Impact of CD40 Expression by Flowcytometry on Outcome of Patients with Non-Hodgkin's Lymphoma

[1]Mohamed A. Soliman, [1]Amr Ahmed Fathy, [1]Amira Alkilani, [2]Naser Abd El-Bary, [1]Fathai El-Bassal

Departments of [1]Clinical Pathology and [2]Oncology, Faculty of Medicine, Menofia University, Menofia, Egypt.

Lymphoid malignancies represent a wide variety of disease entities characterized by malignant proliferation of lymphoid cells which have distinct clinical features, cellular morphology, immunophenotype, cytogenetic changes and histologic features. CD40 is a member of the tumor necrosis factor receptor super-family. It was first identified and characterized in B cell, signaling through the CD40 receptor was found to play an important role in multiple events in T-cell dependent antibody response including B-cell survival and proliferation, memory B-cell formation and immunoglobulin isotype switching. The aim of this study is to detect the expression of CD40 on B lymphocytes in patients suffering from Non-Hodgkin's Lymphoma and correlate the results with the patients' response to treatment protocols. This study was carried out on 114 patients, of them only 100 patients completed 4 cycles of chemotherapy *and* were valuable. Their age was ranged from 17 to 63 years old. Fifteen age and gender matched individuals were, also, selected as a control group. CD40 expression was measured on peripheral blood samples by flowcytometry at patient's presentation as well as after 4 cycles of chemotherapy. This study showed that there's significant decrease in the mean values of % of CD40 on B-cell in patients with NHL in all stages when compared with normal control group. Also the study showed that there's statistical significant correlation between percent of CD40 on B-lymphocytes and stage of lymphoma i.e., the more advanced stage, the lower the % of CD40 on B-cell. After receiving a corresponding treatment, the CD40 expression is increased in significant correlation with the response to treatment. (This is a preliminary result after 4 cycles of CHOP treatment). We concluded that CD40 Lymphocyte development occurs in discrete functional steps that are defined by the onset of expression is highly expressed in healthy subjects and its expression on B-lymphocyte is decreased with advanced stage of NHL. Percent of CD40 on B-lymphocyte can be considered as an evaluation marker for outcome of treatment in NHL patients as its expression is increased in responding patients

The lymphomas are a diverse group of malignant disorders that vary with respect to their molecular features, genetics, clinical presentation, treatment approaches, and outcome (Cheson, 2004)

Lymphocyte development occurs in discrete functional steps that are defined by the onset of expression of specific molecules. Predictably many of these defining factors are required for proper development, Staging of lymphopoiesis is thus done primarily by determining the expression or lack of expression of different stage-specific molecules (Echeverri *et al.,* 2002). Monoclonal antibodies and FCM have enabled scientists and clinicians not only to easily separate B and T cells but also to define precise developmental and functional lymphocyte populations. In addition, aberrant cell types in disease states can be classified with respect to both cell type and developmental stage by use of this approach (Echeverri *et al.,* 2002).

CD40 was identified in 1985 as 45-50 KDa type 1 trans-membrane glycoprotein that belongs to the TNF receptor family with average homology of 25% (Clark and Ledbetter, 1986). CD40 is expressed by multiple cell types, in the hematopoietic cells, it is present on hematopoietic progenitors, early B cells, mature B, plasma cells, macrophage, monocytes, dendritic cells, eosinophils, basophils and T lymphocytes (Younes, 2001).It is also expressed on many

different non-hematopoietic cells such as endothelial cells, fibroblasts and epithelial cells (Van Kooten and Bancherau, 2000) as well as many other organs (Navarro *et al.,* 1997). CD40 expression is also observed on malignant hematopoietic cells including: all cases of Reed Sternberg (RS) cells of Hodgkin's disease, most mature malignant B, some early B cell acute lymphoblastic leukemia (Van Kooten and Bancherau, 2000) and T cell lymphomas(Younes and Carbone).

