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Effect of Interleukin-10 -1082 A/G and Interleukin-12p40 1188 A/C Polymorphism on Susceptibility of Non- Hodgkin's Lymphoma

A Thesis

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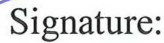

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Dedication

To

All Those Who Shed their Blood for Iraq

My Father Who Taught Me Walking, Writing and Thinking

.

My Mother Who Bears Me, Carries Me and Learns Me How to Smile.

My Brothers The Light By which I Could See my Life Path.

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Summary

Non-Hodgkin's lymphomas (NHLs) are a diverse group of mature lymphoid neoplasms with a wide range of cellular, immunological, histological, and molecular heterogeneity. Numerous environmental and genetic factors have been found to be associated with the incidence of these neoplasms; however the exact causes are beyond the current knowledge and there was inconclusive sole risk factor. as known to play a crucial role in the regulation of essential pathways.

Blood samples were collected from 55 confirmed patients with NHLs. Similar samples were collected from unrelated, age-matched 40 apparently healthy individuals to represent the control group. Informed consents from all participants were obtained which included age, gender, smoking status, diabetes mellitus presence, residence , first relative family history of NHLs and body mass index.

DNA was extracted from blood samples using ready purchased kit. Three sets of primer pairs were used to amplify IL-10, IL-12p40, and TLR2 (Toll-like receptor-2, as internal control) genes by using conventional PCR technique. Genotyping was performed by allele specific-PCR method. The levels of IL-10 and IL-12p40 in sera of NHLs patients and controls were measured by using ready purchased ELISA kit.

The polymorphism of IL-10 -1082A/G appeared in three genotypes: AA, AG and GG which represent 36.36%, 45.45% and 18.18%, respectively, among NHL patients compared to 32.5%, 35% and 32.5%%, respectively, in the control group. The differences between patients and control, however, were insignificant neither among genotypes nor at the allelic level.

On the other hand, the SNP IL-12p401188A/C had three genotypes which were AA, AC and CC. These genotypes represented 32.73%, 38.18% and 29.09%, respectively, among NHL patients compared to 52.5%, 37.5% and 10%, respectively, among controls. There was a significant difference for the homozygous mutant genotype CC {OR (odd ratio) = 4.667, 95%CI (95% confidence interval) = 1.319-16.512, *P value* = 0.017}. At allelic level, the mutant of C allele was more frequent among NHL patients (48.18%) than in controls (28.75%) with a highly significant difference (OR = 2. 304, 95%CI = 1.25-4.249, *P* = 0.007).

The serum levels of IL-10 and IL-12p40 were significantly higher among NHL patients (458.31±241.126pg/ml and 156.44±54.188 pg/ml, *P* = 0.011 and 0.0001, respectively) than the control group (323.46±242.344 pg/ml and 107.34±56.957 pg/ml, respectively) as measured by an ELISA technique. The elevation of IL-10, although it was not significant, was associated with the AG genotypes. While the elevation of IL-12p40 was linked to CC genotypes. These data indicate that IL-10 SNP is not directly linked to the risk of NHLs. On the other hand, SNP in IL-12p40 showed such correlation and influenced by specific genotype.

Seven other risk factor were studied. These are: age, family history, gender, body mass index (BMI), smoking, residency (urban vs rural), and type 2diabetes mellitus (DM-2). Out of these, four factors showed to be significantly associated with the risk of NHLs. These factors were gender (*P*= 0.009), body mass index (BMI) (*P*=0.028), smoking (*P*= 0.005) and urban residence (*P*=0.007).

In conclusion, this study showed that IL-10-1082A/G polymorphism has no significant effect on either the risk of NHLs or serum levels of IL-10.

In contrast, the allele C of the SNP IL-12p401188A/C could be considered as a risk factor for NHLs.

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List of Abbreviations

Abbreviation	Full Name
NHL	Non-Hodgkin Lymphoma
IL-10	Interleukin-10
IL-12	Interleukin-12
Th1	Type1 helper T-cell
SNP	Single nucleotide polymorphism
TLR2	Toll-like receptor2
PCR	Polymerase chain reaction
ELISA	Enzyme linked immune sorbent assay
BMI	Body mass index
BL	Burkitt's Lymphoma
IG	Immunoglobulin
TCR	T cell receptor
REAL	Revised European-American Lymphoma
WHO	World health organization
HTLV-1	Human T-cell Lymphotropic Virns-1
AIDS	Human lmmunodeficiency syndrome
HCV	Hepatitis C-virus
HBV	Hepatitis B-virus
HLA	Human leukocyte antigen
MHC	Major histocompatibility complex
SSP	sequence specific primer
3' UTR	3'-untranslated region
FL	Follicular Lymphoma
PCA	Principal component analysis
CNS	Central nervous system
SVCO	Superior vena caval obstruction
FCM	Flow cytometry

CD	Cluster differentiation
IHC	Immunohistochemistry
NK	Natural Killer cell
ATLL	Adult T-cell Leukemia Lymphoma
EBV	Epstein- Barr Virus
MALT	Mucosa- associated lymphoid tissue
PTCL	Peripheral T-cell lymphoma
LBL	Lymphoblastic lymphoma
DLBCL	Diffuse Large B- cell lymphoma
ALCL	Anaplastic Large cell lymphoma
TCF-3	Transcription factor3
ID3	Inhibitor of differentiation3
ASHM	Aberrant Somatic hypermutation
AID	Activation–induced deaminase
ALK	Anaplastic lymphoma kinase
PTN	Pleiotrophin
MK/MDK	Midkine
HHV-8	Human herpes virus-8
HIV	Human immunodeficiency virus
KSHV	Kaposi sarcoma herpes virus
PEL	Primary effusion lymphoma
FISH	Fluorescent in situ hybridization
CGH	Comparative genomic hybridization
MCL	Mentle cell lymphoma
HRP	Horse radish peroxidase
SPSS	Statistical package for social sciences
OR	Odd ratio
CI	Confidence interval
BCRFI	*Bam*-HI C fragment rightward reading frame

CHAPTER

ONE

Introduction and

Literature Review

1.1 Introduction

Lymphomas are kinds of tumors derived from lymphocytes. Non-Hodgkin lymphomas (NHLs) are a diverse group of mature lymphoid neoplasms with a wide range of cellular, histologic presentations, cells of origin and etiologies (Kim, 2014). The NHLs represent 4% of all cancers in the United States with an overall annual incidence of 19.7 cases and with 5.3 deaths per 100000 adults / year which makes these neoplasms ranked fifth most common cancer (Lozano *et al*., 2012). In the United Kingdome, NHLs are a common disease ranking sixth among all malignancies and causing 4700 death annually (NHL Statistics, 2014). In Iraq, it also ranks sixth with approximately 1129 annual cases (Iraqi Cancer Registry, 2014).

Despite treatment advances in the last three decades with the use of combination chemotherapy, a significant proportion of patients relapse or are refractory to these treatments (Chao, 2013). This imposes an intensive research program to find out the probable of causes of NHLs in order to overcome these neoplasms.

Numerous environmental and genetic factors have been shown to be associated with the incidence of NHLs, however, the exact causes are beyond the current knowledge (Hartge and Smith, 2007). Regardless of causes, the integrity of the immune system represents the cornerstone in the resistance or progression of the disease. Grulich *et al*. (2007) reported many disorders of this system such as immune deficiency and autoimmune disease like rheumatoid arthritis and systemic lupus erythematosus to be predispose to NHLs. Immune deficiency is usually (but not always) manifested by an insufficient quantity of one or more of the immune components. However, in many cases such components are

present in sufficient quantities but they cannot achieve their duties due to defect in their synthesis resulting from genetic disorder.

