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Evaluation of the Role of NPM/ALK as a Marker in Lymphoid Malignancies

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ABSTRACT

Introduction: Conventional methods of NHL diagnosis are based principally on morphology and immunophenotyping, which have a significant shortage in diagnosis of large cell lymphoma. The discovery of anaplastic lymphoma kinase gene has permitted the definition of a distinct molecular genetic subtype of NHL.

Aim of the Work: Was to determine the fraction of ALK +ve lymphoma among lymphoid malignancy cases. Also to detect the relation of ALK expression to age, sex, pathologic types and staging. Correlation of ALK positivity to the overall survival was studied, to determine its significance as a prognostic marker.

Patients and Methods: This study included 114 cases of lymphoid malignancies (44 ALL and 70 NHL cases), divided according to the sampling technique: 44 cases of bone marrow aspiration samples, 20 cases of bone marrow biopsy and 50 cases of lymph node biopsy samples.

Patients of the study group were subjected to full clinical examination, complete blood count, bone marrow morphological examination (for group I and II only), cytochemistry and immunophenotyping by flowcytometer. All cases were subjected to immunostaining using the monoclonal antibody ALK-1. It recognizes an epitope in both the 80-KD NPM-ALK chimeric and 200-KD wild type ALK protein. Cytoplasmic and nuclear labeling was seen in the t (2;5) and also in other ALK variant proteins.

Results: ALK positivity was detected in 25 cases out of the 114 studied lymphoma and leukaemia cases.

ALK positivity showed a wide morphological spectrum. Positivity was more encountered in large cell lymphoma (10/47), immunoblastic (1/3), peripheral T cell (1/1), Lennert's (1/1) and lymphoblastic lymphoma (1/3).

ALK expression was positively correlated with disease stage. This correlation was statistically significant (p=0.02).

A clear finding of a significantly better survival in ALK +ve cases was found, compared to ALK -ve cases (p<0.02).

Conclusion: The definition of ALK lymphoma on the basis of ALK protein expression has the great advantage that it is more conclusive than morphological examination. The diagnosis of ALK +ve lymphoma is of great importance because of their apparent improved prognosis compared with ALK -ve cases. Immunostaining provides a mean of detecting ALK +ve cells even if few tumor cells are present, so important in detecting minimal residual disease. So, ALK protein immunohistochemistry is recommended to be included in the diagnostic workup to identify this distinct lymphoma entity.

Key Words: *NPM/ALK – Lymphoid malignancies.*

INTRODUCTION

Lymphomas are a heterogeneous group of lymphoproliferative malignancies. Their classification has evolved from a scheme utilizing purely morphologic assessment (the working formulation) to a system that attempted to integrate morphologic, immunophenotypic and genotypic data (the REAL classification) and finally to a classification that integrate all these features into clinically relevant categories of equal use to pathologists and clinicians (the WHO classification).

Anaplastic large cell lymphoma is an example, initially described as a neoplasm characterized by large pleomorphic lymphoid cells that expressed the lymphoid activation antigen, CD30. However, variants composed of smaller cells are also recognized **[1]**. Anaplastic large cell lymphoma (ALCL) harbors the reciprocal chromosomal translocation t (2;5) (p23;q35) in approximately 80% of the cases.

With the discovery of t (2;5) as a cytogenetic marker of ALCL and characterization of the implicated genes (NPM and ALK), there is now convincing literature that ALCL expressing the anaplastic lymphoma kinase (ALK) protein is considered as a distinctive group **[2]**. This group

can be called anaplastic large cell lymphoma, primary systemic form, ALK+ or given the acronym (ALKoma) **[3]**. It is a homogenous entity with the following characteristics:

- Young age, bimodal age distribution with one peak in adolescents and young adults and other in eldery patients.
- Patients with primary systemic disease, frequent expression of epithelial membrane antigen (EMA) and no association with Ebestien Barr virus (EBV) and a highly favourable prognosis **[4]**.

The genes involved which are nucleophosmin (NPM) and anaplastic lymphoma kinase (ALK) and the resulting chimeric NPM-ALK protein is thought to play a key role in the pathogenesis of t (2;5) positive ALCL **[5]**. The anaplastic lymphoma kinase (ALK) is the oncogene of most anaplastic large cell lymphomas (ALCL), driving transformation through many molecular mechanisms **[6]**.