CD40 ligand was identified in 1992 as 32-33 KDa 261 amino acid belongs to the TNF family (Fanslow *et al.,* 1994; Gruss & Dower, 1995). Within the hematopoietic cells, CD40L is expressed on activated T, more frequently on CD4+ than CD8+ cells, but not on resting T cells (Younes, 2001). CD40/CD40L interaction plays important role in both humoral and cellular immunity (Erickson *et al.,* 2002)

As CD40 expressed during all stages of B cell development CD40/CD40L interaction exerts varying effects at different stages of B-lymphocyte maturation, differentiation and plays a pivotal role in B-cell responses (Younes, 2001). *In vitro*, CD40 ligation activates resting B cells, mediates its survival and responsiveness to other signals (as cytokines), subsequent proliferation, induction of Ig and secretion of a panel of cytokines which may act as autocrine and paracrine growth and differentiation factors of all components of the immune system (Mizuno and Rothstein, 2005). It may be possible to alter the biological behavior of malignant B cells by pharmacological agents or biological response modifiers that influence CD40 pathway either to eradicate these cells directly or to render them more vulnerable to elimination by conventional chemotherapeutic agents or by immune system (Khalidi *et al.,* 1999). Interestingly, CD40 activation of B cells also results in induction of Fas expression, and renders cells susceptible to Fas-induced apoptosis (Carbone etal.,1995) .In fact, together with B cell antigen receptor cross-linking, these three receptors (Fas, CD40 and the B cell antigen receptor) generate a complex network of positive and negative signals whereby the response of the B cell activation or death is determined by its differentiation stage(Rathmaell *et al.,* 1996)

Treatment of non Hodgkin's lymphoma (NHL) depends mainly on the histological type and stage. Many of the improvements in survival have been made using clinical trials that have attempted to improve on the best available accepted therapy. Even though standard treatment in patients with lymphomas can cure a significant fraction, numerous clinical trials that explore (Chosen *et al.,* 1999)

CHOP is still the backbone therapy for the management of intermediate and high grade NHL (compared to other combination chemotherapies that showed the same results with higher toxicities) with cure rate over 50%

The Group of d'Etude des Lymphomes de l'Adulte (GELA) was the first to prove that combination of rituximab and CHOP shows improvement in event free survival (EFS) and overall survival (OS) compared to CHOP alone (EFS 57% versus 38%, P = 0.002 and OS 70 % versus 57%, P=0.007, at 2 years) in CD20 +ve elder patients (Coiffier *et al.,* 2002). Many other studies had proved that the addition of rituximab to CHOP results in better outcome; and now CHOP-R is considered the standard treatment in the management of intermediate and high grade NHL that is CD20+ve.

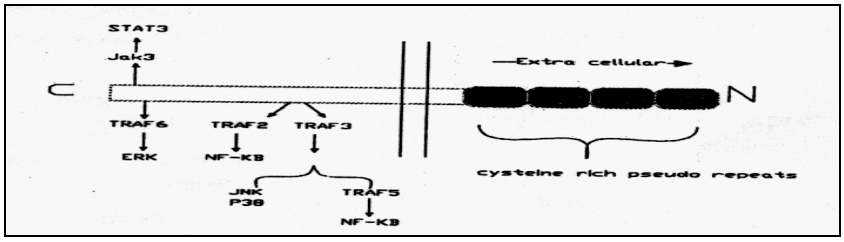

Figure 1. Structure of CD40 (Younes, 2001)

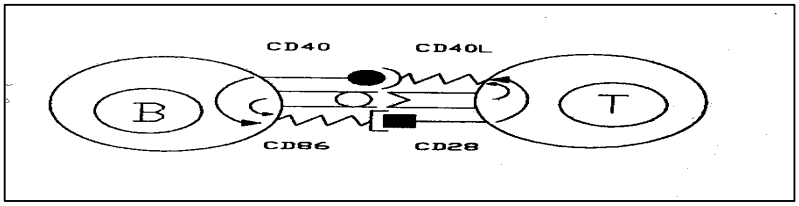

Figure 2. B, T cell interaction (Clark and Law, 1995)

Patients and Methods

The institutional review board approved this study and informed consents were obtained from all patients.

Population

These analyses were performed on peripheral blood of 100 NHL patients (46 males and 54 females) with age range from 17 to 63 years. Fifty seven patients were suffering from diffuse large cell lymphoma (DLCL), mixed small and large cell lymphoma 28%, large follicular cells 9%, and other uncommon types 6%.