Interleukin 10 (IL-10) and interleukin 12 are among the main players cytokines of the immune system. Interleukin-10, first recognized for its ability to inhibit activation and effecter function of T lymphocyte, monocytes, and macrophages, is a multifunctional cytokine with diverse effects on most hemopoietic cell types. The principal routine function of IL-10 appears to be to limit and ultimately terminate inflammatory responses. It plays a key role in differentiation and function of T regulatory cell, which may prominently in control of immune responses (Moore *et al*., 2001). Interleukin-12 is a multifunctional cytokine acting as a key regulator of cell-mediated immune responses through the differentiation of naïve CD4+ T cells into type 1 helper T cells (Th1) producing interferon-γ (Mendez-Samperio, 2010). Factors influencing the functions of these cytokines are expected to influence the efficiency of the immune system especially the cellular arm, and subsequently alter the individual susceptibility to NHLs.

Single nucleotide polymorphism (SNP) is a variation occurring commonly within a population in which a single nucleotide in a certain gene differs between members of a biological species or paired chromosomes (Nachman, 2001). Huge number of SNPs are present in almost every gene including *IL-10* and *IL-12* genes, but certain SNPs have been found to influence the biological activity of these cytokines. Not only do these SNPs affect the levels of gene expression of the cytokine but also they can alter its binding with its receptor and subsequently its activity. Hence, it is reasonable to assume that certain SNPs in *IL-10* and *IL-12* gene may influence the individual susceptibility to NHLs.

1.1.1 Aims and Research Strategy

To the best of our knowledge, there is no previous study in Baghdad about the association of polymorphism of Th1 (IL-12) and Th2 (IL-10) cytokines with the susceptibility of local patients with NHLs. Therefore, this study is aimed to uncover the relationship between the cytokine polymorphism in NHLs samples obtained from specialized Medical centers in Baghdad city.

Specifically, the current research strategy is designed to investigate:

1- The association of genetic polymorphisms in two single nucleotide polymorphism (SNP) of IL-10-1082A/G and the SNP IL-12p40 1188A/C, with the incidence of NHLs among Baghdad patients.
2- The effect of these SNPs on the serum levels of IL-10 and IL-12.
3- The effect of different risk factors on the susceptibility to NHLs among Baghdad patients.

To execute this strategy, the following research plan was set up:

1. Subjects Recruiting: patients with NHLs from medical Centers in Baghdad area with similar number of age matched of apparently healthy individuals as a control group. Blood samples are to be collected.
2. DNA was extracted from blood samples
3. Allele specific polymerase chain reaction (AS-PCR) technique was used for genotyping of the two SNPs using specific primers.
4. Cytokines (IL-10 and IL-12p40) levels in sera of patients and healthy individuals by using a commercially available ELISA kits.

1.2 Literature Review

1.2.1 Historical Perspective

Thomas Hodgkin was the first to recognize that lymphadenopathy could occur as a primary disorder rather than secondary to infection or carcinoma in his 1832 paper entitled, "On Some Morbid Appearances of the Absorbent Glands and Spleen" Brill and Symmers described follicular lymphoma (FL) in the 1925 and named it as a malignant, albeit indolent, disorder (Smith, 2013). In 1941, Gall and Mallory developed a classification scheme for NHL on the basis of both clinical and histopathologic significance. The histopathologic phase of NHL culminated in the 1956 classic work of Rappaport who developed a morphologic classification which stratified cases according to the pattern of growth (Jaffe *et al.*, 2008).

In 1974, Lukes and Collins classified NHL on the basis of the cell of origin within the immune system (Lukes and Collins, 1974). Subsequently, monoclonal antibodies to lymphocyte differentiation antigens have been able to detect sequential stages in the development of B and T cells and to identify subtypes of NHLs. Recurrent cytogenetic translocations involving the immunoglobulin (Ig) gene locus on chromosome 14 were identified in Burkitt's lymphoma (BL) in 1976 and in FL in 1979 (Jaffe *et al.*, 2008). In the 1980s, the lymphoid origin of NHLs was confirmed at the molecular level with the identification of specific Ig gene and T cell receptor (TCR) gene rearrangements in B and T cell lymphomas, respectively. In 1994, a Revised European-American Lymphoma (REAL) classification was proposed to identify specific types of lymphomas of B and T cell origin (Harris *et al.*, 1994).

1.2.3 Epidemiology of NHLs

There were an estimated annual incidence of 356000 new cases and 192000 deaths with NHLs worldwide (Ferlay *et al.*, 2008). These numbers put the disease the eighth most commonly diagnosed cancer in men and eleventh in women. It accounts for about 5.1% of all cancer cases and 2.7% of all cancer deaths (Boffetta, 2011). NHLs are most common in developed countries, with the United States having the highest rate worldwide. The lowest NHLs rates are found in eastern and south central Asia (2 to 3 per 100,000 populations). Certain endemic geographic factors appear to influence the development of NHL in specific areas. Human T-cell lymphotropic virus-1 (HTLV-1)-associated adult T-cell leukemia/lymphoma (ATLL) occurs more frequently where HTLV-1 is endemic in southern Japan and the Caribbean. It occurs sporadically in Brazil, sub-Saharan Africa, the Middle East, and the southeastern United States. The sero-prevalence in southwest Japan is 16%, although the lifetime risk of ATLL for those individuals is 2% to 6% (Burton *et al.*, 2010).

The incidence of BL in Africa (Nigeria and Tanzania) is 6% to 8%, compared with 0.4% in the United States. Moreover, the clinical features of BL in Africa differ from those of cases reported to the American BL Registry. Etiologic endemic factors include malaria as a source of chronic B-cell antigenic stimulation and Epstein-Barr Virus (EBV)-induced immortalization of B lymphocytes. Heavy-chain disease is a disorder of B-lymphoid cells characterized by diffuse thickening of the small intestine due to a lymphoplasmacytic infiltrate with secretion of incomplete IgA heavy chains. Pathologically, it is a mucosa-associated lymphoid tissue (MALT) lymphoma of the small bowel. This

clinicopathologic entity is rarely encountered in individuals other than those of Mediterranean ethnic origin (Boffetta *et al*., 2011).

Follicular lymphomas (FL) are more common in North America and Europe but are rare in the Caribbean, Africa, China, Japan, the Middle East, and Latin America. PTCLs are more common in Europe and China than in North America. They represent about 4% of lymphomas in the United States (Burton *et al*., 2010).

1.2.4 Molecular Pathogenesis of NHLs

Genetic lesions of NHLs are attributed to disorders in the expression of proto-oncogenes and tumor suppressor genes. Proto-oncogenes are conserved genes which normally promote cell growth and survivals. Disorder in the expression of these genes transforms these genes into constitutively active variants which are called oncogenes (Rosen, 1995). On the other hand, tumor suppressor genes are group of genes which normally encode for proteins that inhibit cell growth (Levine, 1995). In NHLs, the most common mechanism of proto-oncogene activation is the chromosomal translocation involving the site of the proto-oncogene on one chromosome (Gaidano *et al*., 1995). These translocations result in deregulation in oncogene expression.

