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AN APPROACH TO DIAGNOSIS OF T-CELL AND NK-CELL NON - HODGKIN LYMPHOMAS BY FLOW CYTOMETRIC IMMUNOPHENOTYPING ON FINE NEEDLE ASPIRATE OF LYMPH NODE

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ABSTRACT

Introduction: Lymphoid Neoplasms are defined as clonal tumors of mature and immature B cells, T cells or natural killer (NK) cells at various stages of differentiation. Because NK cells are closely related and share some immunophenotypic and functional properties with T cells, these two classes of neoplasms considered together. Distinct T-cell/NK-cell NHL subtypes have unique characteristics and often warrant individualized diagnostic and therapeutic treatment strategies. **Objective**: The aim of this study was to diagnose T-cell/NK-cell Non-Hodgkin lymphoma by Flow cytometric immunophenotyping (FCI) on fine needle aspirate (FNA) of lymphnode following immunophenotypic diagnostic criteria based on expression of CD markers. **Method:** All samples were preliminary assessed by fine needle aspiration cytology (FNAC) as NHL or lymphoproliferative disorder (LPD). FCI was performed with a complete panel of antibodies (CD3, CD4, CD8, CD5, CD7, CD10, CD19, CD20, CD23, CD22, CD25, CD30, CD45, CD79a, CD79b, CD95, CD56, FMC7, CD40, CD15, Kappa, Lambda and Bcl-2) by dual color flow cytometry. FCI data were interpreted to diagnose and subclassify NHL according to WHO classification. Wherever possible the diagnoses were compared with available immunohistochemistry (IHC) histopathology reports. **Result:** During one year period (from March 2016 to February 2017) 31 cases of Non-Hodgkin lymphoma were diagnosed by FCI of which 13 (41.9%) cases were T-cell type of Non-Hodgkin lymphoma and 2 (6.5%) cases were Aggressive Natural killer cell leukemia. Among T-cell type NHL, 3(23.1%) cases were Peripheral T-cell lymphoma (NOS), 3(23.1%) cases were T-lymphoblastic lymphoma, 4(30.8%) cases were Angio-immunoblastic T-cell lymphoma, 2(15.3%) cases were Anaplastic large cell lymphoma, and 1(7.7%) case was Adult T- cell lymphoma. CD45 and both membrane and cytoplasmic CD3 were positive in all types of T-cell NHL except T-lymphoblastic lymphoma, in which membrane CD3 negative but cytoplasmic CD3 was positive. There were variation in CD4, CD8, CD5, CD7, CD10, and CD30 markers. In case of Aggressive NK-cell NHL, CD56 and BCL2 was found to be positive. Identification by FCI is 23.4% higher in T-cell lymphoma than IHC and is 27.8% higher than histopathology. No NK-cell type of Non-Hodgkin lymphoma was detected by histopathology. **Conclusion:** By histopathology, it is almost impossible to detect all subtypes specially T-cell and NK-cell type of Non-Hodgkin lymphoma but flow cytometric immunophenotyping can directly recognize the different subtypes associated with disease progression by detecting specific CD markers.

KEYWORDS: Flow cytometry, Immunophenotyping, T-cell/NK-cell Non-Hodgkin lymphomas, Fine needle aspirate.

INTRODUCTION

Lymphoma is a cancer that originates in immune system cells, called lymphocytes, according to the American Cancer Society. According to WHO Classification of Tumors of Hematopoietic and Lymphoid Tissue, Lymphoid Neoplasms are defined as clonal tumors of mature and immature B cells, T cells or natural killer (NK) cells at various stages of differentiation. Because NK cells are closely related and share some immunophenotypic and functional properties with T cells, these two classes of neoplasms considered together.[1]

T-cell lymphomas represents approximately 10% of Non-Hodgkin lymphomas.[2] According to WHO 2016, T-cell/NK-cell Non-Hodgkin lymphoma subtypes are about 29.[3] The major subtypes are - 1) Peripheral T-cell Lymphoma-NOS(25.9%) 2) Angioimmunoblastic

lymphoma(18.5%) 3) Extranodal Natural killer/T-cell lymphoma(10.4%) 4) Adult T- cell leukaemia/lymphoma(9.6%) 5) Anaplastic large cell lymphoma ALK+(6.6%) 6) Anaplastic large cell lymphoma ALK-(5.5%).[1] Various geograqphic frequencies of T-cell NHL have been documented ranging from 18.3% of NHLs diagnosed in Hong Kong to 1.5% in Vancouver, British Columbia, Canada. This may in part reflect increased exposure to pathogenic factors such as Human T-cell leukemia virus -1 (HTLV-1) and Epstein Barr virus (EBV) in Asian nations.[4] The broad spectrum of pathologic subtypes with varied clinical behavior poses a challenge to the systemic study of these diseases. Furthermore, these distinct T-cell NHL subtypes have unique characteristics and often warrant individualized diagnostic and therapeutic treatment strategies.[5]