The hybrid gene, NPM-ALK encodes the formation of 80-KD ALK protein in which 40% of N terminal portion of NPM is fused to the complete intracytoplasmic and nuclear compartment of the cell. Heterodiamers form between NPM-ALK and normal NPM, which are translocated to the nucleus **[7]**. A novel function of NPM-ALK was suggested recently. It acts through phosphorylation and activation of JNK and cJun, which may contribute to uncontrolled cell-cycle progression and oncogenesis.

The frequency of marrow involvement of ALCL is 10-17% on morphologic assessment but increases to 36% if immunostaining is performed on the marrow biopsies to highlighten the dispersed neoplastic cells among the haemopoietic cells **[8]**.

Immunostaining with the monoclonal antibody ALK-1 is a quick and efficient method as a marker for the diagnosis of ALK +ve lymphoma. Because ALK protein is normally absent in all normal tissues, ALK-1 staining represents significant advances in the detection of NPM-ALK anomaly **[9]**.

MATERIAL AND METHODS

This study included 114 cases of lymphoid malignancies, divided according to the sampling technique: 44 cases of bone marrow aspiration samples, 20 cases of bone marrow biopsy and 50 cases of lymph node biopsy samples.

Patients of the study group were subjected to full clinical examination, complete blood count, bone marrow morphological examination (for group I and II only), cytochemistry and immunophenotyping by flowcytometer. All cases were subjected to immunostaining using the monoclonal antibody ALK-1.

**Sample preparation:*

1- Bone marrow aspiration samples were subjected to staining by leishman stain for morphological examination.

2- One ml of bone marrow aspirate was collected on a preservative free heparin (50 I.U./ml blood) and processed to cytopreparation for immunostaining.

To purify leukemic cells, BM samples were mixed with an equal volume of RPMI-1640 medium and centrifuged on Ficoll Hypaque. Also mononuclear cells from the control group were separated using Ficoll Hypaque. Drops of MNCs from patients and the control group were blotted on slides.

3- Bone marrow biopsy samples were subjected to:

• *Fixation:* The obtained core biopsies were immediately preserved in 10% buffered formol-saline for at least 24 hours.

• *Decalcification:* Specimen were placed in large quantities of formic acid-sodium citrate for 48 hours, Washed in running water for 4-8 hours then transferred directly to 70% alcohol to continue dehydration, clearing and impregnation.

• *Processing:* The decalcified core was transported into a wire-mesh basket where it was dehydrated in an ascending concentration of alcohol (70%, 80%, 90%), cleared in xylene and embedded in paraffin wax.

• *Sectioning:* By using the rotating microtom (Shandon-SA325) sections (4-5 micron thick) were obtained and transerfered on adhesive coated glass-slides using a floating warm water bath. The sections were then dried in a hot air oven (60ºC for 1 hour).

4- Lymph node biopsy samples were subjected to paraffin impregnation and paraffin embedding. Sectioned tissue (5µ thick) from paraffin embedded blocks were collected on clean glass slides. For increased adhesion of tissue sections during immunohistochemistry (IHC) staining procedures, poly-L-Lysine coated slides were used.

**Immunoperoxidase:* Staining with monoclonal antibody against ALK-1 (DAKO Envision TM system, Peroxidase (DAB) Mouse).

Before immunostaining, both bone marrow and lymph node biopsy samples were subjected to:

a- Deparaffinization and rehydration:

- Slides were placed in xylene bath for 5 min.
- Excess liquid was removed and slides placed in absolute ethanol and incubated for 5 minutes. Bath is changed and this step was repeated for another 5 minutes.
- Excess liquid was removed and slides were then placed in 90% ethanol, incubated for 5 minutes. Bath was changed and this step was repeated for another 5 minutes.
- Excess liquid was removed and slides were then placed in distilled water for 1 minute.

b- Target retrieval:

- Tissue sections were placed in citrate buffer (DAKO Target retrival solution, 10X concentrate) and heated in an autoclave at 121ºC for 9 minutes (3 min x 3 times).
- Slides were left to cool for 15 minutes and then washed thoroughly with phosphate buffered saline. All samples were then subjected to immunoperoxidase staining **[10]**.

**Interpretation of results:*

Normal lymphoid cells do not contain detectable ALK protein and a positive immunostaining reaction usually indicates that a lymphoma expresses the NPM-ALK fusion protein as a result of the t (2;5) anomaly. Positive staining pattern is characterized by the brown-stained nucleus and cytoplasm (Fig. 1) **[11]**. However, a new pattern of ALK protein expression, mixed membranous and cytoplasmic was recently reported **[1]**.

