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Immunophenotype, Histopathology and Clinical Stage: Their Predictive Value in the Prognosis of non-Hodgkin's Lymphomas

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ABSTRACT

The relationship between immunophenotype, histopathology and clinical stage in influencing prognosis was evaluated in 99 cases of non-Hodgkin's lymphoma (NHL). All cases were histopathologically classified according to the system of the international conference on working formulation (WF), immunologically analysed by flow cytometry with a panel of monoclonal antibodies and clinically staged by the Ann Arbor scheme. Eighty eight percent of T- and 87.7% of B-phenotype NHLs respectively received combination chemotherapies with or without radiotherapy. Early stages I and II showed higher response rates (86% for T- and B-NHL) compared to the advanced ones III and IV (58% for T-NHL and 65% for the B-NHLs), $p<0.05$. Higher overall survival rates were observed in low and intermediate grade NHLs, $p<0.05$. The early stages further showed relatively higher survival rates in T- than in B-phenotype of intermediate grade NHL. Low grade NHL had the highest survival rates in both early and advanced stages, whereas the survival rate was the lowest in high grade NHL irrespective of the clinical stages. Immunophenotype, pathological grade and clinical stage jointly displayed varied predictive values in the prognosis of NHL. Since the present prognostic models are based on histological and staging criteria only, the results suggest that, phenotype should be included in the classification systems or prognostic models for the NHLs, thus facilitating the establishment of effective lineage specific therapies.

Key words: *Immunophenotype, Histology, Clinical stage, Prognosis of NHL*

Non-Hodgkin's Lymphomas (NHLs) are morphologically, immunologically and clinically heterogeneous diseases classifiable by the morphologic or cytological criteria employed by several systems[13,-31,38], including that of the International Conference on Working Formulation (WF)[45]. Although lymphomas are generally known to be neoplasms of the immunocompetent cells, only a few systems, such as the Lukes-Collins classification[31] satisfactorily address this concept. In addition, various immunologically well established clinicopathological entities of NHLs[19,43,46,49] are not sufficiently considered in these systems.

Clinical staging in the Ann Arbor criteria[5] which includes B-symptoms and site involved, age of the patient, therapy applied and several other factors, has been shown to influence the response and survival in NHL further to the effect of histological subtype[11,12,14-16,27,34,36,37]. However, most classification systems[13,38,45] and prognostic models[5] are based on histological or staging criteria only. Likewise, since the contribution of immunophenotype on prognosis is still not obvious, it is not considered in these systems at all. Besides this, therapy in NHL is determined by histological subtype[2-4,11,12,14,15,32,33] and clinical stage[3,25,34,44]. While histological classification still preserves its influence on the choice of therapy, staging based on the Ann Arbor criteria is being challenged by the recently proposed prognostic models[10,24,42,48], and the role of immunophenotype continues to be controversial[7,27,28,30,38,41].

In this study, the joint effect of immunophenotype, histopathological grade and clinical stage on prognosis of NHL was evaluated.

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PATIENTS AND METHODS

1. **Patient population.** Ninety nine patients histologically diagnosed as having non-Hodgkin's lymphoma were studied. All were attending the clinic of the Department of Internal Medicine (Hematology-Oncology) at Hiroshima University between 1980-1988. Patients with adult T-cell leukemia/lymphoma[46] and aggressive natural killer cell leukemia/lymphoma[19,20] were excluded because these entities are not diagnosed histologically.

2. **Histopathology.** Fresh tumor or involved lymphnode biopsy specimens obtained prior to initial therapy were used for the histological diagnosis after obtaining consent from the subjects. The results were adjusted to the classification of the WF[45].

3. **Immunophenotyping.** Fresh cells from the above specimens were used in immunofluorescence either by flow cytometry for the cells which reacted with monoclonal antibodies (MoAbs) or by fluorescence microscope in order to detect cytoplasmic immunoglobulins (cIgs)[21,23]. Spontaneous sheep erythrocyte rosette formation (E), Fc gamma and mu receptors (EA) and complement receptor (EAC) assays by phase contrast microscope[21], and an indirect cytoplasmic immunofluorescence test for detecting terminal deoxynucleotidyl transferase (TdT), were performed in 10% of the cases according to the previously described method of Imamura et al[23].