Lymphoblastic lymphoma (LBL), BL, diffuse large B-cell lymphoma (DLBCL) and anaplastic large cell lymphoma (ALCL) are the four major pathogenic subtypes of NHL. While LBL is known to be initiated from immature B or T lymphocytes, each of BL, DLBCL and ALCL arises from mature B or T cells neoplasms (Nelson, 2016).

Eighty percent of LBL commonly have a *t* (8;14) translocation, while the other 20% are *t* (2;8) or *t* (8;22). Patients with DLBC may have a *t* (8;14)

translocation and aneuploid karyotype. Most patients with ALCL have a *t* (2;5) translocation which leads to a fusion gene encoding constitutively active nucleoplasmic-anaplastic lymphoma tyrosine kinase. A large percentage of BL patients have breakpoints at 14q11.2 or *t* (5;14) translocation (Nelson, 2016). In LBL, a relatively high percentage of translocation involves loci in T-cell receptor gene resulting in juxtaposition of T-cell receptor promoter and enhancer elements with various transcription factors, such as HOX11/TLX1, TAL1/*SCL*,*TAL2*, and *LYL1*, which lead to high levels of expression in precursor thymocytes (Tauchi *et al*., 2008; Smock *et al*., 2008).

In approximately 70% of BL, translocation involving c-*myc* gene is the most predominant lesion (Schmitz *et al*., 2012). However, this result was reversed by Lover *et al*. (2012) as c-*myc* mutation alone is not sufficient for induction of BL. The transcription factor TCF-3 was found to be central for the pathogenesis of BL. Schmitz *et al*., (2014) stated that this factor could be constitutively activated by two mechanisms: somatic mutations that cause inactivation of ID3 factor which is a negative regulator of TCF-3, and somatic mutations in TCF-3 gene itself can block the binding of ID3 to TCF-3.

Accounting for about 40% of total NHLs cases, DLBCL is caused by more sophisticated genetic mechanisms. Beside chromosomal translocation, aberrant somatic hypermutation (ASHM) and sporadic somatic mutations could be involved in the molecular pathogenesis of this malignancy (Campo *et al*., 2011). Activation-induced cytidine deaminase (AID) has been suggested to contribute to the induction of DLBCL by facilitating the translocation process and ASHM (Schneider *et al*., 2011). That is because AID can bind genes in every chromosome throughout the genome. Through this binding it can induce double-stand DNA breaks

(Gu *et al.*, 2012). However, the exact mechanisms by which AID induces these genetic alterations are not clear.

About half of the patients with ALCL have abnormal oncogenic anaplastic lymphoma kinase (ALK) fusion proteins resulted from chromosomal translocation (Amin and Lai, 2007). In normal tissues, ALK is restricted and functions as a dependence receptor regulatory cell survival by interacting with pleiotrophin (PTN) or midkine (MK) (Pulford *et al.*, 2004). Chromosomal translocation in ALK gene results in high expression of ALK in ALCL. Activated ALK can recruit and activate SH2 domain-containing tyrosine phosphatase 2, activates *Ras-Erk* pathway (which in turn upregulates cyclin-A and cyclin-D1), down regulates the cyclin kinase inhibitor (P21) and many other pathways. Thus, abnormal ALK accelerates cell proliferation and inhibiting apoptosis (Shi *et al.*, 2011).

1.2.5 Risk Factors

The exact etiology of NHL has not been explained, however, intensive studies revealed a clear association of the disease with many risk factors.

1.2.5.1 Immune Modulation

Immune suppression in either innate or adaptive immune response is considered as the most important risk factor for NHLs (Rabkin *et al.*, 1997). Many syndromes like Wiskott-Aldrich, common variable hypogammaglobulinemia, X-linked lymphoproliferative and acquired immunodeficiency syndromes are known to cause immune suppression. Not only do these syndrome increase risk of NHLs, but they also associate with high grade and extranodal cancer (Grulich *et al.*, 2007). Moreover, almost all autoimmune diseases have been documented to

increase such risk. Of these, systemic lupus erythematosus and rheumatoid arthritis are well known to increase the incidence of B-cell lymphomas (Ekstrom-Smedby *et al*., 2008). However, it is not clear whether this effect is due to the autoimmune disease itself or to the immunosuppressive drugs used to treat this disease. In the same context, patients who receive immunosuppressive drugs for any cause have 30-50 folds chance to have NHLs (especially DLBCL, marginal zone lymphoma and lymphoplasmacytic lymphoma) compared with healthy control (Clarke *et al*., 2013). Finally, the risk of NHLs also increases (although in less pattern) in patients having treatment involving chemotherapy or radiation (Kim *et al*., 2013) although there is no certain subtypes of NHLs associating with these regimes.

1.2.5.2 Infections

This is one of the most obvious risk factors for NHLs. Several infectious agents have been incriminated in this issue. These can be categorized into viral and bacterial infections.

1.2.5.2.1 Viral Infections

Viruses may function as co-factors in lymphomagenesis, whereby they may exert their effect through genomic integration, which leads to alterations in gene expression, and/or by directly affecting cellular proliferation. EBV, HTLV-I, and human herpesvirus–8 (HHV-8), in particular, have well-established oncogenic roles in subtypes of NHLs. EBV is mostly associated with BL. It appears to have a direct oncogenic role in lymphomas of immunocompromised patients, such as those infected with human immunodeficiency virus (HIV) and in the post-transplant period (Saha and Robertson, 2011). HTLV-I has a direct role in

ATLL, but carriers have only a 2% to 5% lifetime risk of developing disease with a latency period of 30 to 40 years. HHV-8, also known as Kaposi sarcoma herpes virus (KSHV), has been associated with primary effusion lymphoma (PEL), which is a rare B-cell lymphoma that occurs primarily in highly immunosuppressed patients with acquired immunodeficiency syndrome (AIDS) who are often co-infected with EBV (Kaplan, 2013).

Another less prominent association was found with chronic hepatitis C infection which associated with splenic marginal zone lymphoma, lymphoplasmacystic lymphoma and DLBCL. The causal relationship here is thought to be continuous stimulation of lymphocyte receptor by viral antigen (Peveling-Oberhang *et al.*, 2013). However, there is no convince explanation why HCV but not HBV introduces such effect while both of them exert continuous stimulation of lymphocyte receptors.

1.2.5.2.2 Bacterial Infections

An emerging evidences indicate that certain bacteria which cause chronic inflammation in the mucosal associated lymphoid tissue (MALT) can be a risk factor for gastric MALT lymphoma. The first bacterium in this regard is *Helicobacter pylori*. Other bacteria such as *Campylobacter jejuni* and *Borrelia burgdorferi* were also linked with rare types of NHLs (Hjalgrim and Engels, 2008).

1.2.5.3 Gender

The overall incidence of NHLs is about 50% higher in men (23.9 per 100.000) than women (16.4 per 100.000) in the United State (Hawlader *et al.*, 2014). This predominance involves most NHLs subtypes with BL and

mantle cell lymphoma have distinguished excess among men (Hartge *et al.*, 2006). Globally, research conducted in many western countries on adolescents revealed an overall 2.7:1 ratio for male/female in the incidence of NHLs. Some subtypes (especially BL) showed stronger predominance of 4.5:1; while DLBCL showed lower ratio (1.7:1) (Burkhardt *et al.*, 2005).