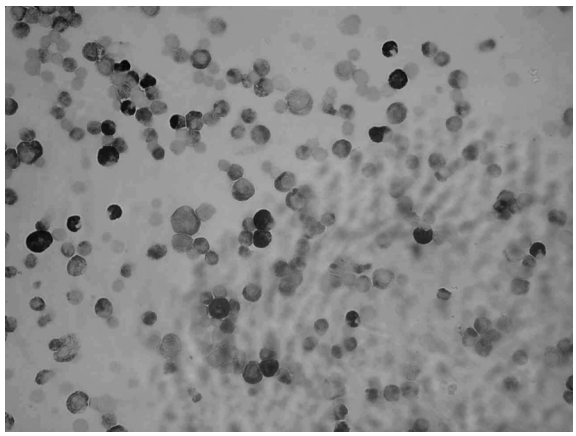

Fig. (1): A case of disseminated lymphoblastic lymphoma showing ALK positivity (by immunocytochemistry).

RESULTS

This study involved 3 groups of lymphoid malignancy cases, received at the pathology and Clinical Pathology Departments, National Cancer Institute, Cairo University. Groups were divided according to the type of sample examined:

- Group I, Bone marrow aspirate group (44 cases). Their age ranged from 1.5-65 years with a mean of 12 years. This group included 32 (72.7%) males and 12 (27.3%) females.
- Group II: Bone marrow biopsy group (20 cases). Their age ranged from 16-71 years with a mean of 49 years. This group included 16 (80%) males and 4 (20%) females.
- Group III: Lymph node biopsy group of 50 cases. Their age ranged from 15-86 years with a mean of 47 years. This group included 29 (58%) males and 21 (42%) females.
- Peripheral blood samples were taken from 10 normal individuals and used for preparation of mononuclear cells to be used as normal control.

Patients of the study group were subjected to full clinical examination, complete blood count, bone marrow morphological examination (for group I and II only), cytochemistry and immunophenotyping by flowcytometer. All lymphoma cases and the control group were subjected to immunostaining using the monoclonal antibody ALK-1. It recognizes an epitope

in both the 80-KD NPM-ALK chimeric and 200-KD wild type ALK protein. Cytoplasmic and nuclear labeling was seen in the t (2;5) and also in other ALK variant proteins.

Laboratory data of the three-studied groups are represented in Table (1).

Immunocytochemical and histochemical results:

ALK positivty was detected in 22.7% in group I (with a mean age of 8.22 years), 14.3% in group II (with a mean age of 25 years) and 24% of cases in group III (with a mean age of 39.5 years). There was no statistically significant difference between the 3 groups regarding ALK positivity (p=0.65). All the samples of the control group were negative for ALK-1.

The mean age of ALK positive cases was slightly lower than that of ALK negative cases (28.3 compared to 35.7 years respectively). However this difference was statistically insignificant (p=0.15).

ALK positivity showed a wide morphological spectrum. Positivity was encountered in large cell lymphoma (10/47), immunoblastic (1/3), peripheral T cell (1/1), Lennert's (1/1) and lymphoblastic lymphoma (1/3).

**Correlation studies of ALK positivity:*

It was correlated to:

1- Age:

Out of the 114 cases of the whole study group, the mean age of ALK positive cases was 28.3 years compared to 35.7 years in ALK negative cases with no statistically significant difference (p=0.15). ALK positivity showed age distribution from 5-20 years in the form of a plateau. Positivity was minimal between 26-44 years. In older age, ALK positivity peaked at 45 years with age distribution between 45-65 years. Positivity was decreasing between 65-80 years.

2- Haemogloin percent and platelet count:

For all groups together, the mean Hb level and platelet count were higher in ALK +ve cases compared to ALK -ve cases. However, this difference was statistically insignificant (p=0.32, 0.54 respectively).

3- Cell morphology:

ALK positivity was detected in 25 cases out of the total of 114 cases. The +ve NHL cases were non- anaplastic in morphology.

4- Bone marrow cellularity:

ALK positivty was detected in 16 out of 64 cases with normocellular marrow (25%) and in 8 out of 44 cases with hypercellular marrow (18.2%). It was detected in 1 out of 6 cases with hypocellular marrow (16.7%).

5- Immunophenotyping of the 1st group (bone marrow aspirate group):

ALK positive cases were 1/7 of C-ALL phenotype cases (14.3%), 1/7 of mature B cell phenotype cases (14.3%) and 8/30 of T cell phenotype cases (26.7%).