4. **Cell Separation.** Solid biopsy specimens obtained prior to therapy from consented patients, were finely minced in RPMI 1640 and filtered through a nylon mesh to prepare cell suspensions. Mononuclear cells from marrow, peripheral blood or effusion were isolated by a density gradient technique (Ficoll/sodium metrizoate $d=1.077 g/cm^3$). Cells (2×10^5) were washed twice and resuspended in the same medium ready for reaction with MoAbs.

5. **Monoclonal Antibodies.** The monoclonal antibodies (MoAbs) used in this study are shown in Table 1 according to cluster designation (CD). All were obtained either commercially or through the courtesy of Ortho Diagnostic Systems K.K. (Tokyo), Japan Scientific Instrument Co. (Tokyo), and Fujisawa Pharmaceutical Co. (Osaka).

6. **Indirect Immunofluorescence Testing.** Cells were incubated with 10μl of MoAbs for 45 min at 4°C, washed twice with RPMI 1640 to remove unbound antibodies, re-incubated with fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ fragment of sheep antimouse Ig reactive with gamma or mu (Tago, Burlingame, CA) for 30 min at 4°C, then washed again twice ready for analysis. Non-specific binding of FITC-conjugated $F(ab')_2$ fragment of antimouse Ig was checked by preincubating cells with medium or non-reactive MoAbs.

Table 1. Monoclonal antibodies used and their specificity

Cluster Designation (CD)	Monoclonal Antibodies	Distribution
CD1	T6, OKT6	Thymocyte
CD2	T11, OKT11, Leu5b	Pan-T
CD3	T3, OKT3, Leu4	Mature-T
CD4	T4, OKT4A, Leu3a	T helper/inducer
CD5	T1, OKCLL, Leu1	Pan-T
CD7	Leu9, Tp40	Pan-T
CD8	T8, OKT8, Leu2a	T supperessor/ cytotoxic
CD9	BA2	Common-ALL
CD10	J5, OKBcALLA, BA3	Common-ALL
CD11	Mo1, OKM1, Leu15	G/M suppressor NK/K (C3bi)
CD16	Leu11, OK—NK	NK/K, Granulocyte (Fcγ)
CD19	B4	Pan-B
CD20	B1	Pan-B
CD21	B2, OKB7	Mature-B (CR2)
CD24	OKB2, BA1	Pan-B
	PCA-1	Plasma cell
CD25	IL-2R1, Tac, Ta60b	Activated T & B
	Ia, OKDR	HLA-DR
CD71	OKT9	Transferrin R
CD38	OKT10	Precursor cells, Thymocytes Activated T and B

G: granulocyte M: monocyte NK: natural killer cell K: killer cell

7. **Flow cytometric analysis.** Positive cells were analysed by a fluorescence-activated cell sorter (FACS IV, Becton Dickinson, Mountain View, CA,) with a focused 488 nm Argon Laser (Spectra-Physics, Mountain View, CA). Fluorescent signals above 520 nm were collected by the analysis of 10,000 cells. Red cells were excluded by size distribution. Positive reaction was defined when the expression was observed in more than 50% of the tumor cells. Percentage of positive cells was adjusted by substracting cells with non-specific fluorescence, usually less than 1%, as described previously[17-20,22].

8. **Clinical and demographic data.** Physical, blood and bone marrow examinations were supported by chest X-ray, gallium scintillation (^{67}Ga), computed tomographic scanning or lymphography. Ann Arbor criteria[5] was used in clinical staging of the disease at presentation.

Protocols included the following agents; daunorubicin, cyclophosphamide, vincristine and prednisolone. CVP[35] and/or involved site radiotherapy (RT) was applied in all low grade and early stage (I and II) NHLs. CHOP[14,33] with or without extended

field RT was used in intermediate grade and advanced stage (III and IV) NHLs as well as large cell immunoblastic (IBL) of the high grade. N^4-behenoyl-1-D-arabinofuranosylcytosine (BHAC)[47] was included in modified CHOP protocol for the treatment of lymphoblastic lymphoma (LBL) which has a tendency of turning leukemic in its early state[32,45]. Two cases in this group had bone marrow transplantation following their first complete remission. Both died after four years, one from a relapse and the other from sepsis while in complete remission. Methotraxate was used with CHOP as CHOP-M[1,14] for the small non-cleaved cell (SNC)[1,32]. Both LBL and SNC have a poor prognosis, and have been immunologically shown to possess exclusively T- and B-phenotypes, respectively[18]. They are also grouped as high grade NHLs by the WF[45].