Adult T-Cell Leukemia/Lymphoma (ATLL) is a rare and often aggressive form of T-cell lymphoma that can be found in the blood (leukemia), lymph nodes (lymphoma), skin, or multiple areas of the body. In leukemic form of ATLL Flow cytometric immunophenotyping (FCI) is critical diagnostic method. Histology from lymph node, spleen and other sites is rarely available as the diagnosis is based on blood and bone marrow FCI in case of T-cell prolymphocytic leukemia (T-PLL) and T-cell LGL leukemia. Immunohistochemistry (IHC) is critical in the the diagnosis of Anaplastic large cell lymphoma (ALCL) variants. There is an overlap between Aggressive NK-cell leukemia and extranodal NK/T-cell lymphoma, nasal type.[6] So accurate diagnosis of lymphoma is challenging and requires ancillary testing because classification of lymphoma is a little bit complex. To reduce difficulties and death, lymphoma cases should be diagnosed at initial stage. Different methods are used to diagnose lymphoma like fine needle aspiration cytology (FNAC), histopathology, Immunohistochemistry (IHC) and Immunophenotyping by flowcytometry (FCI). But FCI is a useful tool in diagnosis of lymphoma, especially in T cell lymphoma. FCI has become a widely used laboratory procedure for diagnosis and subtyping of lymphoma. It is an objective and quantitative diagnostic tool that allows quick multiparametric analysis of a very large number of cells (20,000-50,000 cells per sample) which could be obtained from small tissue sample(0.1 cm3 or even smaller).[7,8,9] Meanwhile analysis of such small sample is facilitated by applying dual and triple markers that permit in a single experiment and the detection of expression of combination of 2 or 3 antigens respectively on the same cell.[10,11] In this technique, distinct cell populations are defined by their size (forward light scatter) and granularity (side light scatter), weakly expressed surface antigens may be detected, two simultaneous hematological malignancies may be detected within the same tissue.[12] FCI can detect abnormal cell population against reactive background. Further, current techniques allow detection of intra cytoplasmic antigens. These features significantly improve the diagnostic sensitivity in lymphoma diagnosis.[13] The aim of this study was to assess the effectiveness of Flow cytometric immunophenotyping for diagnosis of T-cell /NK-cell Non -Hodgkin lymphomas on fine needle aspirate of lymph node.

PATIENTS AND METHODS

Flowcytometric immunophenotyping (FCI) was done on fine needle aspirates (FNA) of lymphnode diagnosed by fine needle aspiration cytology (FNAC) as lymphoproliferative disorders (LPD) during the period from March 2016 to February 2017 at the department of Microbiology and Immunology of Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka with approval of the institutional review board (IRB) of BSMMU.

Fluorescently Labeled Antibodies and Isotype control studies

FCI was performed on 3 lasers, 8-color Becton Dickinson FACS verse flow cytometer. Among the 3 lasers (405nm-violet laser; 488-nm blue laser; 633-nm red laser) 2 lasers (Blue laser and red laser) and 6-color was used in this study. The specific fluorescently labeled anti-human monoclonal antibodies used in this study were obtained from Abcam Biotechnology Company and Becton Dickinson (BD). Monoclonal Antibodies used for Hodgkin and Non-Hodgkin lymphoma panel were CD45-APC-H7, CD19-PECY7, CD3-PerCpCy5.5, CD20-APC-H7, CD79a-PE, CD15-FITC, CD30-APC, CD40-PerCpCy5.5, CD95-PE, CD5-APC, CD22-PerCpCy 5.5, CD23-PE, CD79b-PerCpCy5.5, Bcl-2-APC, FMC7-FITC, CD10-APC, CD25-PerCpCy5.5, CD4-PE, CD8-FITC, CD7-FITC, CD56-APC, Kappa-FITC, Lambda-PE. Defining 6-color FC tube was used in this study. Appropriate isotype control studies to determine background fluorescence were also used.

Sample Collection

Fine needle aspirates were collected from the lymph node of size >2 cm by expert pathologist. Fine needle aspiration cytology (FNAC) using Haematoxylin and Eosin (H&E) stain was made by a cytopathologist in the pathology department of BSMMU. One part of the aspirate was used to prepare smears for FNAC and the other part of the aspirate was flushed in to 500ùl phosphatebuffer solution (PBS) used for flowcytometric immunophenotyping.

Flow cytometry analysis and interpretation

Fine needle aspirate samples were processed as soon as possible mostly within 2-3 hours of collection for better result. A “stain and then lyse/wash” technique was used for processing of samples according to BD FACS Verse™ Manual 2013.

For identification of surface markers

100ùl of sample was taken in each tube to ensure approximate concentration of 10 / ml. 2 ml BD FACS lysing solution was taken in each tube, vortexed and incubated in dark at room temperature for 10-20 minutes.

Then the cells were spuned at 200-300g for 3-5 minute and supernatant fluid was discarded. Cells were washed with sheath fluid, vortexed, spuned and supernatant was discarded. Pre-titrated volume of fluorochrome antibody were added in each tube, vortexed, incubated in dark at room temperature for 10-15 minutes, washed twice with sheath fluid, vortexed, spuned and supernatant discarded. Cells were resuspended in 0.5 ml sheath fluid or PBS with 2% paraformaldehyde. Then the prepared samples were run on a precalibrated flow cytometer. For identification of intracellular markers pretreated volume of surface antibody CD45 and CD19 was added in to the tubes before adding lysing solution. After lysing, vortexing and incubating, permeabilizing solution was added and incubated in dark at room temperature.

