphoma.

Materials and Methods

Formalin-fixed, paraffin-embedded tissue samples from 114 cases were retrieved from the Stanford University (Stanford, CA) surgical pathology archives. Included in the study were neoplasms diagnosed as NK- or NK/T-cell lineage and hematolymphoid malignancies expressing CD56. Of the cases, 58 were originally from San Juan General Hospital, Guatemala City, Guatemala. H&E-stained sections were reviewed by 2 of us (E.S. and Y.N.) for each case in conjunction with immunohistochemical studies to confirm the original diagnosis or, where necessary, reclassify the tumor according to the WHO classification scheme.[13]

Two 600-μm cores were selected from each case using H&E and immunoperoxidase sections to maximize the number of viable tumor cells. TMAs were then manufactured using a tissue arrayer (Beecher Instruments, Silver Spring, MD) as previously described.[31] Sections of the microarray were cut at 4 μm, placed on slides, deparaffinized in xylene, and hydrated in a graded series of alcohol. Sections were stained with antibodies to CD56, CD2, CD3, CD5, CD43, linker for activation of T cells (LAT), CD20, CD30, BCL2, Ki-67, cutaneous lymphocyte antigen (CLA), CD29, CD54, CD62L, CD183, granzyme B, perforin, and TIA1. Immunohistochemical studies were performed using a modified avidin-biotin peroxidase complex amplification and detection system[32] or the EnVision+ System (DAKO, Carpinteria, CA). The antibody sources, dilutions, and epitope retrieval procedures used before incubation with the primary antibody are listed in **Table 1**. Appropriate positive and negative control experiments were performed for each antibody. In situ hybridization for EBV RNA was done on 1 section using an oligonucleotide complementary to the *EBER1* gene and an automated staining machine (Ventana Medical Systems, Tucson, AZ).

Table 1
Reagents and Conditions Used for Immunohistologic Studies*

Antibody	Clone	Vendor	Antibody Dilution	Antigen Retrieval	EnVision+
CD2	AB75	Novocastra	1:100	MW/EDTA	No
CD3	Polyclonal	DAKO	1:100	MW/citrate	Yes
CD5	4C7	Novocastra	1:100	MW/Tris	Yes
CD20	L26	DAKO	1:50	MW/Tris	Yes
CD29	7F10	Novocastra	1:20	MW/EDTA	Yes
CD30	Ber-H2	DAKO	1:80	MW/citrate	Yes
CD43	L60	BD PharMingen	1:50	MW/citrate	No
CD54	23G12	Novocastra	1:10	MW/EDTA	Yes
CD56	123C3	Zymed	1:50	MW/citrate	Yes
CD62L	9H6	Novocastra	1:25	MW/EDTA	Yes
CD183	1C6/CXCR3	BD PharMingen	1:80	MW/citrate	Yes
BCL2	124. (3).	DAKO	1:1,000	MW/Tris	Yes
CLA	HECA-452	BD PharMingen	1:20	MW/citrate	Yes
Granzyme B	GrB-7	CHEMICON International	1:40	MW/citrate	Yes
Ki-67	IVAK-2	DAKO	1:1,000	MW/Tris	Yes
LAT	Polyclonal	Santa Cruz	1:300	MW/citrate	No
Perforin	5B10	Novocastra	1:40	Heat/citrate	Yes
TIA1	2G9	Coulter	1:200	MW/citrate	Yes

CLA, cutaneous lymphocyte antigen; LAT, linker for activation of T cells; MW, microwave; Tris, tris(hydroxymethyl)aminomethane.
* Antigen retrieval involved the following: MW for 15 minutes; heat, 121°C for 5 minutes; EDTA, =1 mmol/L, pH 8.0; citrate, 10 mmol/L, pH 6.0; Tris, 0.5 mol/L, pH 10.0. Vendor locations are as follows: BD PharMingen, Franklin Lakes, NJ; CHEMICON International, Temecula, CA; Coulter, Beckmann-Coulter, Miami, FL; DAKO, Carpinteria, CA; Novocastra, Vector Laboratories, Burlingame, CA; Santa Cruz Biotechnology, Santa Cruz, CA; Zymed Laboratories, South San Francisco, CA.