1.2.5.4 Age

The incidence of most histologic subtypes of NHL rises exponentially with increasing age. In persons older than 65, the incidence was 91.6 (112.3 males and 76.9 females) per 100,000 persons in 2007-2011. Except for high-grade lymphoblastic and BL (the most common types of NHLs seen in children and young adults), the median age at presentation for all subtypes of NHLs exceeds 50 years.

1.2.5.5 Race/Ethnicity

The incidence of NHL varies by race/ethnicity, with non-Hispanic whites (20.6 per 100,000 persons) at higher risk than blacks (14.3 per 100,000), Asian/Pacific Islanders (13.4 per 100,000), and Hispanics (17.7 per 100,000) during 2007-2011. Most subtypes, particularly low-grade small lymphocytic and follicular lymphomas, are more common in whites than in blacks. Only peripheral T-cell lymphoma (PTCL), mycosis fungoides, and Sézary syndrome are more common in blacks than in whites.

1.2.5.6 Body Mass Index (BMI)

Obesity seems to affect only certain types of NHLs. In a meta-analysis study,(Larsson and Wolk,2011) found that BMI positively associated with the risk of DLBCL but not with other NHLs subtypes. A relative

risk for each excessive 5 kg/m^2 was associated with 1.07-fold increase in the incidence of NHLs. Recently (Kane et al ,2015) found that BMI> 30 Kg/m^2 has 1.33-fold increase in DLBCL risk. However no link was found with other subtypes of NHLs.

1.2.5.7 Alcohol Drinking

Several studies have investigated the effect of alcohol drinking on NHLs. However, the results were inconsistent where De Stefani *et al*, (1998) did not find any association. Moreover, Lim *et al.*, (2007) reported low risk in contrast to Chang *et al.*, (2010) who reported a significant risk of alcohol consumption with NHL.

1.2.5.8 Occupational Exposures

A number of occupations were found to increase the risk of getting NHLs. Among these are benzene workers, farmers, rubber workers, pesticide applicators, dry cleaners, petroleum refinery workers, chemists and firefighter (Hartge *et al.*, 2006; Ekstrom-Smedby, 2006). High risk of DLBCL was reported in individuals working as crop farmer, embroiderer and driver who handles equipment operators (Cerhan *et al.*, 2014). Marginal zone lymphoma was reported by Bracci *et al*, (2014) to be increased among metalworkers, while Mbulaiteye *et al*, (2014) found an increase incidence of BL among cleaner workers. The mechanism by which these occupations can influence specific subtype of NHLs is not well understood.

1.2.5.9 Diet

A positive association of NHLs with red meat consumption was frequently reported (Chiu *et al.*, 1996; Zhang *et al.*, 1999; Aschebrook-

Kilfoy *et al.*, 2012) in contrast to fish consumption which was found to reduce the risk (Daniel *et al.*, 2012; Charbonneau *et al.*, 2013). Furthermore, an increase in NHLs risk was attributed to high consumption of food rich with different kinds of fat (Aschebrook-Kilfoy *et al.*, 2012).

1.2.5.10 Smoking

A controversial conclusions were reported about the effect of smoking on the incidence of NHLs. Heavy smoking was found to be associated with increased risk of FL (Schollkopf *et al.*, 2005) and DLBCL (Stagnaro *et al.*, 2004). However, a recent study by Diver *et al.*, (2014) indicated that exposure to tobacco smoke increases risk for FL but reduced the risk for DLBCL in adults. It seems likely that the other risk factors interfere with the effect of cigarettes and a solid conclusion could not be drawn.

1.2.5.11 Family History

This factor is one of the most prominent one as a risk factor for NHLs. Two- to three-fold increase in NHLs was reported in individuals who have close relatives suffering from other hematolymphoid malignancies (Casey *et al.*, 2006). In particular, DLBCL and follicular lymphoma were found to be increased in individuals who have a family history (especially brothers) with NHLs (Cerhan *et al.*, 2014; Linet *et al.*, 2014)

1.2.5.13 Genetics

The importance of genetic factors in NHLs is firstly suggested by the racial differences in the incidence of these neoplasms. Large number of

studies on different genes reported a significant influence of certain genetic variants on the susceptibility to NHLs. Among the most investigated genes are that of cytokines, oxidative stress, innate immunity, apoptosis, DNA repair pathways and human leukocyte antigen (HLA).

1.2.6 Interleukin-10 Gene

The human IL-10 gene illustrated in (Figure 1-1) is located on chromosome 1 and encodes for 5 exons with molecular weight (5.1 kb) (Spits and De Waal Malefyt, 1992).

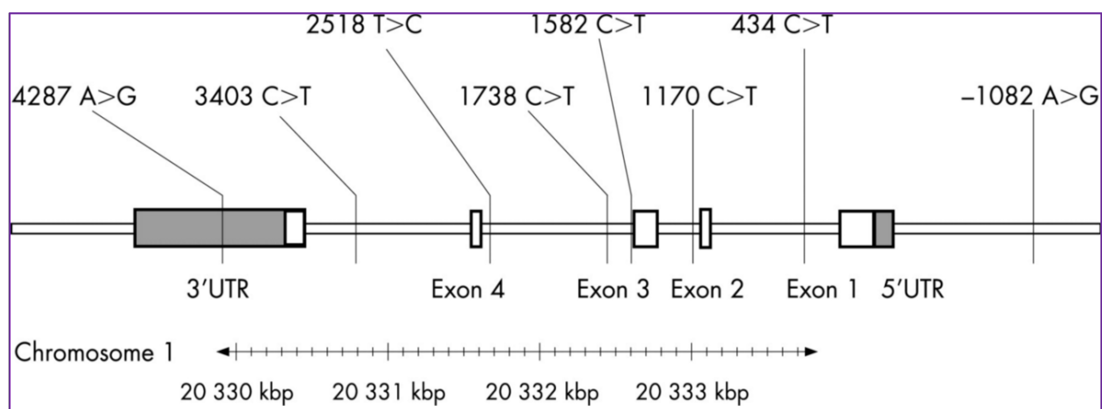


Figure (1-1). The most important single nucleotide polymorphisms in IL-10 gene (adapted from Malarstig *et al*., 2008).

Interleukin 10 (IL-10) is an important pleiotropic immunoregulatory cytokine mainly secreted by macrophages, but also by T helper 1 (Th1) and Th2 lymphocytes, dendritic cells, cytotoxic T cells, B lymphocytes, monocytes and mast cells. IL-10 inhibits the capacity of monocytes and macrophages to present antigen to T cells via an inhibitory effect on expression of major histocompatibility complex (MHC) class II, costimulatory molecules such as CD80 (B7.1) and CD86 (B7.2) and

therefore downregulates the expression of many cytokines (Trifunovic *et al*., 2015).