6- Pathologic subtypes in the 2nd group (bone marrow biopsy group):

The ALK positivity was high in large cell lymphoma (33.3%), peripheral T cell (30%) and lymphoblastic lymphoma (30%) types. This results is of borderline significance (p=0.08). No ALK positivity was detected in either small cell or mixed cell types.

7- Pathologic subtypes in the 3rd group (lymph node biopsy group):

Seventy five percent of ALK positive patients were of large cell lymphoma type (21 cases), compared to 8.3% positivity in Immunoblastic (3 cases), Lennert's lymphoma (1 case) and other undefined cases. Although highest expression in large cell lymphoma, this correlation was statistically insignificant (p=0.38).

8- Nodal and extranodal presentation in the 3rd group (lymph node biopsy group):

ALK +ve expression was detected in 25.6% of nodal cases and in 24% of the extranodal cases. This correlation was statistically insignificant (p=0.52).

9- Liver enlargement in the whole study group:

In the present study, hepatomegaly in ALK +ve lymphoma was minimal. In cases with either mild or moderate liver enlargement, ALK positivity was detected in 20% of cases. No positivity was detected in cases with massive hepatomegaly.

10- Lymphoma stage in the 3rd group (lymph node biopsy group):

Patients in stage I and II together showed ALK positivity in 12.9% of cases, while patients in stage III and IV, together showed positivity in 42.1% of cases. This higher positivity in stage III and IV was statistically significant (p=0.02).

11- Overall survival:

Out of the 114 studied cases, only 51 cases were followed up for 5 years to detect the overall survival. In positive cases, the overall survival was 91.9% (22 out of 24 positive cases) compared to 70.4% (19/27) in ALK negative cases. This difference was statistically significant (p<0.02) (Fig. 2).

Table (1): Laboratory data of the three-studied groups.

	Group I	Group II	Group III
	Immunophenotyping *C-ALL, 7 cases (15.9%) *Mature B cell, 7 cases (15.9%) *T cell 30 cases (68%)	Pathological types *Small cell, 4 cases (20%) *Large cell, 6 cases (30%) *Mixed cell, 6 cases (30%) *Peripheral T cell, 1 case (5%) *Lymphoblastic, 3 cases (15%)	Pathological types *Large cell, 41 cases (82%) *Immunoblastic, 3 cases (6%) *Mixed cell, 2 cases (4%) *Lennert's, 1 case (2%) *Other types, 3 cases (6%)
Hb gm/dl	8.29±2.3*	8.05±2.7*	10.98±1.8*
Platelet count x10^9/L	92.7±100.1*	130.6±124.5*	229.3±188.3*
BM cellularity:			
Hyper	27 (62.8%)	12 (60%)	5 (10%)
Normo	14 (32.6%)	6 (30%)	43 (86%)
Hypo	2 (4.6%)	2 (10%)	2 (4%)

*: Mean±SD.

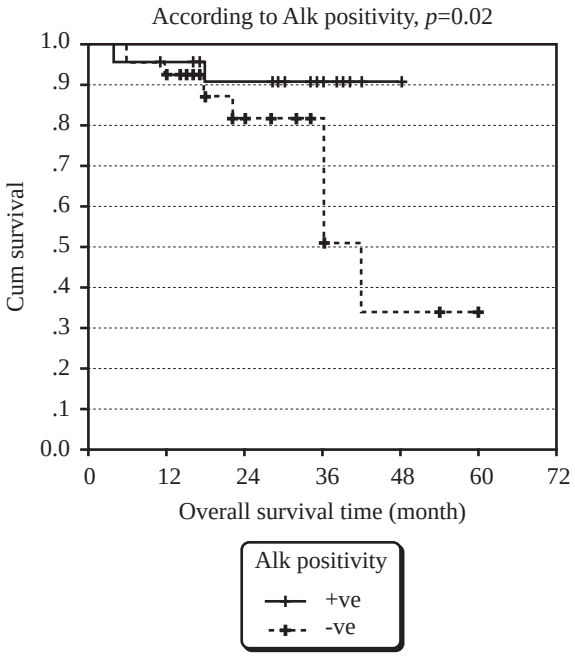

Fig. (2): The overall survival in the studied NHL patients in correlation to ALK positivity.

DISCUSSION

Acquired chromosomal anomalies (most commonly translocations) in leukemias and lymphomas usually result in either activation of a quiescent gene and expression of an intact protein product or creation of a fusion gene encoding a chimeric protein. Thus immunohistochemical detection of the products of many rearranged genes in leukemias and lymphomas can be clinically informative and provide information on cellular and subcellular protein expression that cannot be inferred from studies based on messenger RNA **[13]**.