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Lesional tissue was scored semiquantitatively based on the percentage of positive tumor cells as follows: negative, fewer than 10%; partial expression, 10% to 50%; and strong expression, more than 50%. For inclusion in the final numeric analysis, each case was required to show a positive result for CD56 staining or EBV in situ hybridization to ensure that lesional tissue was present on the microarray levels because these markers have been considered the most specific for NK/T-cell lymphoma; 93 cases met this criterion.

The immunohistochemical data were then converted into scoring schemes that are used in the Stanford TMA database, such that strong expression is given a score of 3, partial expression a score of 2, and negative a score of 0. A score of 1 is given to tissue that is uninterpretable, when, for example, no lesional cells are present. When discordant scores were obtained for the 2 cores of a single case, the higher score was used. The data were entered into an Excel (Excel, Microsoft, Redmond, WA) database and reformatted using TMA-Deconvoluter software (http://genome-www.stanford.edu/TMA) as previously described.[33] Hierarchical clustering and display of clustered output were generated using Cluster and Treeview software tools (http://rana.lbl.gov/EisenSoftware.htm) originally developed to analyze cDNA microarray data. Hierarchical cluster analysis groups tumors in 1 dimension based on similarity of staining pattern and, in a second dimension, groups antibodies based on the degree of relatedness in immunoreactivity across all samples examined. Strong expression was displayed as bright red, partial expression as dark red, and lack of expression as green. Data that were uninterpretable were displayed as a gray block.

A total of 5,000 digital images of all original immunostained tissue microarray cores of lymphomas used in this article are shown on the following open-access Web site: http://tma.stanford.edu/tma_portal/NK.

Results

The study included 114 cases divided into the following categories: nasal-type NK/T-cell lymphoma, 96 cases; unspecified peripheral T-cell lymphoma, 6; NK/myeloid neoplasm, 3; anaplastic large cell lymphoma, 3; aggressive NK-cell leukemia, 2; blastic NK-cell lymphoma, 2; NK-cell large granular lymphocytic leukemia, 1; and hepatosplenic T-cell lymphoma, 1. As indicated in the "Materials and Methods" section, inclusion in the final numeric analysis required that each case show a positive result for CD56 staining, EBV in situ hybridization, or both to ensure that tumor cells were present on the microarray levels. The 93 cases that met this criterion were distributed as follows: nasal-type NK/T-cell lymphoma, 84; unspecified peripheral T-cell lymphoma, 2; NK/myeloid neoplasm, 1; anaplastic large cell lymphoma, 1; aggressive NK-cell leukemia, 1; blastic NK-cell lymphoma, 2; NK-cell large granular lymphocytic leukemia, 1; and hepatosplenic T-cell lymphoma, 1.

The 84 cases of nasal-type NK/T-cell lymphoma that met criteria for inclusion showed the following site distribution: upper aerodigestive tract with or without other sites of involvement, 60; primary cutaneous, 11; gastrointestinal tract, 4; breast, 1; liver, 1; and mixed sites not involving the upper aerodigestive tract, 7. These cases were submitted from California (n = 28), other US states (n = 3), and Central America (n = 53). Patient surnames were categorized by the investigators as Asian (n = 5), Hispanic (n = 59), or other (n = 20).

Immunohistochemical stains were performed for a variety of markers, including NK- and T-cell surface antigens, molecules involved in cytotoxicity, and adhesion and homing receptors. Selected examples are illustrated in **Image 1**. The results of immunohistochemical and in situ hybridization studies are summarized in **Table 2** and **Table 3**. Hierarchical cluster analysis showed that in most cases, both cores from the same tissue sample exhibited similar staining patterns and clustered tightly.

We looked to see if cases with different sites of manifestation would cluster together based on adhesion molecule expression **Image 2**. We found that this was indeed the case, with primary cutaneous cases present predominantly in the right side of the dendrogram owing to a lack of CD54 expression. Of the 10 scorable primary cutaneous NK/T-cell lymphomas, only 3 (30%) were positive for CD54 compared with 55 (77%) of 71 cases manifesting in other sites **Table 4**. None of the primary cutaneous tumors showed strong expression of this antigen.