Boqunia-Kubik *et al*. (2008) studied the association of three SNPs in IL-10 gene (-1082A/G, -819C/T and -592A/C) with the incidence of NHL in Polish patients. They found that the frequency of the IL-10 low-producing -1082 AA homozygous genotype was significantly higher in patients with aggressive NHL as compared with patients with indolent forms of the disease and controls, Furthermore, the presence of the AC genotype was more frequently detected among patients with more aggressive disease than in those with indolent forms and healthy controls. In multivariate analyses, the AA homozygosity and ACC genotype appeared as independent risk factors of more aggressive manifestation of disease. In Egypt, Talaat *et al*. (2014) studied the influence of the two SNPs (−1082 G/A and −819 C/T) on the susceptibility of Egyptians to DLBCL using SSP-PCR. They reported that GT haplotype was significantly elevated in NHL patients. A significant linkage disequilibrium between the −1082 and −819 SNPs with $D' = 0.596$ and *r*2 = 0.1032 was demonstrated. In the same regard, Zhang *et al*. (2015) conducted a meta-analysis included 12 publications with more than 5500 NHL cases and 7000 controls. Stratification analyses demonstrated a significant decreased risk associated with the *IL10* -819C>T polymorphism (homozygous:, and recessive model: and *IL10*-592C>A polymorphism, and recessive model: among patients with DLBCL.

1.2.7 Interleukin 12 Gene

IL-12 is encoded by two separated genes, IL-12A (P35) located on 3p12-q13.2, and IL-12B (P40) located on 5q31-33 (Figure 1-2). Several SNPs have been identified in these two genes, of which IL-12p40118A/C most

widely investigated. This SNP, located in the 3'-untranslated region (3'UTR) of the IL-12B gene, has been found to be correlated with IL-12 secretion (Seegers *et al*., 2002).

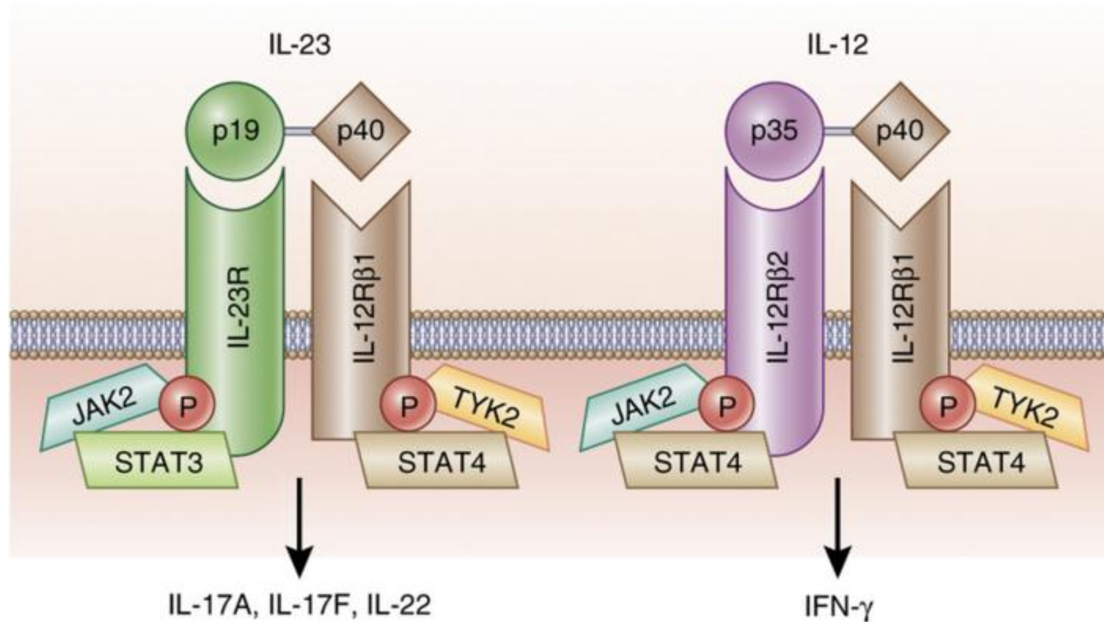


Figure (1-2). Structure of IL-12 cytokine (adapted from Teng *et al*., 2015).

Using a meta-analysis of 17 articles, (Yang *et al.*, 2013) reported a significant association of this SNP with overall cancer. When the authors stratified the results by regions, the A allele of the SNP was significantly associated with a decreased risk of overall cancer among the Asian population, but not among Americans and Europeans. A somewhat different result was reported by Lan *et al.*, (2006) who did not found such association with NHL among American population; whereas Lan *et al.*, (2011) revealed a strong association between the SNP IL-12A rs485497 with the disease among Australian population with AA genotype predisposes for NHL (OR= 1.51, 95%CI= 1.15-1.990, *P*=0.0033).

1.2.8 Immune Response in NHLs

As the incidence of these group of neoplasms increases in individuals with various types of immune-deficiencies, it is supposed to be a great role of immune system against NHLs. This role of immune system may be related to the fact that high percentage of NHLs are associated with infection especially viral infection. Dysregulation in cytokines profile (especially those related to cell-mediated immune response) is the characteristic of several subtypes of NHLs. Serum levels of several cytokines have been shown to be elevated in NHLs, and for some of these cytokines, circulating levels predict disease outcome (Gu et al, 2012). However, temporal fluctuation in cytokine levels suggests that measurement of these cannot be reliable to interfere with etiology or confirm diagnosis of the disease.

As early as 1993, the levels of IL-10 (Th2 cytokines) in serum was investigated (Blay *et al*., 1993) using an ELISA, which detects human IL-10 in patients with active NHL and healthy volunteers. They described the detection of IL-10 in serum from about 50% of these patients but none of the control blood donors. IL-10 was detectable with a similar frequency in all subtypes of NHL and in all clinical stages, as well as in both EBV-seropositive and EBV-seronegative patients. One year later Stasi *et al*, (1994) demonstrated similar results obtained in patients with aggressive NHL.

Serum concentration of IL-12p40, a subunit of IL-12, which is an important cytokine for Th1 development (Watford *et al*., 2003), was positively associated with both FL and DLBCL in single cytokine and PCA analyses. This cytokine was also examined in three of the prior nested case-control studies of all NHLs and no associations were found with NHL risk (Rabkin *et al*., 2011), raising the possibility that IL-12p40 may be elevated as the result of lymphoma, either by lymphoma cells

themselves or other cells in the tumor microenvironment.This is Probably due to its ability to enhance innate and cellular immunity.This cytokine has been demonstrated to be a useful mediator of anti-tumor response (Lasek et al, 2014). However, a recent study found that IL-12 induced T-cell exhaustion and decreased immune function in follicular lymphoma (Yang *et al*., 2012).

For the other cytokines, Gu *et al.*, (2010) studied the association of fifteen cytokines with NHLs. Only five of these were found to be affected. Three of them are Tumor necrosis factor α (TNF-α), soluble tumor necrosis factor-receptor-2 (sTNF-R2) and soluble interleukin 2 receptor (sIL-2R) were found to be positively associated with the disease. On the other hand, IL-13 and IL-5 were negatively associated.

1.2.9 Disease Site

The usually arise or present in lymphoid tissues, such as lymph nodes, spleen, and bone marrow, but they may arise in almost any tissue. The most frequent sites for extranodal lymphomas, which constitute about 20% to 30% of all lymphomas are the stomach, skin, oral cavity and pharynx, small intestine, and central nervous system (CNS) (Yuen and Jacobs, 1999). Although primary CNS lymphomas are rare (3% to 4% of CNS), there has been a threefold increase in incidence from the 1960s to the mid-1990s, even if patients with HIV infection and other types of immunosuppression are excluded. However, there has been an overall decline in the incidence of primary CNS lymphomas in the United States since 1995, driven mainly by the changing incidence in young and middle-aged men (Marawa and Ragan, 2007). The reason for the preferential involvement of specific extranodal sites at recurrence is not clear, but it is likely that this is closely linked to the homing process

regulated by homing receptors or lymphoid cells and ligands on high endothelial venules (Advani *et al*., 1990).