Complete remission (CR) referred to the disappearance of all known disease maintained for one month, and partial remission (PR) as a reduction of over 50% of the disease also maintained for one month. Restaging was carried out to confirm the CR or PR state. Distribution differences for the prognostic factors and their association with response were analysed unilaterally by Chi-square test (χ^2)[8].

Censored cases were included in the analysis of actuarial survival, which was defined as the time between date of first evaluation following induction therapy and the date of last follow-up or of recordable death. Survival curves were plotted by the method of Kaplan and Meier[26], and compared by log-rank test[9] to assess the effect of each prognostic factor.

RESULTS

1. **Histology and Phenotypic features.** Table 2 shows immunophenotypic distribution of each histopathology for the 99 cases of NHL. All subtypes of NHLs which histologically had a follicular pattern and those with SNC were exclusively B-phenotype, whereas LBL was exclusively T-phenotype. Cells of all cases defined as B-NHLs tested positive for the pan B and/or mature B-cell antigens; CD19, CD20, CD21 and CD24 respectively, including either of the sIgs and/or cIg. Both light chains κ and λ were equally common, whereas μ and γ were more common than the other heavy chains. EA and EAC assays were carried out in 10% of the cases. Cells of all cases defined as T-NHLs were positive for either pan T or mature T-cell antigens; CD2, CD3, CD5, CD7 and CD4 or CD8. E-rosette was tested in 10% of the cases. One case of small lymphocytic cell type had a strongly positive (more than 90%) E-rosette test. LBL type was positive for immature antigens, CD1 and TdT. Ia like antigens were positive in NHL cases with B-phenotype more than those with T-phenotype, whereas the reverse was true for CD38 antigen positivity in T-NHL more than in the B-NHL cases. Two cases of follicular mixed cell type (B-phenotype) were also weakly positive for several pan and mature T-cell antigens. None of these cases were positive for the anti-HTLV-1 antibody.

Table 2. Phenotype according to histopathology by the WF

Histological Subtype	Phenotype T-50	B-49
Low grade		
Small lymphocytic	1	3
Follicular small cleaved	—	4
Follicular mixed	—	6
Intermediate grade		
Follicular large	—	1
Diffuse small cleaved	10	7
Diffuse mixed	8	5
Diffuse large	16	8
High grade		
Immunoblastic	13	11
Lymphoblastic	2	—
Small non-cleaved	—	4

WF: working formulation

Table 3. Characteristics of patients by phenotype

Features	Case distribution T n	%	B n	%
	50	50.5	49	49.5
Male: Female	2:1		1.5:1	
Age-Range	12-93		27-89	
Mean	53.5		62.5	
Stage I or II	14	28.0	14	28.6
III or IV	36	72.0	35	71.4
Pathology-Low	1	2.0	13	26.5
Intermediate	34	68.0	21	42.9
High	15	30.0	14	30.6
Curative chemotherapy	44	88.0	43	87.7
+ Radiotherapy	10	20.0	5	13.8

2. **Clinical characteristics.** Table 3 shows distribution of clinical features for the NHL according to T- and B-phenotype. Ninety three percent of the cases with low grade NHL were B-phenotype. Intermediate grade NHL comprised the majority of the studied population with T-NHL having more cases than B-NHL. Cases of high grade NHL were equally distributed between those with T- and B-phenotype. T- and B-NHLs comprised of an equal number of cases having early stages (I and II) and advanced stages (III and IV), whereas cases of NHL with advanced stages were more than those with early stages in both T- and B-NHLs. Eighty eight percent of T- and 87.7% of B-NHLs received combination chemotherapy of curative intent.