Discussion

Nasal-type NK/T-cell lymphoma is an uncommon neoplasm accounting for 2% to 8% of non-Hodgkin lymphomas in Asia, where this entity shows its highest prevalence.[34-36] In Europe and North America, nasal-type NK/T-cell lymphoma represents fewer than 2% of non-Hodgkin lymphomas.[34] Our goals in the present study were to construct a TMA to allow screening of antibodies on large numbers of this uncommon malignancy to better define immunologic profiles because only a few reports in the literature examine a large number (>50) of cases and to determine if expression of any immunohistochemical markers correlated with primary site of disease.

Our immunohistochemical results generally fall within the broad ranges reported in prior studies[5,12,29,35-49] **Table 5**. CD56 expression seems to be slightly lower in our series (58%). This is likely due to a lack of detection of focal staining on the limited amount of tissue available for evaluation on

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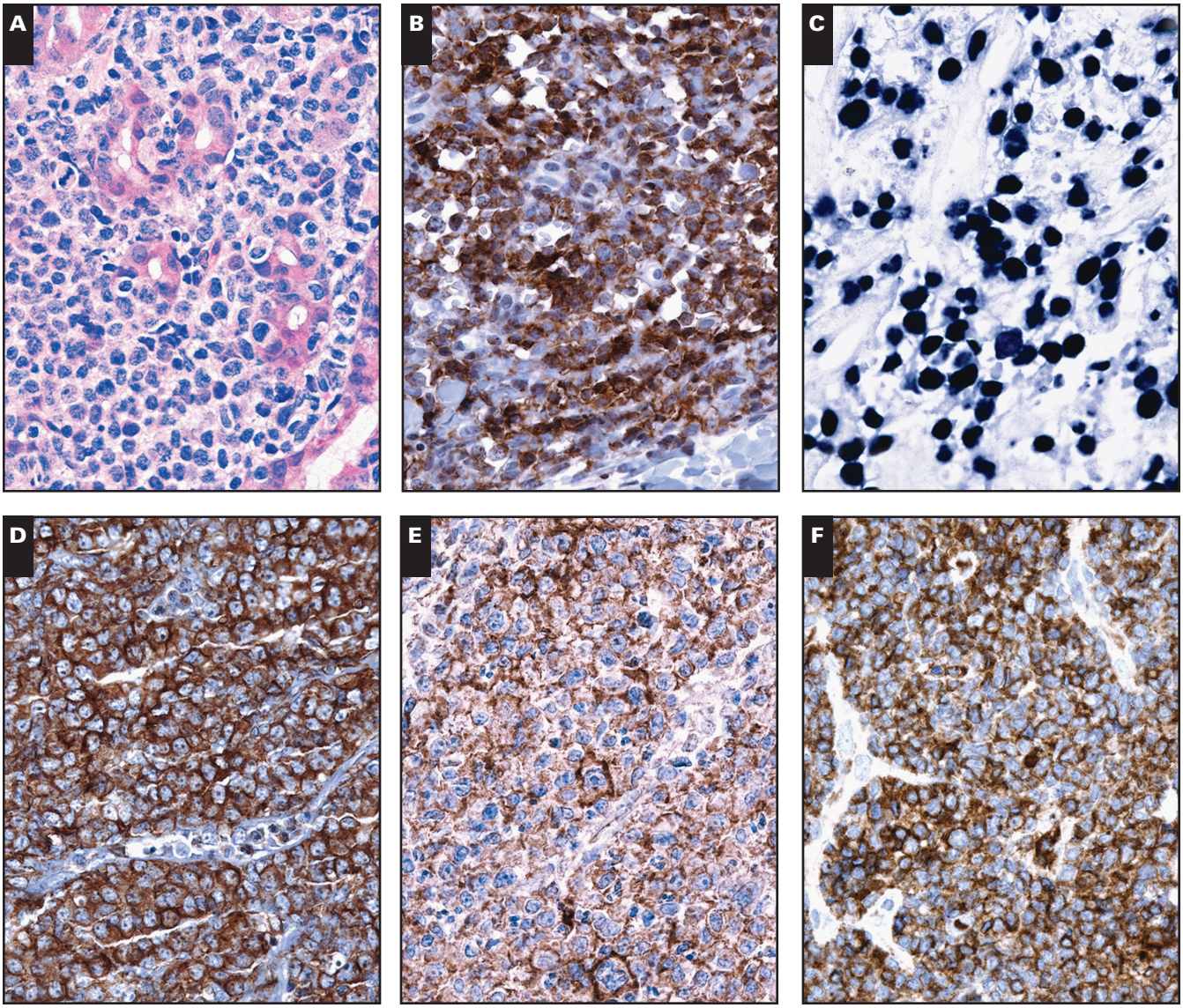