1.2.10 NHLs Presentation

1.2.10.1 Nodal Lymphoma

Painless enlargement of a lymph node is the most frequent presentation of NHL, and the most common site is the neck. Sometimes the nodes fluctuate in size, which can lead to delay in diagnosis. NHLs tend to be present at more unusual lymph node sites, for example, Waldeyer's ring. (Nelson, 2016). Although the presentation is usually straightforward, the enlarging lymph node mass may cause initial symptoms due to compression, for example, swelling of the arm or leg, causing deep venous thrombosis or superior vena caval obstruction (SVCO). Retroperitoneal lymph node enlargement can result in backache and obstructive renal failure, and nodes in the porta hepatis may cause obstructive jaundice (Greer *et al*., 2014).

Widespread infiltration of the liver is usually accompanied by weight loss, anorexia and fever. Even without liver involvement, constitutional symptoms are not infrequent, with weight loss, night sweats and fever. Intra-abdominal lymphoma may present with fever of unknown origin, especially if there is hepatic or bone marrow involvement.

1.2.10.2 Extranodal Presentation

NHLs may present at a variety of extranodal sites, and when they do they often pose particular problems in diagnosis and management. In addition to presentation at an extranodal site, there may be involvement of these sites later in the course of the disease, at a time when a nodal lymphoma

relapses and disseminates. In this latter circumstance, this is usually part of a generalized spread of the disease (Greer *et al*., 2014).

Lymphomas may occur at any site in the gastrointestinal tract but the stomach and the small intestine are most frequently involved. The presentation of gastric lymphoma is similar to that of adenocarcinoma of the stomach, with nausea, anorexia and upper abdominal discomfort as the chief symptoms and occasionally haematemesis or chronic iron-deficiency anemia as associated features. Barium meal shows a large gastric ulcer or appearances similar to adenocarcinoma of the stomach. On endoscopy, a malignant ulcer is usually seen, but occasionally the appearance can simulate a benign gastric ulcer (Nelson, 2016).

NHL not infrequently invades the marrow and may sometimes cause localized bone lesions with pain, vertebral collapse and pathological fractures. Occasionally, NHL presents as a primary bone lesion, and most of these cases are of large-cell lymphoma (Zhang and Foucar, 2009). Sometimes the lesion is localized with no evidence of NHL at other sites, even after extensive investigations.

1.2.11 Laboratory Diagnosis of NHLs

Biopsies obtaind from mass or bone marrow are mostly used as favorable sample for investigation for NHLs. Laboratory diagnosis and classification mainly depends on four types of investigations:

1.2.11.1 Flow Cytometry (FCM)

Immunotyping of different cells using FCM relies upon the detection of individual cells in liquid phase by using specific fluorescently labeled

antibodies for certain antigens. These antibodies are then excited by laser to emit light at varying wavelengths.

In B-cell NHL, the expression of Ig κ and λ light chains is evaluated. When the κ:λ ratio is greater than 4:1 or less than 1:2, it is highly suggestive for NHL (Kaleem, 2001). Many other cluster differentiation (CD) markers are widely used especially for classification purposes. Of these CD5, CD10 and CD23 are mostly exploited. The range of these markers could be extended for T-cell and NK-cell lymphomas to involve CD1, CD2, CD4, CD5, CD7, CD8, CD16, CD56 and CD57 (Kaleem, 2006).

1.2.11.2 Immunohistochemistry (IHC)

Similar to FCM, the aim of IHC is the detection of antigen expression on cells in the specimens. However, FCM is superior to IHC because the analysis in the latter evaluates single antigen usually at one time whereas large numbers of antigens could be detected at one time using FCM. Anyhow, there is a wide range of markers for IHC, choosing of which depends on the morphologic differential diagnosis (Sharma *et al*., 2014). For B-cell, the most commonly used marker is CD20 which is expressed from naïve B-cells until the final stages of B-cell development. In B-cells from lymphoma, CD20 is positive (Torlakovic *et al*., 2002). The other important marker is Pax-5 (also known as B-cell-specific activator protein). This marker is expressed in the early stages of B-cell development. In certain subtypes of NHLs especially those associated

with *t* (8;21) translocation, there is a strong expression of Pax-5 (Desouki et al., 2010).

The most commonly used CD markers for T-cells is CD3 which is expressed at the second stage of the thymic differentiation on T-cells and NK cells. This marker may be lost in certain subtypes of NHLs especially ALCL (Bonzheim *et al*., 2004). CD5, on the other hand, is a T-cell antigen which is not expressed on NK cells. Loss of CD5 expression on T-cells is highly suggestive for T-cell lymphomas (Higgins *et al*., 2008).

1.2.11.3 Cytogenetic Analysis

Fluorescent *in situ* hybridization (FISH), blot analysis and sequencing were frequently used for detection of genomic lesion in NHLs. However, these methods suffer from several limitations such as the dependence on the availability of dividing cells, resolution restriction, and application to candidate region of the genome (Najfeld, 2003).

New methods were introduced to overcome these limitations. Comparative genomic hybridization (CGH) and SNP microarrays are of particular importance in this regards. These methods provides the opportunity for detection of several types of genetic lesions by progressively expanding the resolution of DNA analysis (Sato-Otsubo *et al*., 2012; Gowda and Dovat, 2013; Iacobucci *et al*., 2013).
Using SNP microarrays,(Takeyama et al, 2008), for example, found a SNP locus in chromosomal location 15q15 encoding for P53-Binding protein1 in only single copy in 14.5% cases of DLBCL, while Conde *et al* , (2014) revealed a duplication in chromosomal region 11q25 in 6.2% of this malignancy.

1.2.11.4 Molecular Testing

Procedures using PCR have many advantages. They require only a small amount of DNA or RNA, are relatively rapid, can detect abnormalities at a very low levels, and can be performed on paraffin-embedded tissues as well as fresh and frozen tissues (Arber, 2000). However, these methods can only employed for diagnosis of only certain subtypes of NHLs.

Using real-time PCR, Medeiros *et al*., (2002) assessed the level of cyclin D1 overexpression for diagnosis of mautle cell lymphoma. This protein results from *t* (11;14) translocation which is prevalent in 50 %-60% by PCR and 70%-75% by cytogenetics in this subtype of NHL , They found that MCL had higher cyclin D1 mRNA levels than other subtypes of NHLs or nonneoplastic specimens without any overlap.

Of note, hematological alterations have a very limited benefit for NHL diagnosis. When there is bone marrow involvement, anemia or thrombocytopenia are present, but, of course, they have no diagnostic importance. However, anemia may be adverse prognostic factor in some subtypes of NHL (Moullet *et al*., 1998).

CHAPTER TWO

Materials and Methods

Materials and Methods

2.1 Equipment and Instruments

Equipment and instruments used in this study are listed in Table (2-1).

Table (2-1). Equipment and instruments used in the study with their manufacturers and country of origin.