The mature lymphocyte gating strategy included using dot plots of CD45 expression versus side scattering (SSC) and CD19 versus SSC and also a second gating strategy using forward scattering (FSC). A total of 30,000 events were acquired in target gate. Any antigen maker was considered positive if 20% or more of the cells reacted with a particular antibody. Data acquisition and analysis was done using BD FAC suite software version 1.0.3. The diagnostic criteria were used for flow cytometric immunophenotyping of lymphoma according to revised WHO classification of tumors of hematopoietic and lymphoid tissues.[3]

RESULT

Among 31 Non-Hodgkin lymphoma cases identified by FCI, 13(41.9%) cases of T-cell type and 2(6.5%) of Aggressive Natural killer cell leukemia type of Non-Hodgkin lymphoma were identified during one year period (from March 2016 to February 2017). All cases were screened for atypical lymphocytes by FNAC which suggested the cases as lymphoproliferative disorder (LPD) or non-Hodgkin lymphoma (NHL). The Age range was between 22 years to 80 years with 13 male and 2 female.

Sub types of T-cell lymphoma by Flow cytometric immunuphenotyping are depicted in Table-1 where among 13 cases of T-cell lymphoma, 3 cases (23.1%%) were Peripheral T-cell lymphoma (NOS); 3 cases (23.1%) were T-lymphoblastic lymphoma(T-lbly); 4 cases (30.8%) were Angioimmunoblastic T –cell lymphoma; 2 cases (15.3%) were Anaplastic large cell lymphoma; and 1 case (7.7%) was found to be Adult T–cell lymphoma.

Flow cytometric immunophenotyping criteria for classification of T/NK-cell NHL has been shown in Table-2. CD45 and both membrane and cytoplasmic CD3 were positive in all types of T-cell NHL except T-lymphoblastic lymphoma, in which membrane CD3 negative but cytoplasmic CD3 was positive. There were variation in CD4, CD8, CD5, CD7, CD10, and CD30 markers. In case of Aggressive NK-cell NHL, CD56 and BCL2 was found to be positive.

Figure-1 shows relationship of flowcytometric immunophenotyping (FCI) and histopathology(n=29) for T cell type of Non-Hodgkin lymphoma where by FCI T-cell type was 47.8% and by histopathology T- cell type was 20%. NK –cell type was 1(4.40%) by FCI but none was detected by histopathology.

Correlation between FCI and IHC were done on 17 available IHC results. FCI findings showed, 6 (35.2%) cases were T-cell type Non-Hodgkin lymphoma(NHL) and 1(5.9%) was NK-cell NHL. Whereas on IHC, 2 (11.8%) cases were T-cell type NHL. Identification by FCI is 23.4% higher in T-cell Non-Hodgkin lymphoma than IHC (Figure-2).

T-lymphoblastic lymphoma (T-Lbly) in this study showed strong reaction to CD45, cytoplasmicCD3, CD5, CD7 and variable reaction to surface CD3, CD4, CD8, CD10 (Figure-3).

Peripheral T cell lymphoma (NOS) showed strong positive reaction to CD45, CD3, CD4, CD5, CD7, BCL-2 and negative reaction to CD8, CD10 (Figure-4).

Angioimmunoblastic T-cell lymphoma showed strong reaction to CD45, CD3, CD5, CD7 with predominance of CD4 over CD8 and variable reaction to CD10; medium reaction to CD19, CD20, CD79a which indicate reactive B lymphocytes (Figure-5).

Anaplastic large cell lymphoma (ALCL) showed strong reaction to CD45, CD3, CD5, CD30, CD56; medium reaction to CD4, CD8, CD7, BCL-2 but negative to CD10. A CD30 expression along with one or more T-cell markers is characteristics of ALCL (Figure-6).

Aggressive Natural Killer cell Leukemia in this study showed strong reaction to CD56 and BCL-2 but negative reaction to all B and T- cell markers (Figure-7).

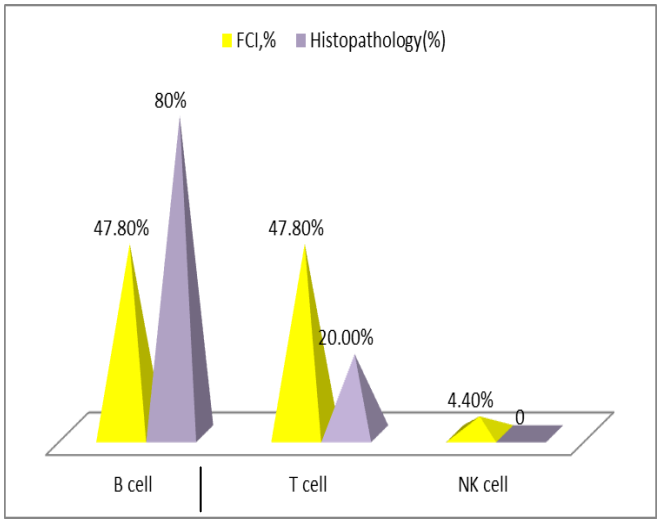

Figure 1: Relationship of flowcytometric immunophenotyping (FCI) and histopathology(n=29).