Image 1 Immunohistologic staining of selected markers in nasal-type extranodal natural killer (NK)/T-cell lymphoma. Representative images of nasal-type extranodal NK/T-cell lymphoma are shown. **A**, H&E, ×400. **B**, CD56, ×400. **C**, Epstein-Barr virus in situ, ×400. **D**, Linker for activation of T cells, ×400. **E**, CD54, ×400. **F**, CD62L, ×400.

the tissue cores. Indeed, CD56 was positive in 70 (86%) of 81 cases in which CD56 was performed when the original tissue sections were examined.

On the original tissue sections, EBV in situ hybridization was positive in 48 (79%) of 61 cases. EBV RNA was present in 87% of cases as scored on the tissue cores. Although positivity varied geographically, with 21 (68%) of 31 US cases positive vs 52 (98%) of 53 Central American cases, this difference seems to be accounted for by the presence of samples from non-Asian non-Hispanic patients in the US samples. These were positive for EBV RNA in only 11 (55%) of 20 cases. Samples from Asian and Hispanic patients were positive in 5 (100%) of 5 and 57 (97%) of 59 cases, respectively.

Among the T-cell antigens that were examined, CD2, CD3, and CD43 stained the majority of cases (93%, 84%, and 96%, respectively) and are sensitive but nonspecific markers of nasal-type NK/T-cell lymphoma. CD5 stained 27% of cases. Although normal NK cells do not express this T-lineage antigen,[50,51] it has previously been demonstrated that NK-cell neoplasms lacking T-cell receptor gene rearrangements can aberrantly express this marker.[29] Thus, as is the case with polyclonal CD3, CD5 expression alone does not define T- vs NK-cell lineage.

The recently characterized marker LAT is a transmembrane protein required for thymocyte development and T-cell signaling that is normally expressed in T cells, NK cells,

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Table 2
Immunohistologic and In Situ Hybridization Results of Lymphoma Subtypes*

Antibody	NK/T	PTL	NK/M	ALCL	NKL	BNK	NKLGL	HTL
CD2	69/74	2/2	1/1	1/1	NA	2/2	0/1	1/1
CD3	68/81	2/2	1/1	1/1	1/1	1/1	0/1	1/1
CD5	22/81	1/1	1/1	0/1	0/1	1/1	0/1	0/1
CD20	0/82	0/2	0/1	0/1	0/1	0/1	0/1	0/1
CD29	75/82	2/2	1/1	1/1	NA	1/1	1/1	1/1
CD30	29/84	0/2	0/1	1/1	1/1	0/1	0/1	0/1
CD43	81/84	2/2	1/1	1/1	1/1	1/1	1/1	0/1
CD54	58/81	1/2	1/1	1/1	1/1	0/1	1/1	0/1
CD56	46/79	2/2	0/1	0/1	NA	2/2	1/1	0/1
CD62L	23/83	0/2	1/1	0/1	1/1	1/1	0/1	0/1
CD183	66/83	2/2	1/1	1/1	1/1	1/1	1/1	1/1
BCL2	33/84	2/2	1/1	0/1	1/1	1/1	1/1	1/1
CLA	21/84	1/2	1/1	0/1	0/1	0/1	0/1	0/1
Granzyme B	70/84	1/2	1/1	0/1	1/1	0/1	1/1	1/1
LAT	60/84	2/2	1/1	1/1	1/1	1/1	1/1	1/1
Perforin	66/77	1/1	0/1	1/1	NA	1/1	1/1	0/1
TIA1	76/84	1/2	1/1	1/1	1/1	1/1	1/1	1/1
EBV in situ	73/84	0/2	1/1	1/1	1/1	0/2	0/1	1/1