3. **Response.** Table 4 shows that 75% of T- and 84% of B-NHLs achieved a CR or PR. NHL with early stages showed higher response rate in both T- and B-NHLs than those of advanced stages, $p<0.05$ by χ^2. Cases of NHL with either T- or B-

Table 4. Response results by phenotype

Features	Reponse (CR+PR) T=33 n	%	B=36 n	%
Stage-I or II	12	86.0	12	86.0
III or IV	21	58.0	24	65.0
Pathology- Low	1	100.0	6	69.0
Intermediate	22	65.0	15	71.0
High	10	66.6	11	78.6
Curative chemotherapy	33	75.0	36	84.0
+ Radiotherapy	4	12.0	5	13.8

Response rates are significantly higher in the early stages compared to advanced ones for the T- and B-NHLs ($p<0.05$)
CR: complete remission PR: partial remission

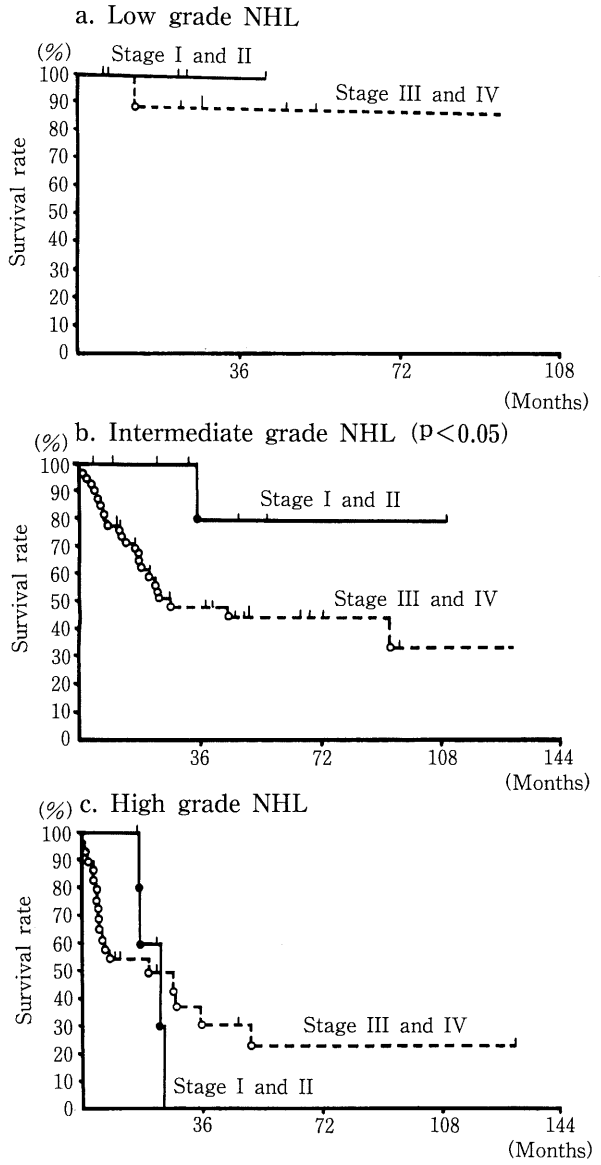

Fig. 1. Overall survival according to clinical stage.
(a) Low grade NHL.
(b) Intermediate grade NHL ($p<0.05$).
(c) High grade NHL.

phenotype in all three pathologic grades showed similarly good response rates. These response rates varied according to the clinical stage more than pathological grade or phenotype.

4. **Survival.** Fig. 1 shows the overall survival in each pathological grade of NHL according to their clinical stage at presentation. Cases having early stages showed the highest survival rate in low grade NHL (Fig. 1a), and the rate was higher in the intermediate grade NHL (Fig. 1b) where median survival was not reached ($p<0.05$ log rank). But survival rate of these cases was very low in high grade NHL (Fig. 1c) where median survival was below 36 months. Cases having advanced stages showed high survival rate in low grade NHL only.

Fig. 2, indicates the overall survival in intermediate grade (Fig. 2a) and high grade (Fig. 2b) NHL according to the immunophenotype. In intermediate grade NHL (Fig. 2a), those with T-phenotype showed a slightly higher survival rate than B-phenotype, especially after 36 months, though not significant by log rank test, whereas in high grade NHL (Fig. 2b) both cases with T- and B-phenotype showed similarly low survival rates and the median was about 24 months.