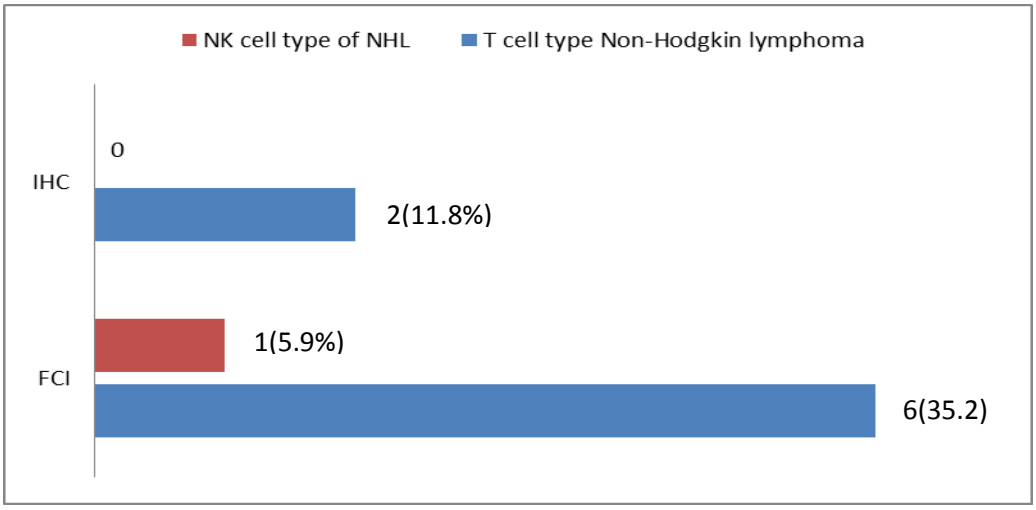

Figure 2: Correlation between findings of flowcytometric immunophenotyping(FCI) and immunohistochemistry for T-cell and NK cell type non-hodgkin lymphoma.

Table 1: Subtypes of T-cell NHL identified by Flowcytometric immunophenotyping(n=13).

Subtypes	Number	Percentage(%)
Peripheral T-cell lymphoma(NOS)	3	23.1
T-lymphoblastic lymphoma(T-lbly)	3	23.1
Angio-immunoblastic T–cell lymphoma	4	30.8
Anaplastic large cell lymphoma	2	15.3
Adult T–cell lymphoma	1	7.7

Table 2: Flow cytometric immunophenotyping criteria for classification of T/NK-cell NHL in study population.

Diagnosis	CD45	CD3	CD4	CD8	CD5	CD7	CD10	CD30	CD56	CD19	CD79b	BCL2
PTCL(NOS)	+++	+++	+++	-	+	+	-	-	-	-	-	++
AITL	+++	+++	+++	-/+	+	+	+/-	-	-	+	+	+
T-lbly	+++	**++cy,-mem**	-/+	-/+	+	+	-/+	-	-	-	-	+/-
ALCL	+++	++	+	++	++	++	-	+	+	-	-	++
ATCL	+++	++	+	-	+	-	-	-	-	-	-	+
Aggressive NK-cell leukaemia	-	-	-	-	-	-	-	-	+++	-	-	++

Note: CyCD3=cytoplasmicCD3, memCD3=membraneCD3, PTCL(NOS):Peripheral T-cell lymphoma(Not other -wise specified), AITL: Angioimmunoblastic T-cell lymphoma, T-lbly: T- lymphoblastic lymphoma**,** ALCL: Anaplastic large cell lymphoma ATCL: Adult T-cell lymphoma.

