# INTRODUCTION

The development of non Hodgkin lymphoma (NHL) is a complex process involving a panoplia of molecular pathways causing transformation of mature lymphoid cells ***(Patel and McMahon, 2007).*** The molecular pathogenic mechanisms underlying the complex process of lymphomagenesis in NHL have been found to be due to genetic aberrations including activation of proto-oncogenes (BCL1, BCL2, BCL6 and C-MYC), inactivation of tumor suppressor genes such as TP53 and retinoblastoma (Rb) genes and infection of the tumor clone by oncogenic viruses such as Epstein -Barr virus (EBV) and human T lymphotropic virus (HTLV)-I/II ***(Willenbrock et al., 2007; Brown and Freedman, 2009)***.

The B-cell lymphoma (BCL) family members are proto-oncogenes that seem to be important regulators of lymphocyte function, differentiation and survival ***(Sanchez-Beato et al., 2003)***.

BCL2 derives its name from B-cell lymphoma 2, as it is the second member of a range of proteins initially described in chromosomal translocation involving chromosome 14 and 18 found in approximately 90% of follicular lymphoma (FL) and 20%-30% of diffuse large B-cell lymphoma (DLBCL) ***(Cleary et al., 1986; Opferman et al., 2003; Wong and Puthalakath, 2008)***.

BCL2 is an anti-apoptotic factor that is important for normal B-cell development and differentiation ***(Vega et al., 2002; Zinkel et al, 2006)***. In addition to its well-defined role as an antagonist in apoptosis, BCL2 may act as an intracellular suppressor of cell motility and adhesion under certain conditions ***(Ke et al., 2010)***.

The BCL2 gene rearrangements especially t(14;18) (q32;q21) brings the gene under the control of immunoglobulin heavy chain gene (IgH) enhancers and leads to over-expression of BCL2 protein ***(Zinkel et al., 2006)***. BCL2 over-expression provides a survival advantage for malignant B-cells and is thought to play a critical role

in resistance to chemotherapy. As a result of these biologic functions, BCL2 over-expression is expected to be of prognostic importance in NHL. However, the prognostic significance of the t(14;18) or over expressing BCL-2 is still unclear and studies have been conflicting ***(Guo et al., 2005; Gu et al., 2009)***.

Another member of the BCL-family is BCL6 (B-cell lymphoma 6) which maps to 3q27. BCL6 protein is a protein that in humans is encoded by the BCL6 gene. This protein acts as a sequence-specific repressor of transcription with pivotal roles in B-cell activation, differentiation, inflammation and cell-cycle control ***(Ohno, 2004; Parekh et al., 2007)***. The presence of BCL6 gene rearrangements can be considered as a marker of lymphomas derived from germinal center related B-cells ***(Guo et al., 2005; Margalit et al., 2006)***.

The multiplicity of effects of BCL6 in regulating normal B- and T-cell function and survival suggests that dysregulated activity will have a marked effect on cell survival and function, with a potential to promote lymphomagenesis ***(Jardin and Sahota, 2005)***. Abnormal BCL6 expression is suggested to be a favorable and independent prognostic marker of DLBCL ***(Zhang et al., 2005)***.

Recent gene expression profiling studies have identified the genetic aberrations of BCL family as the most common type encountered in NHL reflecting either an aggressive or favourable clinical course, aiming to assign molecular targeted therapy in order to improve the overall survival and the disease free survival of lymphoma patients ***(Cottliar et al., 2006)***. The detection of cytogenetic abnormalities in NHL patients with conventional cytogenetic analysis (CCA) is technically difficult, therefore, molecular methods will be increasingly utilized and eventually required as the accepted method of diagnosis and monitoring the disease ***(Nakamura et al., 2007)***.

# AIM OF THE WORK

- Detection of 18q21 (BCL2) and 3q27 (BCL6) genetic aberrations using FISH technique applied on different samplers (BM or LN biopsy or trephine biopsy) in NHL patients.
- Evaluation of the prognostic significance of these genetic aberrations on overall survival of NHL patients.