Anaplastic large cell lymphomas (ALCL) represent a subset of lymphomas in which the anaplastic lymphoma kinase (ALK) gene is fused to several partners, most frequently to the NPM gene **[14]**. The discovery of anaplastic lymphoma kinase (ALK) gene has permitted the definition of a distinct molecular genetic subtype of NHL. Because ALK is not normally

expressed in haemopoietic cells, immunostaining of lymphoma cells with anti ALK antibodies can be used to detect ALK positive lesions. The monoclonal antibody, ALK1 specifically recognize an epitope that is present in both the NPM-ALK chimeric protein (produced by 2;5 chromosomal translocation) as well as by the full length wild type ALK protein **[15]**.

This study included 114 cases of lymphoid malignancies (44 ALL and 70 NHL cases). Cases were divided according to the sampling technique: 44 cases of bone marrow aspiration samples, 20 cases of bone marrow biopsy and 50 cases of lymph node biopsy samples. The number of the whole cases was 114 with age ranging from 1.5 to 86 years. ALL cases were classified according to the immunophenotyping and NHL cases according to REAL classification **[16]**.

In the present study, immunostaining with the monoclonal antibody, ALK1, was used to detect ALK expression in samples of the whole study group. ALK positive cases showed an age range from 2-63 years with a mean of 28.3 years which was slightly lower than that of ALK negative cases (a mean of 35.7 years). This difference was statistically insignificant. However, other researchers have stated that ALK expression occurs in younger populations when compared to lymphoma patients lacking ALK reactivity **[11,17]**.

Also ALK positivity showed age distribution from 5-20 years in the form of a plateau. Positivity was minimal between 26-44 years. In older age, ALK positivity peaked at 45 years with age distribution between 45-65 years. Positivity was decreasing between 65-80 years.

The ALK positive cases were 25 of the whole study group; none of those cases were anaplastic in morphology. These results agree with what was reported earlier that ALK expression is not only associated with large cell lymphomas that have anaplastic morphology **[17,18]**. In recent studies, it was reported that ALK-negative ALCL were found to have a high number of anaplastic cells compared with ALK-positive cases. It was suggested that the use of antibodies specific for ALK protein is a must, for the identification of the tumor entity ALK-positive from the poorly defined morphological category of anaplastic large cell lymphoma **[19,20]**. It was also suggested that immunohistochemistry is superior to molecular analysis in identification of this group, because this is a simpler test and is more sensitive: ALK immunoreactivity is seen even in cases showing variant translocations involving ALK fusion with a partner other than NPM **[4]**.

In the present study, the mean haemoglobin level in ALK +ve cases was higher than that of ALK -ve cases. However, this difference did not reach a statistically significant level. This goes with previous reports **[21]**.

In our study, ALK +ve cases showing hepatomegaly constituted 20% of ALK +ve cases. This is in contrast with previous reports which showed a lower incidence (8%) **[22]**. This increase in frequency of hepatomegaly in Egyptian cases could possibly be due to an already existing liver affection due to prevalent HCV or Bilharziasis.

The ALK positivity was more evident in cases with advanced stage of the disease (sage III & IV) when compared to those with stage I & II. This is in agreement with previous researches **[23]**.

A clear finding of a significantly better survival in ALK +ve cases was found, compared to ALK -ve cases ($p<0.02$). This prognostic difference was previously described documenting that ALK-positive ALCL has a superior outcome **[24,25]**. Recently, it was suggested that ALK-positive ALCL has a good prognosis compared to ALK-negative ALCL, possibly as a result of the immune recognition of the ALK proteins **[26]**.

Due to the selective over expression of ALK in tumour cells, its direct involvement in the process of malignant transformation and its frequent expression in ALCL patients, ALK was suggested as a suitable candidate for the development of molecularly targeted strategies for the therapeutic treatment of ALK-positive lymphomas **[6,27]**.

From this study we conclude that:

* ALK positive expression is not only associated with large cell lymphomas that have anaplastic morphology. So, ALK immunostaining should be tested in all types of lymphoma regardless the morphologic evaluation. ALK

positive lymphomas are suggested to be referred to as ALK lymphoma.

* Immunostaining provides a mean of detecting ALK +ve cells even if few tumor cells are present, so important in detecting minimal residual disease. As minimal BM disease monitoring could identify patients at risk of relapse.

* ALK expression is not detected in normal haemopoietic cells. This suggests that it should be used as diagnostic marker of malignancy as well as a good prognostic marker of different types of lymphomas.

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