Those patients were selected and followed at oncology department, Menoufyia university hospitals at the period between Jan. 2005 to Dec. 2007. Fifteen age and gender matched individuals were also selected as a control group.

All patients were subjected to the following: full history taking, clinical examination, radiological assessment including chest plain X-ray, ultrasound, and computed tomography scan (chest & abdominal), and ECG with EF.

Laboratory assessment included, routine laboratory investigations (CBC, liver function tests, renal function tests, LDH), Bone marrow biopsy, and immunophenotyping including CD40 evaluation.

Patients were divided into 4 groups according to their stages (assessed by Ann-Arbor staging system):Stage I: showed involvement of a single lymph node region or of a single extra nodal organ or site (IE); Stage II: showed involvement of two or more lymph node regions on the same side of the diaphragm, or localized involvement of an extra-nodal site or organ (IIE) and one or more lymph node regions on the same side of the diaphragm; Stage III: showed involvement of lymph node regions on both side of the diaphragm, which may also be accompanied by localized involvement of an extra nodal organ or site (IIIE) or spleen (IIIS) or both (IIISE); Stage IV: showed diffuse or disseminated involvement of one or more distant extra-nodal sites.

Methods

Besides the routine cell markers used to evaluate the NHL patients, CD40 was determined on the peripheral blood samples, using FACS caliber flowcytometer (*BD immune cytometry systems, San Jose, CA)*. The instrument was checked weekly using QC windows beads (*flowcytometry standard, San Juan, PR)*. Monoclonal antibodies are designed to quantitatively determine the percentage of cells bearing these monoclonal antibodies within a population, and quantitatively determine their density on cell surface.

Mononuclear cells separation (Biotest AG, Driesch)

- Sample staining (surface marker analysis)

For each sample, a set of tubes were labeled for all the antibodies used, and the isotopic negative control; The

panel of monoclonal antibodies used include CD5, CD10, CD19, CD20, CD22, CD23, CD79, FMC7, HLA Dr, IgM, in addition to CD40. Ten µl of the monoclonal antibodies were added in matched tube; then, 100 µl of the previously prepared suspension were transferred into tubes with gentle mix; The mix was incubated at 2 - 8° C for 30-45 minutes; Following this incubation, un-reacted monoclone was removed by washing the cells twice in 2ml (PBS) buffer. Finally, the cells were suspended in 200-400 µl of PBS for final flowcytometric analysis. One hundred µl of cells were put in a separate tube as an auto-control (R & D systems inc.).

- Flowcytometric analysis

Data were acquired on a FACS caliber flowcytometer. Forward scatter and side scatter measurements were made using linear amplifiers, where as fluorescence measurements were made with logarithmic amplifiers and flowcytometric two parameters dot plots and quadrant statistics were generated by cell quest software.

Analysis was performed after manual gating around a lymphocyte population on a forward scatter versus side scatter dot-plot. A second gate was subsequently put on the B-lymphocyte population (CD19 positive). In the gated population, the percentages of positive cells for CD40 were made by dual platform technique (Deneys *et al.,* 2001). Results were expressed as percentages of B cells positive for CD40 marker.

Statistical Methods

Data were statistically analyzed using SPSS program version 13 for windows. For all the analyses a *P*value < 0.05 was considered statistically significant; Data are shown as mean or value, and 95% confidence interval (95% CI).