ALCL, anaplastic large cell lymphoma; BNK, blastic NK cell lymphoma; CLA, cutaneous lymphocyte antigen; EBV, Epstein-Barr virus; HTL, hepatosplenic T-cell lymphoma; LAT, linker for activation of T cells; NA, not available; NK, natural killer; NK/M, NK/myeloid neoplasm; NK/T, nasal-type extranodal NK/T-cell lymphoma; NKL, aggressive NK cell leukemia; NKLGL, NK cell large granular lymphocytic leukemia; PTL, unspecified peripheral T-cell lymphoma.
* The numerator represents positive cases and the denominator, total scorable cases.

Table 3
Immunohistologic and In Situ Hybridization Results for Nasal-Type Extranodal NK/T-Cell Lymphoma*

	Score				
Antibody	0	1	2	3	Positive
CD2	5	10	21	48	69/74 (93)
CD3	13	3	30	38	68/81 (84)
CD5	59	3	21	1	22/81 (27)
CD20	82	2	0	0	0/82 (0)
CD29	7	2	55	20	75/82 (91)
CD30	55	0	18	11	29/84 (35)
CD43	3	0	19	62	81/84 (96)
CD54	23	3	55	3	58/81 (72)
CD56	33	5	31	15	46/79 (58)
CD62L	60	1	21	2	23/83 (28)
CD183	17	1	63	3	66/83 (80)
BCL2	51	0	26	7	33/84 (39)
CLA	63	0	21	0	21/84 (25)
Granzyme B	14	0	30	40	70/84 (83)
LAT	24	0	46	14	60/84 (71)
Perforin	11	7	22	44	66/77 (86)
TIA1	8	0	21	55	76/84 (90)
EBV in situ	11	0	6	67	73/84 (87)

CLA, cutaneous lymphocyte antigen; EBV, Epstein-Barr virus; LAT, linker for activation of T cells; NK, natural killer.
* Scores were assigned as follows: 0, negative; 1, uninterpretable; 2, partial expression; 3, strong expression. For positive cases, the numerator represents the positive cases and the denominator, total scorable cases with the percentage of cases positive in parentheses.

mast cells, and megakaryocytes.[38] Reactivity for LAT was present in 71% of cases in our series. Granzyme B, perforin, and TIA1 are proteins involved in target cell death induced by cytotoxic lymphocytes and are normally expressed in NK cells and cytotoxic T cells. Granzyme B is a serine protease stored in secretory lysosomes and internalized by the target cell during its conjugation with the cytotoxic lymphocyte.[52] There it activates caspase-dependent cell apoptosis. Perforin is a pore-forming protein that is also stored in cytotoxic lymphocyte secretory lysosomes and is thought to allow entry of granzymes into the target cell cytoplasm.[53] Its presence is an absolute requirement for target cell death. TIA1 is an RNA binding domain–containing protein that promotes cell apoptosis through unknown mechanisms.[54] Granzyme B, perforin, and TIA1 were present in 83%, 86%, and 90% of NK/T-cell lymphoma cases, respectively.