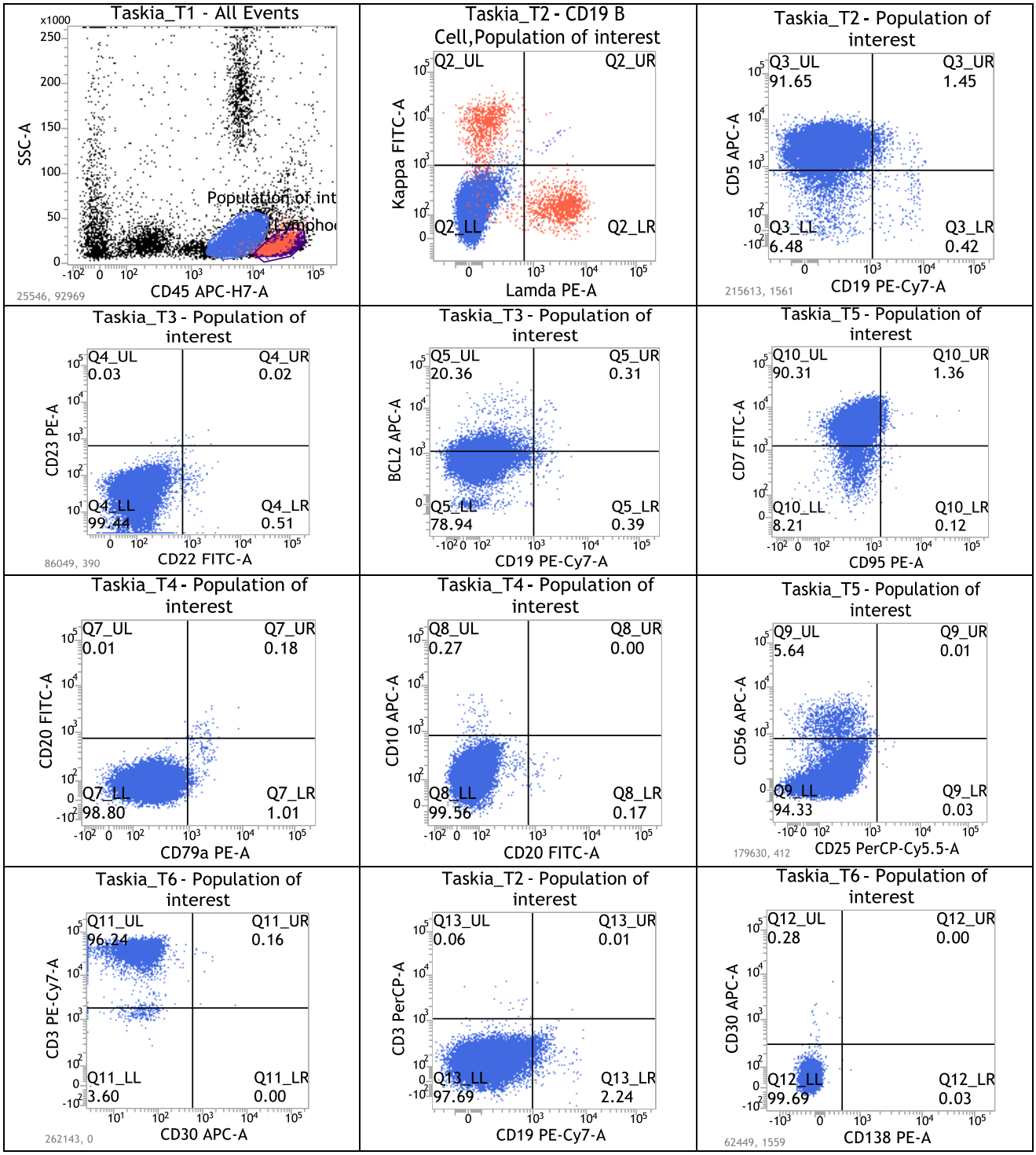

Figure 3: Flow cytometric immunophenotypic findings in a patient with T-lymphoblastic lymphoma (T-Lbly).

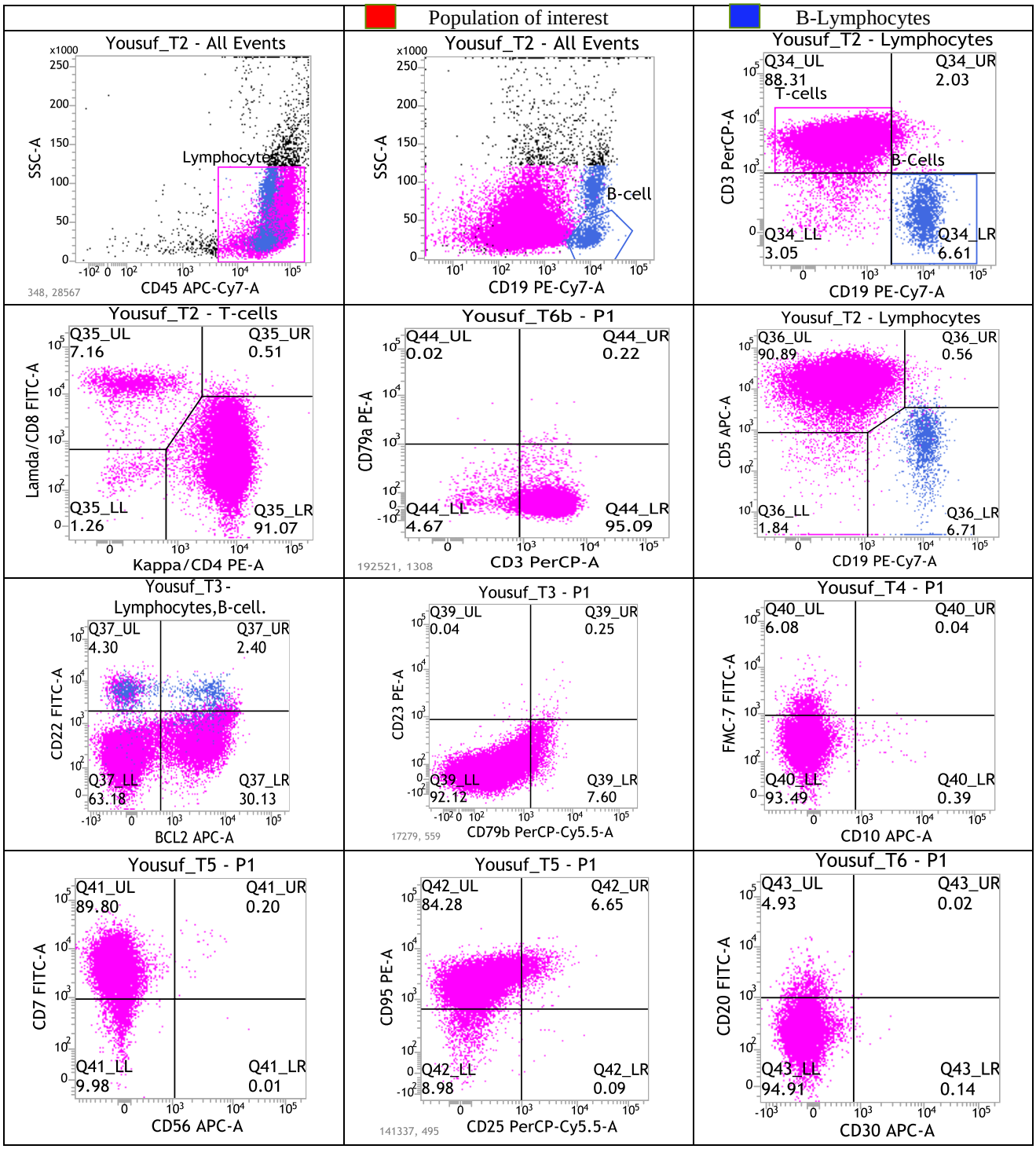

Figure 4: Flow cytometric immunophenotypic findings in a patient with Peripheral T-cell lymphoma (NOS).