# NON-HODGKIN LYMPHOMA

The non-Hodgkin lymphomas (NHLs) encompass a wide spectrum of hematologic neoplasms that entail clinical, morphological and molecular heterogeneity. NHL arises from two distinct lymphocyte types, B or T cells, and the heterogeneity is at least in part related to the many stages of normal differentiation and maturation of these cells ***(Illes et al., 2009).***

## A) Etiology:

The etiology of NHL, as well as its global dramatic rise in incidence during the past decades, remains largely unexplained. Current evidence suggests three common themes underlying the emergence and perpetuation of NHL:

1- An episodic or persistent immunosuppressive state, which may be the result of primary or acquired immunodeficiency or exposure to an immunosuppressive agent such as ultraviolet rays (UVR) or blood transfusion.

2- Chronic antigenic stimulation due to an autoimmune condition, viral infection, or allergic/inflammatory agent.

3- Disruption of normal cell proliferation within this framework, each neoplasm emanates from a genetic mutation, either a random error or a mutation caused by an oncogenic agent, and, ultimately, the growth of the malignant cell into an established tumor is promoted by one or more cofactors that interfere with the usual regulatory mechanisms ***(Ekstrom-Smedby, 2006).***

**Table (1):** Etiological Factors Associated with Increased Risk of NHL

- **Genetic Aberrations**
- **Immunodeficiency (ID)**
  - *Congenital ID Diseases*
    - Ataxia telangectasia (AT) syndrome
    - Wiskott-Aldrich syndrome
    - Severe combined ID
    - Common variable ID
    - Immunoglobulin (Ig) A / IgM deficiency
    - X-linked (Bruton) hypogammaglobulinemia
    - X-linked lymphoproliferative syndrome
  - *Acquired ID States*
    - Solid organ and bone marrow (BM) transplantation with iatrogenic immunosuppression
    - Acquired immunodeficiency syndrome (AIDS)
- **Autoimmune and Other Immunologic Disorders**
  - Sjögren syndrome
  - Hashimoto thyroiditis
  - Castleman disease
  - Celiac disease (gluten-sensitive enteropathy)
  - Hodgkin disease (HD)
- **Infectious Agents**
  - EBV
  - HTLV-I/II
  - Human herpes virus-6/8
  - Hepatitis C virus
  - Helicobacter pylori
- **Drugs**
  - Phenytoin
  - Immunosuppressive agents
- **Occupational Factors**
  - Exposure to: herbicides Jobs in: farming
    Pesticides Forestry
    Wood dust Painting
    Epoxy glue Carpentry
    Solvents Tanning
- **Other Possible Etiological Factors**
  - Hair dyes
  - Sunlight exposure
  - Nutritional factors (↑ protein,↓ vitamins)
  - Family history of NHL

***(Swerdlow, 2003)***

# B) Molecular Pathogenesis:

Significant progress has been made in understanding the pathogenesis of NHL as a malignant (clonal) expansion of B- or T-cells. The most frequent cytogenetic abnormalities have been characterized at the molecular level, leading to the identification of genes that are altered in B-cells or T-cells. Most of these genetic lesions selectively associate with specific NHL subtypes, thus representing markers of potential diagnostic significance ***(Brown and Freedman, 2009).***

Molecular pathogenesis of NHL represents a complex process involving the accumulation of multiple genetic lesions which include: the activation of proto-oncogens such as BCL1, BCL2, BCL6, C-MYC by chromosomal translocation, as well as inactivation of tumor suppressor genes such as TP53 by chromosomal deletion or mutation ***(Kuppers, 2005).***

The progressive and clonal accumulation of multiple genetic lesions, affecting both proto-oncogenes and tumor-suppressor genes can lead to a disturbed balance between cell proliferation, differentiation and death which play the pivotal role in lymphomagenesis ***(Pasqualucci et al., 2003)***.

## 1- Activation of proto-oncogenes:

Proto-oncogenes are highly conserved genes which, in normal conditions, promote cell growth and/or cell survival under the close control of mitogenic stimuli. These genes encode for

several functional classes of proteins. In NHL, proto-oncogenes are grouped into different categories according to their function and include: a) transcriptional regulating factors, b) anti-apoptotic genes, c) cytoplasmic signal tranducers ***(Vega et al., 2002)***.