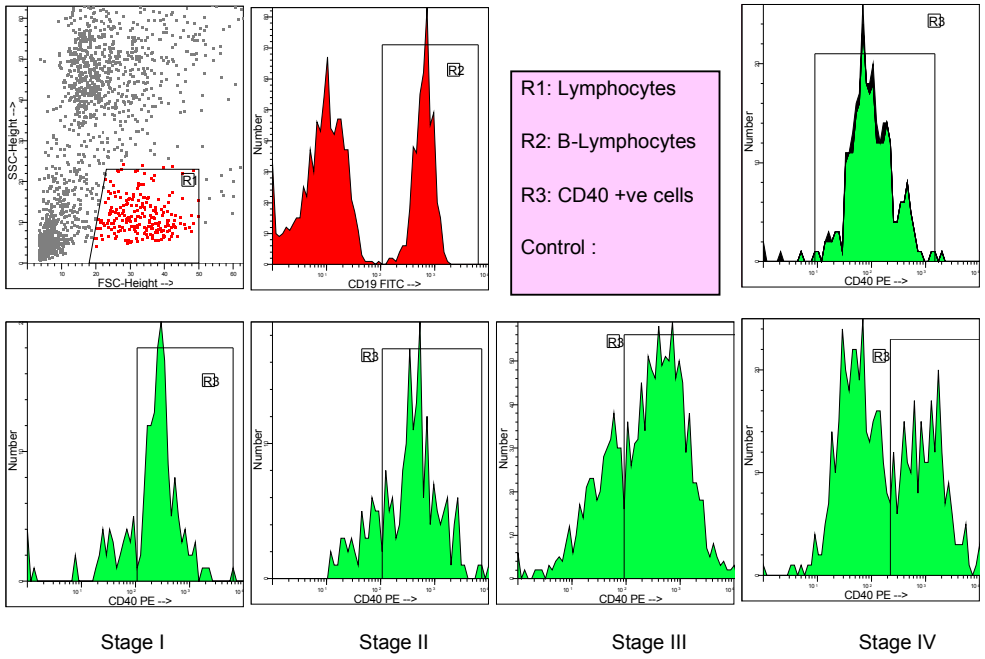

Figure 3. Flowcytomery figures show CD40 expression on B-lymphocytes in control, & patients groups

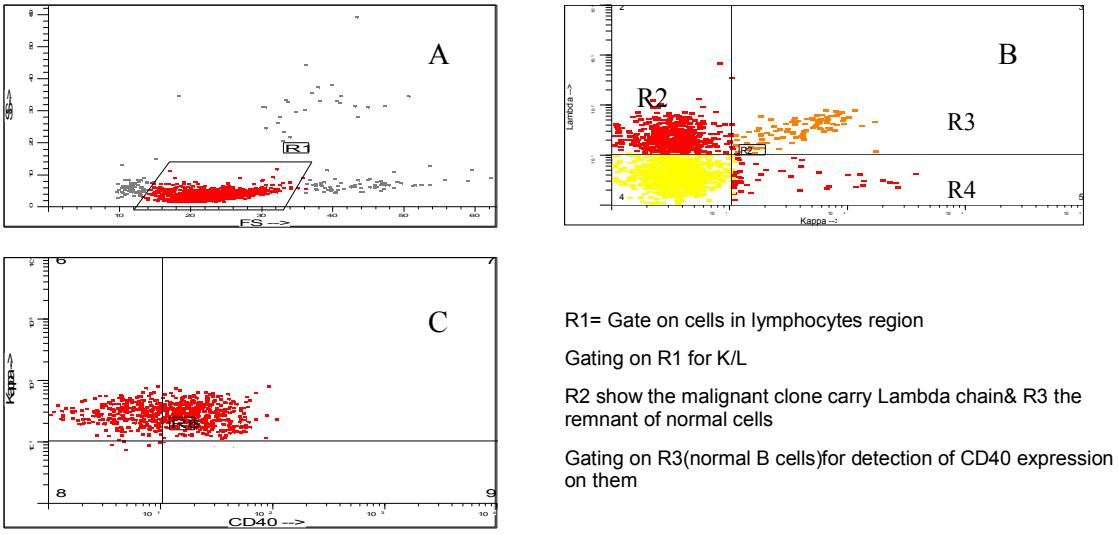

Figure 4. The gates strategy for selection of CD40 positive cells in stage IV NHL

Results

Table 1. Descriptive Table

Item	No. &/or %	Item	No. &/or %
Age		LDH	
Range	17 – 63	Normal	44 %
Mean	43	High	56 %
Gender		Bulky disease	
Male	46	Bulky	23 %
Female	54	Not Bulky	77 %
Stages		Extra-nodal sites	
I	20	Bone Marrow	19
II	24	Liver	3
III	26	Pulmonary	2
IV	30	Others	6

The expression of CD40 on B-lymphocytes shows a great variation on patients with NHL, as there is a decline of the CD40 expression with advanced stage of disease. In stage I the CD40 percent was 81.1 ± 8.2, in patients with stage II 70.1 ± 18.5, and was 66.0 ± 13.0 in stage III while in stage IV it was 54.6 ± 23.2. When the all patients in different groups compared with the normal control group (96 ± 1.6) there was a highly significant difference between the control group and patients groups.