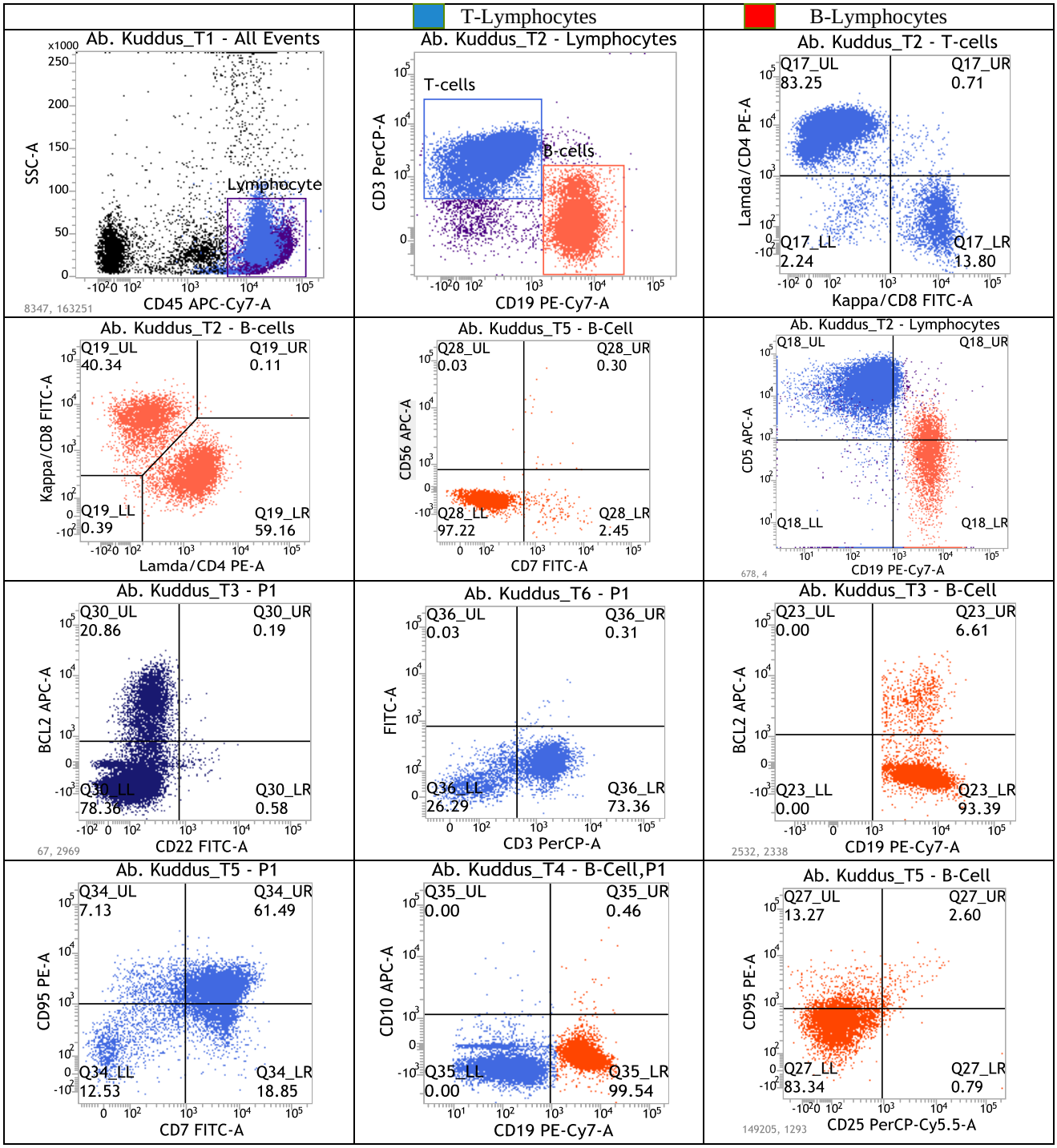

Figure 5: Flowcytometric immunophentypic findings in a patient with Angioimmunoblastic T- cell lymphoma.