In NHL, the main mechanism of proto-oncogenes activation is represented by chromosomal translocation which have been associated with different NHL histologic subtypes (Table 2) ***(Krause and Shahidi-Asl, 2003)***.

**Table (2):** Lymphoma **s**ubtypes with specific molecular markers and proto-oncogenes involved

| Proto-oncogene | Chromosomal translocation | Lymphoma subtype | Proto-oncogene function |
|---|---|---|---|
| PAX-5 | t(9;14) (p13;q32) | Lymphoplasmacytoid | Control of B-cell proliferation and differentiation |
| BCL-1 | t(11;14) (q13;q32) | Mantle cell | Regulation of cell cycle progression |
| BCL-2 | t(14;18) (q32;q21) | Follicular | Prevention of programmed cell death (apoptosis) |
| BCL-6 | 3q27 | Diffuse large cell | Germinal center differentiation |
| C-MYC | t(8;14) (q24; q32) | Burkitt's | Control of cell proliferation, differentiation and death |
| NPM/ALK | t(2;5) (p23;q35) | Anaplastic large cell | Signal transduction |
| API2-MLT | t(11;18) (q21;q21) | Maltoma | Apoptosis inhibition |

***(Krause and Shahidi-Asl, 2003)***

**a. Chromosomal translocation:**

Chromosomal translocations are the genetic hallmark of lymphoid malignancies, where they represent the main mechanism of proto-oncogene activation in NHL. Analogous to most types of hematopoietic neoplasms, chromosomal translocations in NHL represent reciprocal and balanced recombination events between two specific chromosomal sites. These translocations are characterized by recurrence within a specific clinico-pathologic category of NHL and are clonally represented in each tumor case ***(Seto, 2004)***.

*Two main mechanisms of oncogenic activation have been encountered with chromosomal translocations in NHL:*

In the first type, a proto-oncogene is placed under the control of active regulatory elements (gene promoter) of another gene causing deregulated expression of a structurally intact (or occasionally truncated) protein. In most cases, these regulatory elements are those of one of the *Ig genes* (Ig heavy "H", kappa "κ" or lambda "λ" light "L" chain genes) or *T cell receptor (TCR) genes* (alpha "α", beta "β", gamma "γ" or delta "δ") that can promote high level protein expression in B or T lineage cells, respectively ***(Kusec, 2002)***.

The other major class of translocations includes those that create fusion genes encoding chimeric proteins that possess novel structural and functional properties. This type of translocation often fuses a DNA binding domain from one

protein with effector domain, such as a transcriptional activation domain, from the other ***(Brown and Freedman, 2009)***.

Although the precise mechanisms involved in the development of chromosomal translocations in NHL are unclear, yet a clear relationship has been established with the mechanisms that lead to Ig/TCR gene remodeling; including Variable (Diversity) Joining {V(D)J} recombinations, somatic hypermutation and isotype switching. The disruption of this normal molecular sequence of events leads to the development of pathogenic molecular lesions and thereby neoplastic cell transformation ***(Vega and Medeiros, 2003)***.

***i. V(D)J recombination and lymphomagenesis:***

V(D)J recombination is a process by which gene segments coding for the variable region of the Ig/TCR molecules are assembled. This is initiated when two lymphocyte-specific endonucleases, the recombination activating gene (RAG) 1 and 2 proteins, cut the rearranging gene segments at specific recombination signal sequences (RSS) that flank the coding sequences of the V, D and J segments, thus yielding two signal ends and two covalently sealed hairpin coding ends ***(Krause and Shahidi-Asl, 2003).***

The signal ends are precisely joined, releasing the intervening deoxyribonucleic acid (DNA) between the rearranging gene segments as a DNA circle. During this reaction, nucleotides may be removed from the coding ends by

exonucleolytic digestion, and non-germline nucleotides (N sequences) may be added by the lymphocyte-specific terminal deoxynucleotidyl transferase (TdT), further increasing the diversity of variable region genes (Figure 1) ***(Gellert, 2002)***.