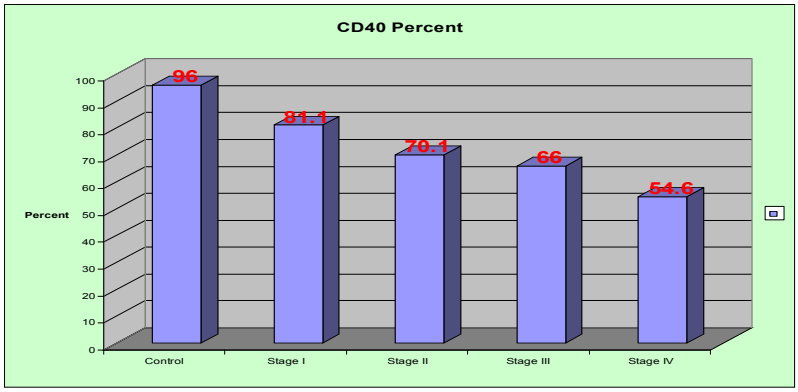

Figure 5. Differences between control & the four stages of NHL

Table (2) showed highly significant difference between the control group and the four stages pre-treatment as regards to percent of CD40 on B-lymphocytes.

Table 2. Association between the four stages of NHL and control group as regards to % of CD40 on B-lymphocytes

Variable	control	S - I	S - II	S - III	S - IV	*p*-value
	N:15	N:20	N:24	N:26	N:30	
	X±SD					
% of CD-40 on B-cells	96±1.6	81.1±8.2	70.1±18.5	66±13	54.6±23.2	*p1*<0.001 *p2*<0.001 *p3*<0.001 *p4*<0.001

P1= S - I Vs S – II., P2= S - I Vs S – III

P3= S - I Vs S – IV., P 4= S - II Vs S – III

Table (3) showed association between the four stages of NHL pre-treatment as regards to percent of CD40 on B-lymphocytes. There was significant difference as regards to percent of CD40 on B-lymphocytes between stage I & stage III and highly significant difference between stage I & stage IV; however, No significant difference was detected between the other stages.

Table 3. Association between the four stages of NHL as regards to percent of CD40 on B-lymphocytes

Variable	S - I N:20	S - II N:24	S - III N:26	S - IV N:30	*P*	Post HOC p
	X ± SD					
Percent of CD40 on B-cells	81.1±8.2	70.1±18.5	66±13	54.6±23.2	< 0.001	*p*1 > 0.05 *p*2 < 0.05 *p*3 < 0.001 *p*4 > 0.05 *p*5 > 0.05 *p*6 > 0.05

P1= S - I Vs S – II., P2= S - I Vs S – III

P3= S - I Vs S – IV., P 4= S - II Vs S – III

P 5= S - II Vs S – IV., P6= S - III Vs S – IV

Table (4) show no significant difference between pre & post treatment in stage 1 and significant difference as regards to stages II &IV and highly significant difference at stage III.

Table 4. Association between the four stages of NHL pre and post –treatment groups as regards to percent of CD40 0n B-lymphocytes

	NHL (I) N (20)		NHL (II) N (24)		NHL (III) N (26)		NHL (IV) N (30)	
	X ± SD		X ± SD		X ± SD		X ± SD	
	Before	After	Before	After	Before	After	Before	After
% of CD40 on B cells	81.10 ±8.2	85.2±5.0	70.10±18.5	81.6±7.6	66.0±13.00	75.7±8.4	54.6±23.20	66.8±19.6
P value	NS		0<.05		0<.001		<0.05	

Response to Therapy

Overall response was observed in 87% of patients, complete response (CR) was 61% while partial response (PR) was 19%. Stationary disease (SD) was detected in 13% of patients and only 7% was showed progressive disease (PD).

All patients were re-evaluated after 4 cycles of therapy as regards to CD40 expression and comparing the results to the treatment responding states.