Fig. 3, shows the comparative survival between T- and B-NHL cases having early stages (Fig. 3a) and

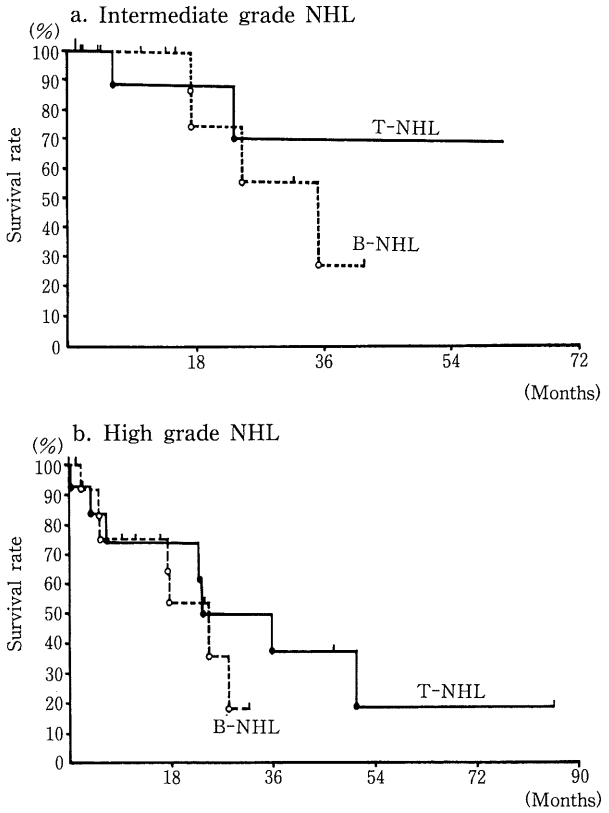

Fig. 2. Overall survival according to T- and B-phenotypes.
(a) Intermediate grade NHL.
(b) High grade NHL.

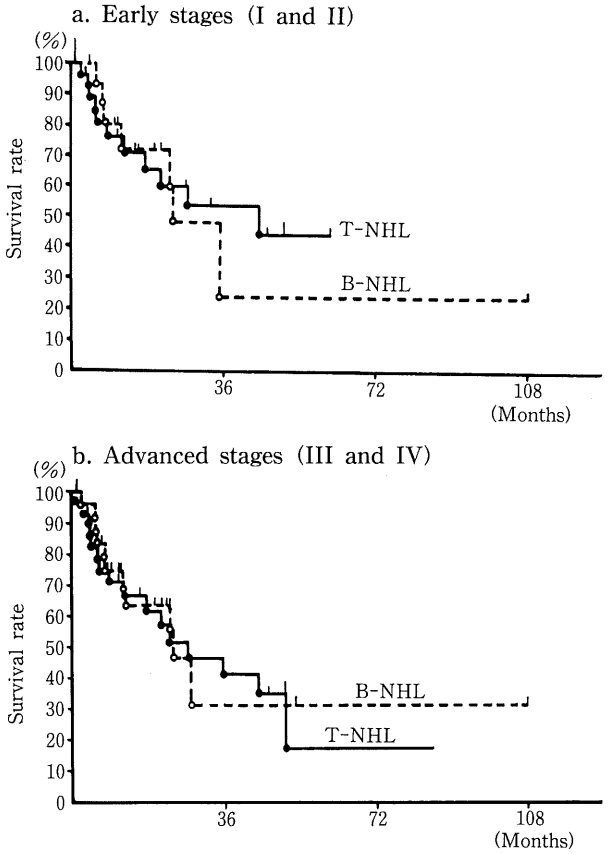

Fig. 3. Overall survival for the T- and B-phenotype in intermediate grade NHL
(a) Early stages I and II
(b) Advanced stages III and IV

those with advanced stages (Fig. 3b) of intermediate grade NHL. For the early stages, T-NHL cases in which mediam survival was being not reached showed a relatively higher survival rate than B-NHL in which the median survival was about 36 months. This difference was not significant by log rank test. In advanced stages (Fig. 3b) both T- and B-NHL cases showed low survival rate.