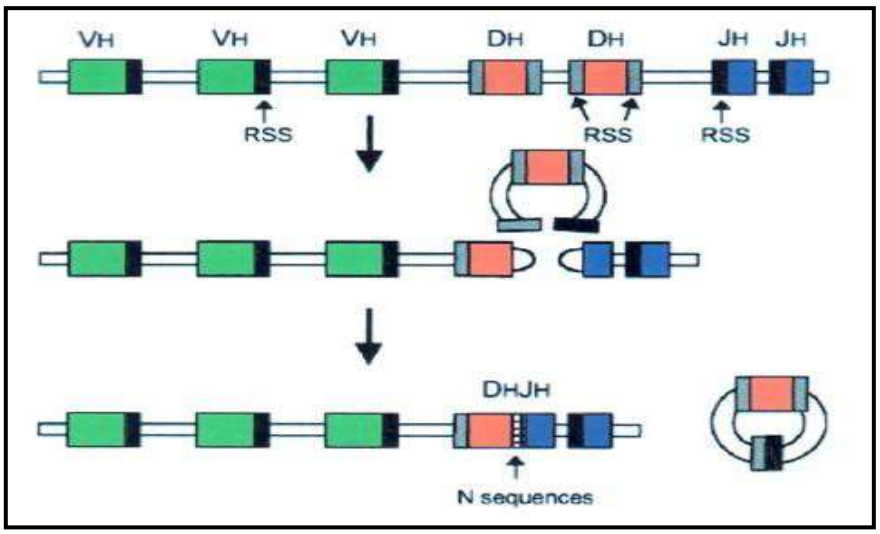


**Figure (1):** Ig gene remodeling (VDJ recombination)
***(Kuppers and Dalla-Favera, 2001).***

Several recurrent chromosomal translocations involving Ig/TCR loci show features suggesting involvement of V(D)J recombination in their generation, such as:

- t(8;14)(q24;q32) C-MYC/IgH translocation in endemic Burkitt lymphoma (BL).
- t(8;14)(q24;q11) C-MYC/TCRαδ locus in T-Lymphoblastic lymphoma/leukemia (T-LBL).
- t(14;18)(q32;q21) Bcl-2/IgH translocation in FL.
- t(11;14)(q13;q32) Bcl-1/IgH translocation in mantle cell lymphoma (MCL).

- t(1;14)(p22;q32) Bcl-10/IgH translocation in mucosa-associated lymphoid tissue lymphoma(MALT).

***(Kuppers and Dalla-Favera, 2001)***

***ii. Somatic Hypermutation and Lymphomagenesis:***

Somatic hypermutation is a B-cell-specific mechanism that targets the rearranged IgV genes during the germinal center (GC) reaction in order to generate antibody diversity and to allow the selection of B-cells producing antibodies with high-affinity to the respective antigen. GC B-cells will undergo several rounds of proliferation, mutation and selection before they finally differentiate into memory B-cells or plasma cells ***(Pasqualucci et al., 2003).***

The process of somatic hypermutation introduces mutations at a very high rate in a region spanning approximately 1-2 kb from the promoter of the rearranged IgV genes. These mutations not only generate nucleotide exchanges, but also a significant frequency of deletions and duplications. As the generation of deletions/duplications is intimately associated with DNA cleavage, it was suggested that somatic hypermutation is accompanied by DNA strand breaks that are potentially recombinogenic. Thereby, somatic hypermutation may be involved in the generation of chromosomal translocations in B-NHL where the majority of them carry hypermutated rearranged Ig genes that are generated during lymphocyte passage through the GC (Figure 2) ***(Muramatsu et al., 2000).***

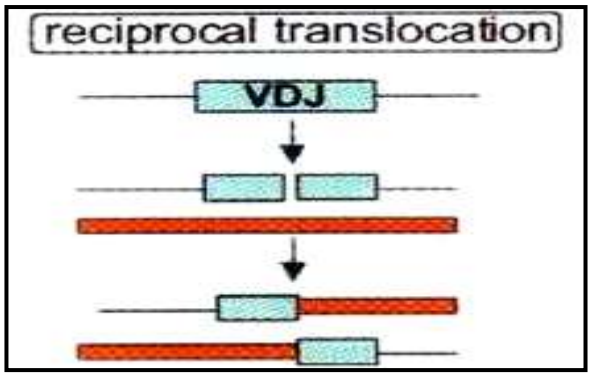


**Figure (2):** Ig gene translocation during the process of somatic hypermutation ***(Kuppers and Dalla-Favera, 2001).***