Table 5. Patients' response to treatment

Patients	S – I	S – II	S – III	S – IV	Total No.	CD-40 expression
	N	N	N	N	N	X ± SD
CR	20	22	16	03	61	84.5% ± 7.09%
PR	/	02	06	11	19	71.3% ± 12.17%
SD	/	/	03	10	13	49.0% ± 11.86%
PD	/	/	01	06	07	39.6% ± 6.00%

Discussion

The present study spots a beam of light on impact of CD40 on B-lymphocytes in patients suffering from Non-Hodgkin's Lymphoma and correlates the results with the patient's response to treatment protocols; in addition to comparing the results of patients with control group.

In the present study, there was highly significant difference between the control group and the four stages pre-treatment as regards to percent of CD40 on B-lymphocytes; the control has the highest percent mean of 96± 1.6. This was in agreement with Deneys *et al.,* (2001) and Lan *et al.,* (2002) as they stated that the expression level of CD40 on B-cell is much lower in NHL than on normal B-cells.

In addition, this study showed a highly significant difference between the four stages of NHL pre-treatment as regards to the percent of CD40 on B-lymphocytes; the more advanced the stage, the lower would be the percent of CD40 on B-lymphocytes, and this also comes with the results of Murugaiyan *et al.,* (2007) as they stated that a low expression level of CD 40 promotes tumor growth, and the higher expression level induces tumor regression. They also found that tumor regression requires activation of T–cells, as the interaction between cell-expressed CD40-ligand and antigen presenting cell-expressed CD40 plays a crucial role in T cell activation. CD40-L- or CD40 deficient mice were susceptible to tumor growth.

As the patients received 4 cycles of CHOP regimen (±R), they were re-evaluated by repeating the clinical and laboratory investigations with focusing on impact of treatment on percent of CD40 on B-lymphocytes.

The present study showed that there was a significant correlation between the response to treatment and the level of CD40 expression on B-lymphocytes. This may be explained by the study of Zhou *et al.,* (2000) who found that agonistic anti-human CD40 monoclonal antibody (5C11) can inhibit proliferation of malignant B-cells by inducing apoptosis or arresting the development of the tumor cells at G2-M interphase.

Funakoshi *et al.,* (1994) also stated that CD40 stimulation by the antibody or soluble ligand directly inhibits human B-cell lymphoma growth. Coming in the same direction is the work of Hock *et al.,* (2006) who stated that many patients with NHL have elevated level of soluble CD40, resulting from shedding of CD-40 from B-cell surface to the patient plasma, and this elevated circulating level of soluble CD40 was associated with poor prognosis. Skibola *et al.,* (2008) also hypothesized that single nucleotide polymorphism (SNP) in TNFRSF5 encoding CD40 protein influence lymphoma risk, particularly (-1C>T, rs 1883832) SNP which is associated with reduced B-cell CD40 expression.

In conclusion, CD40 is shown to be expressed in higher percent in responding than in non responding patients, this may

denote enhanced immune modulation to fight against malignancy, this also may explain the lower expression of CD40 in advanced stages of the disease at presentation, although outcome is improving by adding rituximab to CHOP, yet more efforts are needed to define more targets to be hit as well as critical prognostic indicators. It is early (for this study) to give final conclusion as regard the role of CD40 expression in NHL, but it is the time to conclude that it has a strong prognostic role