Instrument and Equipment	Company, Country
Autoclave	Tomy, Japan
Bench centrifuge	VEB, Germany
Electrophoresis equipment	Consort, Belgium
Laminar flow cabinet (Hood)	Labogene, Denmark
Microcentrifuge	Eppendorf, USA
Microtome	Lieca, Sweden
Micropipette 0.5-10, 20-200, 100-1000 µl	Biobasic, Canada
Nanodrop/UVS-99	ACTGene, USA
Oven	Sanyo, Japan
Plane tubes	Afco-Dispo, Japan
Sensitive balance	Sartorius, Germany
Shaker	Bioneer, Korea
Spectrophotometer	Shimadzu, Japan
Thermocycler	Bioneer, Korea
U. V. transilluminator and camera	Flowgen, U.K.
Vortex	Lab-Kits, Korea
Water bath	Labtech, Korea

2.2 Chemicals and Biological Materials

Chemicals and biological materials used in this study are listed in Table (2-2).

Table (2-2). Various chemical and biological materials used in the study.

Chemical	Company, Country
Absolute ethanol	Pharmacia, Sweeden
Agarose	Biobasic, Canada
DNA ladder (1000bp)	Kappa Biosystems, USA
Deionized water	Bionee, Korea
Ethidium bromide	Biobasic, Canada
Isopropanol	BioBasic, Canada
Loading dye	Biobasic, Canada
Master mix	Bioneer, Korea
Primers	Bioneer, Korea
Tris-Borate EDTA	BioBasic, Canada

2.3 Kits

DNA and ELISA kits used in this study are shown in Table (2-3).

Table (2-3): Kits used in the study.

Kits	Company, Country
DNA extraction from blood	gSYNC™ DNA Mini Kit Whole Blood Protocol, Geneaid, Korea
ELISA kit for estimation of serum level of IL-12p40	Cusabio, China
ELISA kit for estimation of serum level of IL-10	Cusabio, China

2.4 Primer Sets

A total of three primer pairs were used in this study as illustrated in Table (2-4).

Table (2-4): Primer sets sequences and their corresponding genes

Genes	Primers 5'→3'	Fragments	References
IL-10	Consensus Reverse: GTAAGCTTCTGTGGCTGGAGTC Wild-Forward: AACACTACTAAGGCTTCTTTGGGT**A** Variant-Forward: AACACTACTAAGGCTTCTTTGGGT**G**	161 bp	Bhayal *et al.*, 2012
IL-12p40	Consensus F: ATCTTGGAGCGAATGGGCAT R1: TTGTTTCAATGAGCATTTAGCATCT R2: GTTTCAATGAGCATTTAGCATCG	780 bp	Latsi *et al.*, 2003
TLR2 (internal	F: CCTGGCAAGTGGACCATTGAC R: GGCCACTCCAGGTAGGTCTT	254 bp	Chen *et al.*, 2012

control)			

2.5 Study Subjects

This case control retrospective study included 55 patients with ages between six months to seventy five years confirmed NHL during the period from start of January to end of June 2015 were collected from Baghdad hospital/Medical city, Central teaching hospital of paediatric and National center of hematology, Al-Mustansiriya University in Baghdad's city. unrelated, apparently healthy 40 individuals were selected to represent the control group. The mean ages of patients and control were 33.45 years and 36.29 years, respectively. Informed consents from patients as well as control were taken which included age, sex, previous and current occupation, smoking, drinking, residence, and first relative family history of NHL.

case

2.6 Samples

2.6.1 Blood Samples

Five ml of venous blood was collected from each participant; 2 ml of which was kept in EDTA tube and the other 3ml in plane tube. The latter was undergone centrifugation where the serum was obtained and preserved at -20°C until be used.

2.6.2 DNA Extraction

DNA was extracted from blood samples using ready kit (gSYNC™ DNA Mini Kit Whole Blood Protocol, Geneaid, Korea) according to the manufacturer's instructions as follows:

A- A total of 200 µl of blood was transferred to a 1.5 ml microcentrifuge tube.

B- Twenty µl of proteinase K was added to the sample, mixed by pipetting, and incubated at 60°C for 5 min, after which a GSB Buffer (200 µl) was added to the 1.5 ml microcentrifuge tube and mixed by shaking vigorously.

C- The tubes were incubated at 60°C for 5 minutes. During incubation, they were inverted every 2 minutes (it is essential that the sample and GSB Buffer are mixed thoroughly to yield a homogeneous solution). At this time, the required elution Buffer (100µl/sample) was pre-heated at 60°C (for step K DNA elution).

D- Absolute ethanol (200 µl) was added to the sample lysate and immediately mixed by shaking vigorously for 10 sec. If precipitate appears, it was broken up as much as possible with a pipette.

E- The GD Column was placed in a 2 ml collection tube, and the lysate from step C (including any precipitate) was transferred to the GD Column, and centrifuged at 14000 g for 1 minute, or until the mixture passes the GD Column completely.

F- The 2 ml collection tube containing the flow-through was discarded, and the GD column was placed in a new 2 ml collection tube, where W1 buffer was added (400 µl) to the column which was centrifuged at 14000 g for 30 sec.

G- The flow-through was discarded, and the GD column was placed back in the 2 ml collection tube, where wash buffer was added (600 µl) to the GD column, which was again centrifuged at 14000 g for 30 sec.

H- The flow-through was discarded, and the GD column placed back in the 2 ml collection tube.

I- To dry the column matrix, the GD column was centrifuged at 14000 g for 3 min.

J- The dried column was transferred to a clean 1.5 ml microcentrifuge tube, and 100 µl of pre-heated elution buffer was added to the center of the column matrix, and let stand at least 3 min to ensure the elution buffer is absorbed by the matrix.

K- The microcentrifuge tubes containing the GD column was centrifuged at 14000 g for 30 sec to elute the purified DNA.

Eluted DNA was stored at -20°C until be used for PCR.

2.6.3 Measurement of the concentration and purity of extracted DNA

A nanodrop (UVS-99, ACTGene, USA) was used to measure the concentration and purity of the extracted DNA (from blood patients, and control samples) according to the following procedure:

2.6.3.1 Blanking

i- After lifting the sampling arm, 2 µl of elution buffer was pipetted onto the measurement pedestal.

ii- The sampling arm was laid down and the solution was observed bridging the gap between both optical fibers.

iii- When the "Blank" button was clicked, the apparatus measured the solution with both 1mm and 0.2 mm path lengths, and then the system recorded both results automatically.

iv- When the measurement completed, the sampling arm was opened and the blanking buffer was wiped from pedestal using laboratory wipe.

2.6.3.2 Sample Measurement

i- Two µl of the sample was pipetted onto the measurement pedestal while the sampling arm is opened.

ii- The sampling arm was laid down and the solution was observed bridging the gap between both optical fibers.

iii- The button "Measure" was clicked and the measurement result appeared on the screen within 3 sec.

When any sample gives purity less than 1.5 and/or concentration less than 10 ng/µl, DNA extraction from the blood of that sample re-performed until the desired purity and concentration were obtained. Blood samples satisfied these criteria from the first extraction. .

2.7 PCR Protocols

Extracted DNA from blood samples was used in PCR for amplification of *IL-10* and *IL-12p40* genes.

2.7.1 *IL-10* Gene

A pair of primers specific for *IL-10* gene (Table 2-4) were used. The PCR protocol and amplification conditions are shown below in Table (2-5):

Table (2-5). PCR amplification conditions of IL-10 genotypes.