### *iii. Ig Switch Mechanism and Lymphomagenesis:*

A fraction of GC B cells undergo class switch recombination and thereby change the isotype of the expressed Ig. Through class switching, the Constant mu (Cμ) and Cδ genes that are originally expressed by a naïve B cell are subsequently replaced by the Cγ, Cα or C epsilon (Cε) genes. During class switch recombination, DNA strand breaks are introduced into both the switch μ (Sμ) region and a switch region associated with one of the other constant heavy chain (CH) genes. The DNA fragment between the switch regions is removed and they are joined together, such that a new CH gene is placed downstream of the variable region exon (Figure 3) ***(Maizles, 1999)***.

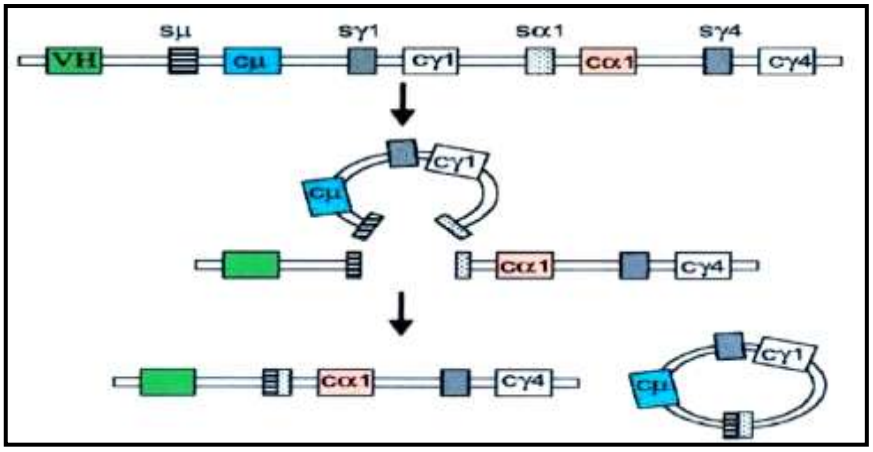


**Figure (3):** Ig gene remodeling (isotype switch) ***(Kuppers and Dalla-Favera, 2001).***

Chromosomal breakpoints located in IgH switch regions (Figure 4) have been detected in a large number of different translocations. These include:

- t(8;14)(q24;q32) C-MYC/IgH translocation in sporadic BL.
- t(3;14)(q27;q32) Bcl-6/IgH translocation in DLBCL.
- t(9;14)(p13;q32) Paired homeobox (Pax)-5/IgH translocation in lymphoplasmacytic lymphoma (LPL) ***(Kuppers and Dalla-Favera, 2001)***.

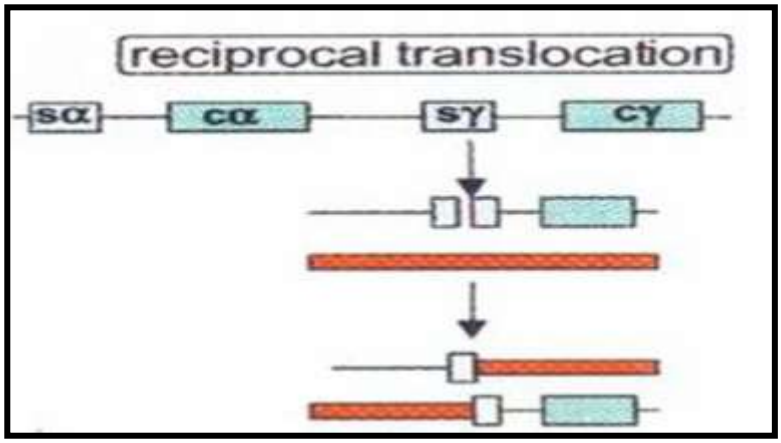


**Figure (4):** Ig gene translocation during the process of class switching ***(Kuppers and Dalla-Favera, 2001).***

## 2- Inactivation of tumor-suppressor genes:

The function of tumor suppressor genes under normal conditions is to inhibit cell growth. Since a single copy of the gene is usually sufficient to exert its physiological role, inactivation of tumor suppressor genes in human occurs biallelically, most commonly through a deletion of one allele and an inactivating mutation of the other. The tumor suppressor