References

1. Carbone A., Gloghini A., Zagonel V. (1995). The expression of CD26 and CD40 ligand is mutually exclusive in human T-cell non-Hodgkin's lymphomas/leukemias. Blood. 15;86(12): 4617-26
2. Cheson BD. (2004). What Is New in Lymphoma? CA Cancer J Clin, 54: 260-72.
3. Chosen BD, Horning SJ, Coiffier B. (1999). Report of an international workshop to standardize response criteria for NHL NCI responsered International working group. J Clin. Oncol.
4. Clark EA and Law CL (1995). Immune accessory molecules and signal transduction in: Williams Hematology, By: E Beutlet, M Lichtman, B Coller and T Kipps. (5th ed). McGraw-Hill. Chapter 98. P 946-955.
5. Clark EA and Ledbetter JA. (1986). Activation of human B cells mediated through two distinct cell surface differentiation antigen, Bp35 and Bp50. Proceedings of the national Academy of science of the united states of America ;83:4494-4498.
6. Coiffier B, et al. (2002). CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. New England J. Med., 346(4): 235–242.
7. Deneys V, Mazzon M, Marques L, Benoit H and Bruyere. (2001). Reference values for peripheral blood B-lymphocyte subpopulation: a basis for multiparametric immunophenotyping of abnormal lymphocytes. Journal of Immun,: 2531(2) : 23-36.
8. Echeverri C, Fisher S, King D., Craig F.E. (2002). Imunophenotypic variability of B-cell NHL. Am. J. Clin. Pathol; 117: 615.
9. Erickson LD, Durell BG, Vogel LA, O'Connor BP, Cascalho M, Yasui T, Kikutani H, Noelle RJ. (2002). Short circuiting long lived humoral immunity by the hightened engagement of CD40. J. Clin. Invest; 109: 613-20.
10. Fanslow WC, Srinivasan S, Paxton R, Gibson MG, Spriggs MK, Armitage RJ. (1994). Structural characteristics of CD40 ligand that determine biological function. Seminars immunology; 6: 276-78.
11. Funakoshi S, Longo DL, Beckwith M, Conley (1994). Inhibition of human B-cell lymphoma growth by CD40 stimulation; 83(10)2787-2794.
12. Gruss HJ, Dower SK. (1995). Tumor necrosis factor ligand superfamily: involvement in the pathology of malignant lymphomas. Blood; 85:3378-3404.
13. Hock BD, Mckenzie JL, Patton NW. (2006). Circulating levels and clinical significance of soluble CD40 in patients with hematologic malignancies; 106(10): 2148-57.
14. Khalidi H.S, Chang KX, Medeiros LJL (1999). Acute lymphoblastic leukemia. Survey of immunophenotype, French-American-British classification, frequency of myeloid antigen expression, and karyotypic abnormalities in 210 pediatric and adult cases. Am. J. din: 23 (9): 12-17.
15. Lan V, Archito T, Linda C, Yoshimura, Piao L, Nicholas T, Pamela S, Richard J. (2002). A CD40 Signalosome Anchord in lipid Rafts leads to constitutive activation of NF-KB and Autonomous cell growth in B cell lymphomas. Haematopathol. Dept. 16(1): 37-50.
16. Mizuno T, Rothstein TL. (2005). B cell receptor (BCR) cross talk: CD40 engagement enhances BCR-induced ERK activation. J. Immunol. 6:369-3376.
17. Murugaiyan G, Martin S, Saha B. (2007). Levels of CD4O expression on dendritic cells dictate tumor growth or regression; J Clin. & exp. Immunol. 149 (1) p 194-202.
18. Navarro PJ, Gauthier DC, Moulon C, Berthier O, Reano A, Gaucherand M, Banchereau J, Schmitt D. (1997). CD40 ligation of human keratinocytes inhibits their proliferation and induces their differentiation. J Immunol; 158:144-152.
19. Rathmaell JC, Townsend SE, Xui JC, Flavell RA, Goodnow CC. (1996). Expansion or elimination of B cells in vivo: dual roles for CD40 and Fas

(CD95)-ligands modulated by the B cell antigen receptor. Cell; 87:319-329.

20. Skibola CF, Nieters A, Bracci PM, Curry JD, Agana L, Skibola DR, Hubbard A,. Becker N, Smith MT, Holly EA (2008). A functional TNFRSF5 gene variant is associated with risk of lymphoma. Blood University of California USA (Epub ahead of print).

Van Kooten CB, Bancherau J. (2000). CD40-CD40 Ligand. J. Leuc. Biol. 67: 2-17.

22. Younes A and Carbone A. (1999). CD30/CD30 Ligand and CD40/CD40 Ligand in malignant lymphoid disorders. Int. J. Biol. Mark. 4:135-143.

23. Younes A. (2001). CD40 ligand (CDI54) and tumor necrosis factor-related apoptosis inducing ligand (APO-2L) in hematological malignancies. British Journal of Hematology; 113: 265-274.

24. Zhou Z, Shi Q, Wang J, Pan J. Zhang X (2000). Anti-CD40 McAb induces CD40+ malignant B cell proliferation arrest and apoptosis Immunology Department ;21(5):237-9.