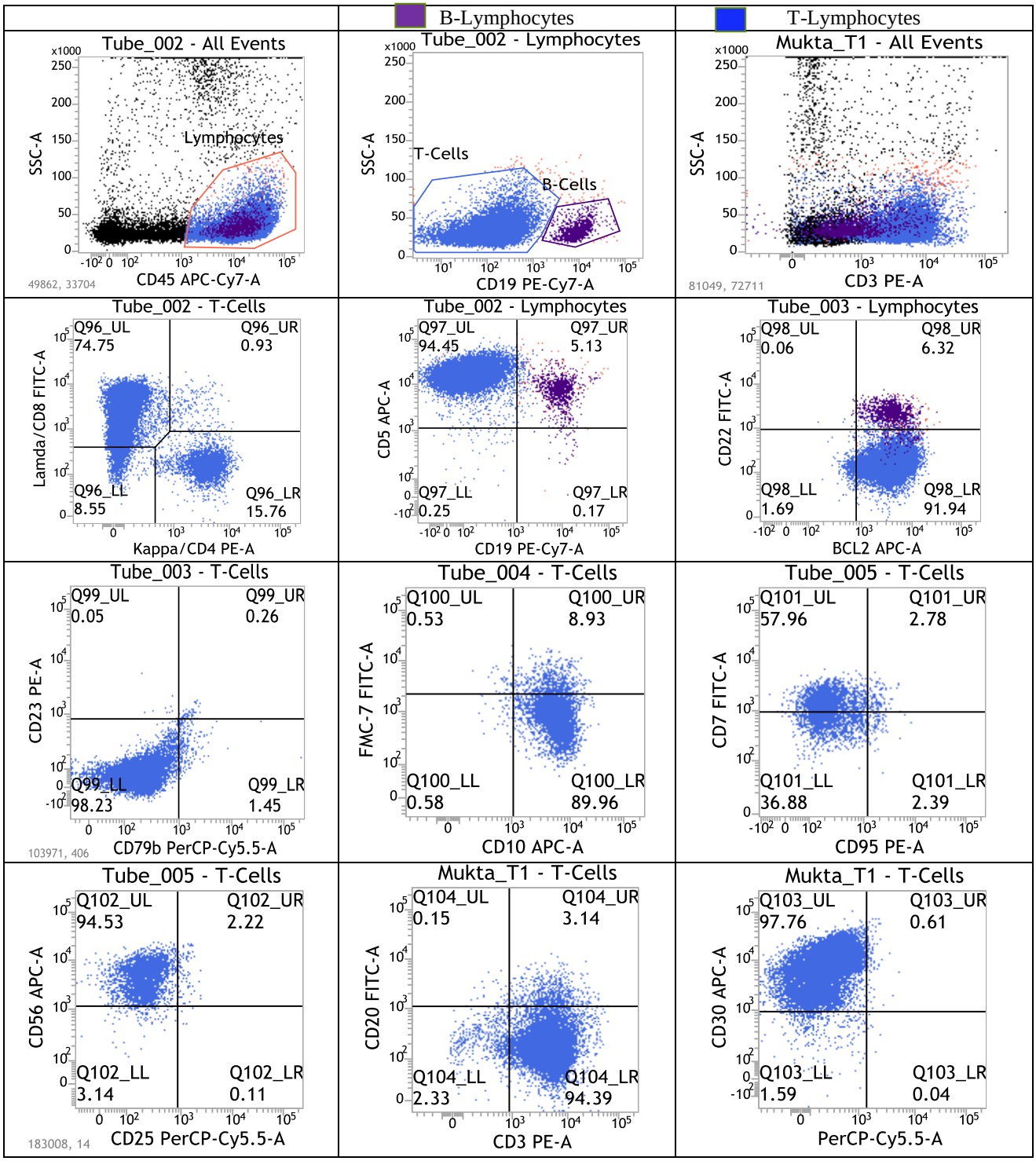

Figure 6: Flow cytometric immunophenotypic findings in a patient with Anaplastic large cell lymphoma (ALCL).