gene most frequently involved in the pathogenesis of NHL is represented by TP53 (Figure 5) ***(Brown and Freedman, 2009).***

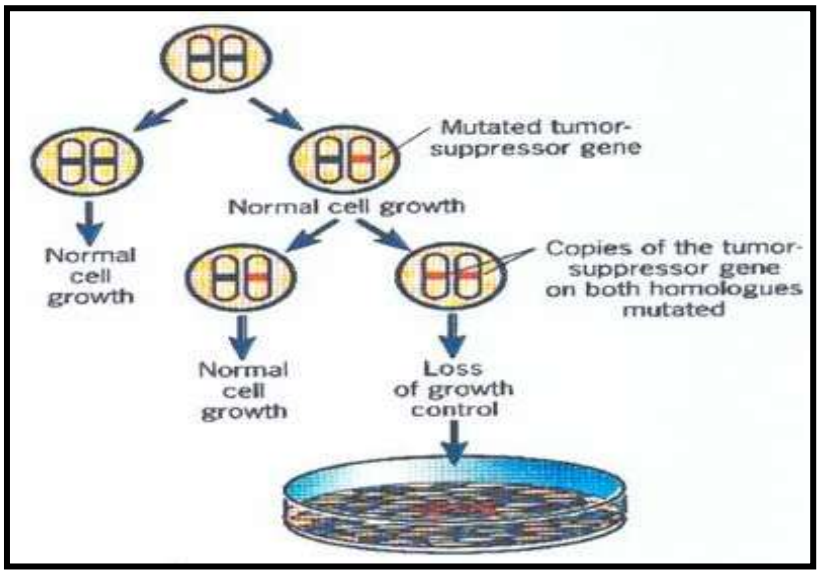


**Figure (5): Mutations in tumor-suppressor genes:** both alleles must be knocked out to induce tumorigenic effect ***(Tam and Dalla-Favera, 2001).***

Different mechanisms by which tumor-suppressor genes are inactivated include; point mutation, gross deletion or hypermethylation ***(Krug et al., 2002).***

**a. Point mutation:**

It arises from nucleotide substitutions affecting the coding region of the gene and result in nonsense or missense mutations. Small deletions and insertions can also occur and lead to frameshift mutations. TP53 is the most frequently mutated tumor-suppressor gene in human cancers which has been found in 30% of high grade B-NHL (rare in low grade NHL) ***(Hill et al., 2006)***.

**b. Gross deletion:**

It results in removal of the entire gene locus leading to gene inactivation especially when associated with nonsense/missense mutation of the other allele. Homozygous deletions can less frequently occur ***(Tam and Dalla-Favera, 2001)***.

The most frequent chromosomal deletion associated with the pathogenesis of B-NHL is the deletion of the long arm of chromosome 6 at regions 6q21-q23 and 6q25-q27, which presumably represent sites of not yet identified tumor-suppressor genes. Moreover, deletion of the long arm of chromosome 13 at 13q14 (Rb gene) represents the most frequent lesion in small lymphocytic lymphoma (SLL), occurring in > 30% of cases ***(Gaidano and Dalla-Favera, 2000)***.

**c. Hypermethylation:**

It affects the gene promoter (p16 & p15) and leads to transcriptional silencing of the gene without any structural alterations. Hypermethylation of promoters result in gene inactivation by affecting intact alleles or by silencing the remaining allele when the other one has been deleted ***(Child et al., 2002)***.

# C) Classification:

The classification of NHLs has been a work in progress and has undergone many revisions to arrive at the current World Health Organization (WHO) classification used today. In