Expression of a number of cell adhesion and homing receptors was also examined. These molecules regulate normal

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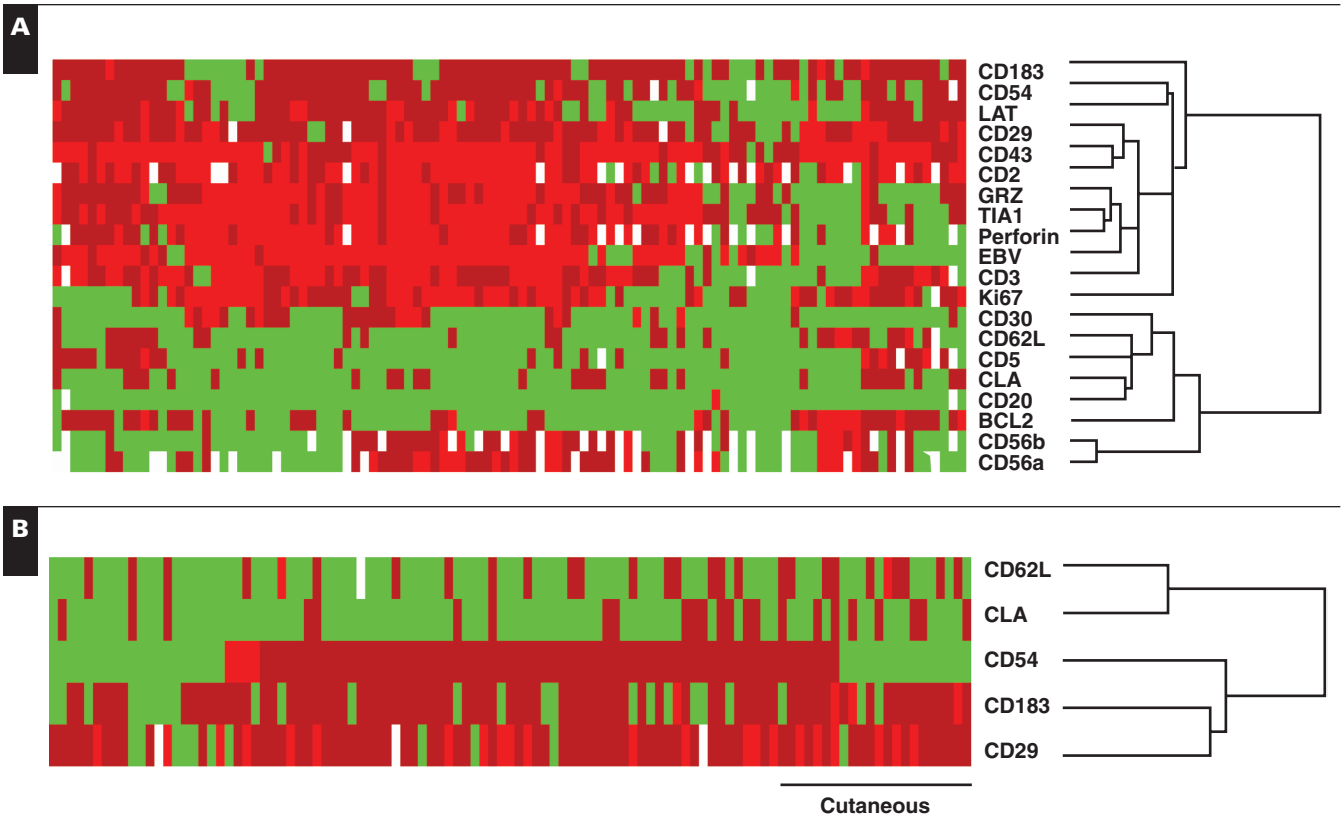

Image 2 Hierarchical cluster analysis of all natural killer (NK)/T-cell lymphomas. **A**, Immunohistologic staining data for all NK/T-cell lymphomas are analyzed by hierarchical cluster analysis and displayed as a dendrogram. The vertical axis of the dendrogram lists the NK/T-cell lymphomas. The horizontal axis of the dendrogram lists the antibodies analyzed. Antibodies that have similar staining patterns cluster next to each other. **B**, Cell adhesion molecules CD62L, cutaneous lymphocyte antigen (CLA), CD54, CD183, and CD29 are analyzed separately by hierarchical cluster analysis. Primary cutaneous cases of extranodal NK/T-cell lymphomas cluster together based on the expression of cell adhesion molecules.