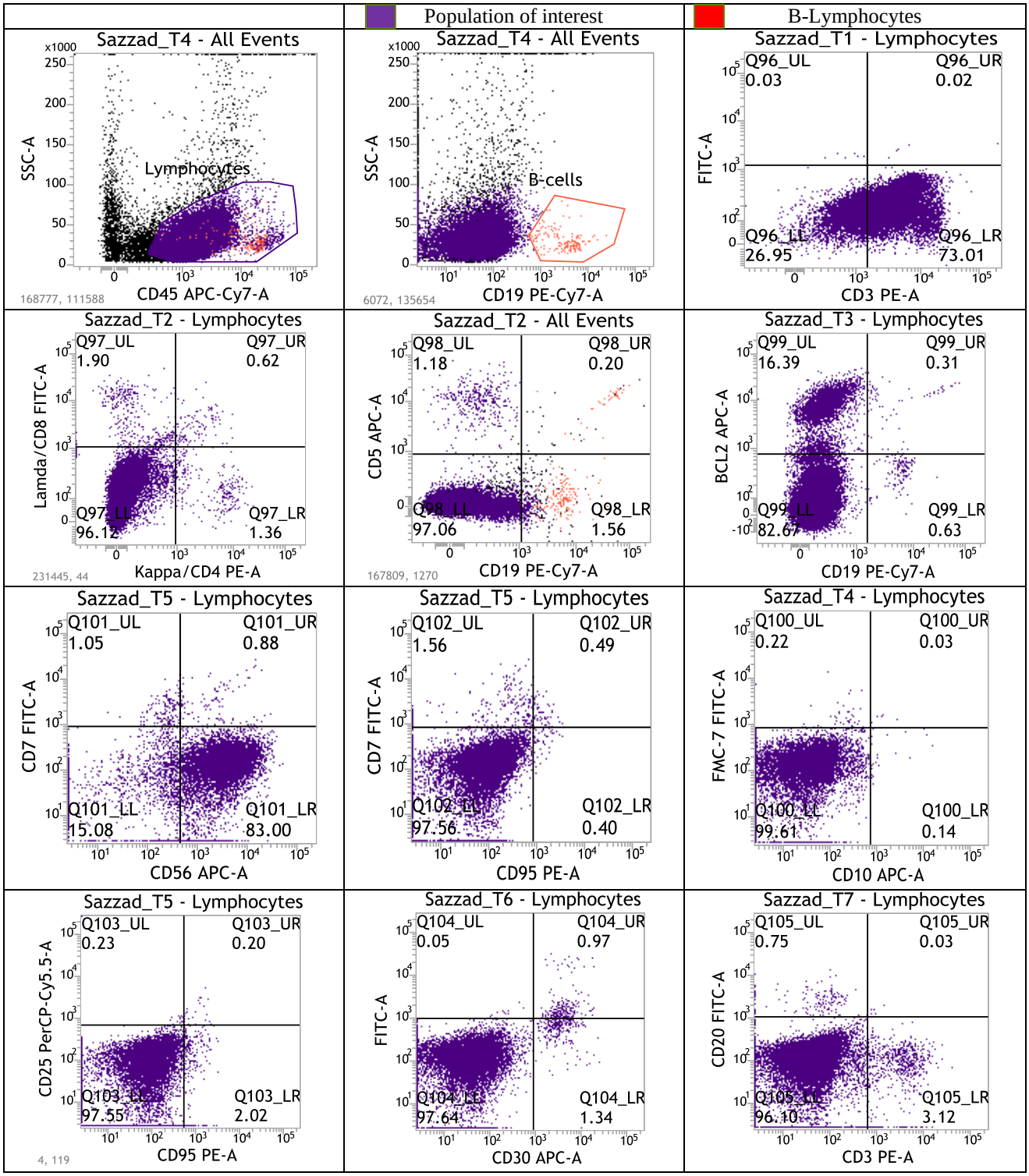

Figure 7: Flow cytometric immunophenotypic findings in a patient with Aggressive Natural Killer (NK) cell Leukemia.

DISCUSSION

In the current study, lymphoma of T-cell origin was 41.9% and Aggressive Natural killer cell leukaemia was 6.5% by flow cytometry immunophenotyping. A study carried out in India reported 34.5% T-cell lymphomas which has a close similarity with ours.[14] In this study, among the 13 cases of T-cell lymphoma, 4(30.8%) cases were Angioimmunoblastic T-cell lymphoma which had strong reaction to CD45, CD3, CD5, CD7 with predominance of CD4 over CD8 and variable reaction to CD10; medium reaction to CD19, CD20, CD79a which indicate reactive B lymphocytes. Predominance of CD4+T cells over CD8+T cells indicates that Angioimmunoblastic T-cell lymphoma is a tumor of follicular T-helper cells.[15] It is the only T-cell lymphoma which have reactive B lymphocytes. Parker et

al., 2010 revealed similar expression of CD markers in Angioimmunoblastic T-cell lymphoma.[6]

In the current study, 3(23.1%) cases were peripheral T cell lymphoma (NOS) showed strong positive reaction to CD45, CD3, CD4, CD5, CD7, BCL-2 and negative reaction to CD8, CD10. Several studies observed similar type expression of CD markers.[6,16] +Out of 13 cases, 3(23.1%) cases were diagnosed as T-lymphoblastic lymphoma (T-Lbly) in this study which showed strong reaction to CD45, cytoplasmicCD3, CD5, CD7 and variable reaction to surface CD3, CD4, CD8, CD10. Many studies reported similar type of CD markers expression in the diagnosis of T-Lbly.[6,14,17] In this study, 2(15.3%) cases were Anaplastic large cell lymphoma (ALCL) following criteria set by Mcpherson and Pincus, 2011; Parker et al., 2010.[6,15] These 2cases of ALCL showed strong reaction to CD45, CD3, CD5, CD30, CD56; medium reaction to CD4, CD8, CD7, BCL-2 but negative to CD10. A CD30 expression along with one or more T-cell markers is characteristics but association of CD56 marker in some cases can confer a poor prognosis in Anaplastic large cell lymphoma.[18] A study carried out by Juco et al. showed similar type of CD markers expression and CD30 was present in 100% cases of ALCL.[19] All these markers can only be identified by FCI but by other methods, identification of these markers are unreliable.

In the current study, 1(7.7%) case was diagnosed as Adult T-cell lymphoma according to diagnostic criteria.[15] This case showed strong reaction to CD45, CD3, CD4, CD5 but negative reaction to CD8, CD7 and CD10. A study in UK observed similar type of CD markers expression in Adult T-cell lymphoma.[6]

Out of 31 NHL cases, 2(6.5%) cases were diagnosed as Aggressive Natural Killer cell Leukemia in this study which showed strong reaction to CD56 and BCL-2 but negative reaction to all B and T- cell markers. Several studies showed similar expression of CD markers in the diagnosis of Aggressive Natural Killer cell Leukemia.[6,18]

Considering the T-cell lymphoma cases in this study their subtypes like peripheral T-cell lymphoma, T lymphoblastic lymphoma, angio-immunoblastic T-cell lymphoma, anaplastic large cell lymphoma and adult T-cell lymphoma were almost equally distributed. Study by Paul et al., 2014 in India has reported almost similar figures regarding this T-cell subtypes.[14] But Maurad et al., 2003 reported that 80% of the T-cell subtypes where T lymphoblastic lymphoma and Anaplastic large cell lymphoma and also 20% of NK cell lymphoma.[20] As this study was done in 2003 the possibility of over diagnosis could not be ruled out which may be due to less availability of monoclonal antibodies to CD markers. The above statement is further supported by the facts that Sayed et al., 2008 from Egypt reported only two variety of T-cell lymphoma.[21] The situation becomes further complicated in cases of Non-Hodgkin lymphoma subtyping because more than 60 subtypes has been identified at present. By histopathology, it is almost impossible to detect all subtypes specially T-cell and NK cell type Non-Hodgkin lymphoma. As well as the subtypes vary in disease progress. Flowcytometric immunophenotyping directly recognize the different subtypes associated with disease progression. But in case of histopathology different grades can be reported which can give idea indirectly about the variety of Non-Hodgkin lymphoma. A large number of lymphoma cases particularly T-cell subtypes initially represent as reactive process by histopathology but flowcytometric Immunophenotyping can solve this controversy by detecting specific markers.

Conflicts of interest

The authors declare no conflicts of interest regarding the publication of this article.

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