The results indicate that pathological grade, clinical stage and immunophenotype support each other in predicting the survival of NHL cases. Influence of immunophenotype was muffled by the broad based pathological grading in low and high grade NHL. To overcome this, evaluation of the effect of immunophenotype based on individual histological subtypes would be essential.

DISCUSSION

Findings from this study emphasize the interrelationship between immunophenotype by flow cytometry applying MoAbs, histophathological grade according to the WF and clinical stage by the Ann Arbor criteria in predicting prognosis for the NHLs.

Institutional variations in the prognosis of NHL have been documented within and outside Japan[6,27,38-41]. The classification system of the WF[45] and the staging model based on Ann Arbor criteria[5] have been applied in order to minimize the prognostic variations and facilitate comparability. Histologic subtypes according to WF, with and without phenotypic predilection are almost equally common inside and outside Japan[1,6,12,13,18,28,30,32,34,38,40,41]. Except for a few studies[6,18,27,28,30,31,38-41], immunophenotyping or the immunological concept of NHL is not taken into consideration by the most widely used classification systems[13,38] including the WF[45] or other prognostic models[5,10,24,42,48] for the NHL. Likewise, other immunologically well established entities of NHL[19,43,46,49] are not adequately represented in most of the widely used classification systems[13,38,45]. This study reviewed the role of immunophenotype in relation to the pathological grade and clinical stage of NHL.

Cases were almost equally distributed between T- and B-phenotype in all pathological grades except for low grade NHL (93% B-phenotype). Advanced stages and intermediate grade NHL comprised a majority of the cases studied. Therapy was initiated according to the histological subtype or pathological grade and clinical stage, and was similar for both T- and B-NHL except in SNC and LBL which have exclusive phenotypes. Overall cases showed good responses to initial therapy. Cases with early stages showed higher response rates than those with advanced stages, $p<0.05$. Responses were good in both pathological grades. On the other hand, cases with low grade NHL (93% B-phenotype) had the highest survival rate compared to the other two grades in both early and advanced clinical stages. Survival rate was the lowest in high grade NHL irrespective of the clinical stage. The high grade NHLs comprised SNC and LBL which have exclusive phenotypes and poor prognosis. In intermediate grade NHL survival rate was higher in the early stages, and was further shown to be higher in T-NHL than B-NHL. Early stage, particularly stage I is rare in T-NHL. Thus the above results thus suggest that: phenotype, pathological grade and initial clinical stage jointly influenced the survival of these NHL cases.

In NHL, pathological grading by the WF and clinical staging according to the Ann Arbor criteria are applied in models which predict the prognosis[5,15,16,25,34,36,45], and in determining therapeutic regimens[1-4,11,13,14,32-34,44]. The WF[45] is solely based on histomorphology whereas systems such as that of Lukes-Collins[31] use morphological or cytological criteria to reflect the immunological concept of NHL. None applies immunophenotyping. On the other hand, investigations of the clinical value of phenotype in NHL have given heterogeneous results[7,28,30,41], making it difficult to develop classification systems or determine initial therapy on the basis of immunophenotype. Recently, several studies have proposed new prognostic

models[10,24,38,42,48] to replace the Ann Arbor staging criteria. Only a few of these studies[6,27,38-40], however, included immunophenotypic analysis.

In this study, phenotypic analysis was carried out by flow cytometry applying a panel of MoAbs. Phenotypic distribution within the WF by histologic subtype or pathological grade was similar to other reports[38,41]. All cases with low grade NHL were B-phenotype, except one case of small lymphocytic cell type which was strongly positive to E-rosette test. Cases were similarly distributed between T- and B-phenotypes for each histological subtype in the intermediate and high grade NHLs, except for the follicular large cell type (B-phenotype: one case), LBL and SNC. Immunophenotype, pathological grade and clinical stage at presentation displayed an interrelationship in predicting prognosis for the NHL. Precise immunophenotyping in NHL will lead to the recognition of the varieties of NHLs[1,18-20,32,34,43,45,46] corresponding to the various categories and maturation stages of normal lymphocytes[6,18-20].

In conclusion, new classification systems based on immunophenotype are needed to encompass the various NHLs and improve the predictability of prognosis. These would be used to determine corresponding therapies which could be lineage specific for the T- and B-NHLs.

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