Table 4
Expression of CD54 in Nasal-Type Extranodal NK/T-Cell Lymphomas by Primary Site*

	Score				
Primary Site	**0**	**1**	**2**	**3**	**Positive**
Skin	7	1	3	0	3/10 (30)
Other	16	2	52	3	55/71 (77)

NK, natural killer.
* Scores were assigned as follows : 0, negative; 1, uninterpretable; 2, partial expression; 3, strong expression. For positive cases, the numerator represents the positive cases and the denominator, total scorable cases with the percentage of cases positive in parentheses.

leukocyte trafficking and are thought to determine the tissue-specific dissemination patterns of lymphoma subtypes.[30] For example, CLA mediates homing to skin via interaction with its ligand E-selectin, which is present on skin endothelium. CLA is more often expressed in cutaneous rather than non-cutaneous T-cell lymphomas.[55,56] In nasal-type NK/T-cell lymphoma, CLA expression has been associated with a worse prognosis.[49] In our series, 25% of cases expressed this antigen.

CD54 or intercellular adhesion molecule-1 is involved with lymphocyte migration through high endothelial venules. Its expression is correlated with an angiodestructive phenotype in NK/T-cell lymphomas involving the skin.[48] Along with CD29 (β1 integrin), which is involved in homing to sites of inflammation, CD54 expression is lost in intravascular large B-cell lymphoma.[57] CD29 and CD54 were positive in 91% and 72% of nasal-type NK/T-cell lymphomas in our series, respectively.

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Table 5
Expression of Various Immunohistologic Markers in Nasal-Type Extranodal NK/T-Cell Lymphomas in the Present Series as Compared With Other Studies Reported in the Literature

Marker	Positive in Present Study (%)	Positive in Literature (%)	References
CD2	93	69-100	5, 29, 45, 48
CD3	84	56-100	5, 12, 29, 37, 40, 41-45, 47, 48
CD5	27	0-42	5, 29, 42
CD20	0	0	5, 29, 37, 41, 42, 44, 47
CD30	35	20-64	29, 40, 41, 43, 44
CD43	96	61-100	5, 29, 37, 44, 47
CD54	72	56	48
CD56	58	50-100	5, 12, 29, 40-45, 47
CD183	80	5-15	39, 46
BCL2	39	7-19	12, 19
CLA	25	56	49
Granzyme B	83	57-100	29, 41, 43, 45, 48
LAT	71	92	38
Perforin	86	36-81	29, 37, 48
TIA1	90	27-100	12, 29, 37, 40-45, 48
EBV in situ	87	13-100	5, 12, 29, 37, 40, 42-45, 47, 48

CLA, cutaneous lymphocyte antigen; EBV, Epstein-Barr virus; LAT, linker for activation of T cells; NK, natural killer.

CD62L or L-selectin is involved in homing to high endothelial venules and is expressed in nodal lymphomas.[58] Staining for CD62L was seen in 28% of cases.

The T-cell chemokine receptor CD183 or CXCR3 is a G protein–coupled serpentine receptor expressed on type 1 T-helper cells, NK cells, macrophages, and dendritic cells.[59,60] Binding of CD183 by one of the several CXC chemokines with which it interacts induces chemotaxis of these immune cells to sites of inflammation. CD183 was present on 80% of NK/T-cell lymphoma cases in our series, which is a substantially higher percentage than that previously reported in the literature.[39,46] Although we used the same antibody clone as both of the previous studies, treatment conditions and dilutions were not listed in these articles. Therefore, it is unclear if differences in these factors were the source of the discrepant results.

Proliferative activity in our series as measured by Ki-67 expression varied widely with 24 cases showing fewer than 10% of cells staining, 22 cases showing 10% to 50%, and 37 cases showing more than 50%.

Hierarchical cluster analysis of our homing receptor data resulted in a segregation of primary cutaneous lymphomas from lymphomas manifesting in other sites. This separation was due to a lower rate of expression of CD54 in the cutaneous cases and was statistically significant (P = .006564; α = .01).

We constructed a TMA and analyzed 84 cases of nasal-type extranodal NK/T-cell lymphoma for expression of 18 immunohistochemical markers and EBV RNA. Hierarchical cluster analysis separated primary cutaneous cases of this uncommon malignancy from cases in other sites based on expression of the integrin CD54.

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