Step	Temperature and duration	
Initial denaturation	95°C for 5 min	1 cycle

Denaturation	94°C for 30 sec	35 cycles
Annealing	63°C for 30 sec	
Elongation	72°C for 30 sec	
Final elongation	72°C for 5 min	1 cycle

2.7.2 *IL-12p40* Gene

For PCR amplification of *IL-12p40* gene, the following PCR protocol and amplification conditions are outlined in Table (2-6).

Table (2-6). PCR amplification conditions of IL-12p40 gene.

Steps	Temperature and duration	
Initial denaturation	95°C for 5 min	1 cycle
Denaturation	94°C for 30 sec	30 cycles
Annealing	61°C for 40 sec	
Elongation	72°C for 1 min	
Final elongation	72°C for 7 min	1 cycle

A ready 50 µl PCR master mix (Bioneer, Korea) was used for amplification for both genes. Components of each master mix are outlined in Table (2-7).

Table (2-7). Components of the Mastermix of PCR reaction.

Component	Quantity/concentration

Taq polymerase	2.5 U
dNTP (dATP, dCTP, dGTp, dTTP)	250 μM
Tis-HCl (pH 9.0)	10 mM
KCl	30 mM
$MgCl_2$	1.5 mM
Stabilizer and tracking dye	No specific concentration

Template DNA (10 μl) from each sample and primers (5 μl from each) were added to each master mix tube. The mixture then put in shaker and spinner for 10 cycles for better mixing. After mixing, the mastermix tubes were transferred to the thermocycler (MyGenie 32 thermal block, Bioneer, Korea) which is previously programmed with the above protocol according to the gene to be amplified.

2.8 Agarose Gel Electrophoresis

Gel was prepared by dissolving 1 gm of agarose (Biobasic, Canada) in 100 ml (1%) of 1X Tris-Borate EDTA (TBE) (Biobasic, Canada), then put in an oven for 5 min. After about 10 min, the gel was poured in the tray of the electrophoresis apparatus. When the gel solidified it was transferred into the tank and enough amount of TBE was poured to just cover the gel. The comb was removed and a 10-μl aliquot of PCR product was mixed with 2 μl loading dye and was loaded into the wells. Power supply was adjusted into 100 volts and run for 1 hour. The gel then was stained with ethidium bromide (Biobasic, Canada) (0.5 μg/ml) for 20 min and examined using UV transilluminator with camera. The amplified products were determined by comparison with a commercial 1000 bp ladder (Kappa Biosystem, USA). In case that any PCR product had negative result in agarose gel electrophoresis,

there will be re-examine of extracted DNA using nanodrop, and DNA re-extraction if the purity is less than 1.5 and/or concentration was less than 10 ng, otherwise the amplification was re-performed and the cause of negativity was attributed to the processing.

2.9 Enzyme-Linked Immunosorbent Assay (ELISA)

A commercial kit (Cusabio, China) was used to measure serum levels of IL-10 and IL-12p40.

2.9.1 Principle of the ELISA Assay

This assay employs the quantitative sandwich enzyme immunoassay technique. Antibody specific for IL-10 or IL-12p40 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any IL-10 or IL-12p40 present is bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for IL-10 or IL-12p40 is added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) is added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of IL-10 or IL-12p40 bound in the initial step. The color development is stopped and the intensity of the color is measured.

2.9.2 ELISA Kit Contents

The components of an ELISA kit for detection of IL-10 and IL-12p40 are outlined in Table (2-8)

Table (2-8). Contents of an ELISA kit used for detection of IL-10 and IL-12p40.

Reagent	**Quantity**
Assay plate (12 x 8 coated Microwells)	1 plate (96 wells)
Standard (Freeze dried)	2 tubes
Biotin-antibody (100 X concentrate)	1 x 120 µl
HRP-avidin (100 X concentrate)	1 x 120 µl
Biotin-antibody Diluent	1 X 15 ml
HRP-avidin Diluent	1 X 15 ml
Sample Diluent	1 X 50 ml
Wash Buffer (25 x concentrate)	1 X 20 ml
TMB Substrate	1 X 10 ml

Stop Solution	1 X 10 ml
Adhesive Strip (For 96 wells)	4
Instruction manual	1

2.9.3 Reagent Preparation

1- All reagents were brought to room temperature (18-25℃) before use within 30 min.
2- Freshly prepared standards were carefully reconstituted to avoid foaming, and were mixed gently until the crystals have been completely dissolved.
3- For Biotin-antibody and HRP-avidin, vials were centrifuged before opening, and 100-fold dilution was done (10 µl of Biotin-antibody+ 990 µl of Biotin-antibody diluents and HRP-avidin diluents, respectively).
4- Twenty ml of Wash Buffer Concentrate was added to 480 ml of D.W. to prepare 500 ml of Wash Buffer.
5- Standards were centrifuged at 6000 rpm for 30 sec and then reconstituted with 1 ml of Sample Diluents. This reconstitution produced a stock solution of 200 pg/ml. The Standard then mixed to ensure complete reconstitution.

2.9.4 Standard Curve

After about 15 minute of standard reconstitution, 250 µl of Sample Diluents was pipetted in each of 7 Eppendorf tubes. A same volume (250 µl) of stock solution was added to the first tube, which then mixed thoroughly before the transfer of 250 µl to the next tube. This procedure resulted in a 2-fold dilution series. The undiluted standard serves as the high standard (200 pg/ml), while sample diluents represents the zero standard (0 pg/ml). The optical density of the seven tubes was measured using microplate reader set at 450 nm.

A standard curve was constructed by plotting the absorbance of each tube on the x-axis against the concentration on the y-axis. The best fit curve was drawn through the points on the graph for IL-10 (Figures 2-1) and for IL-12 (Figure 2-2).

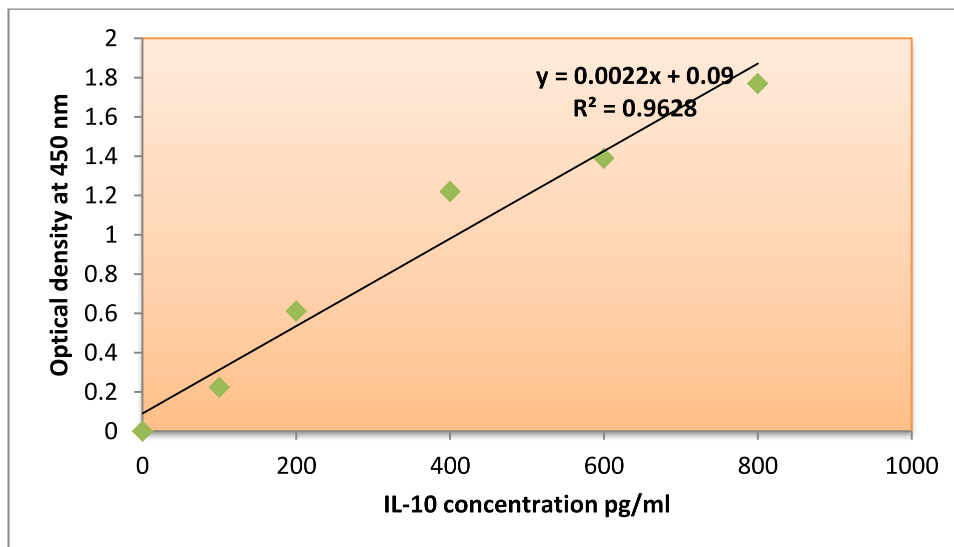


Figure (2-1). Standard curve of IL-10.

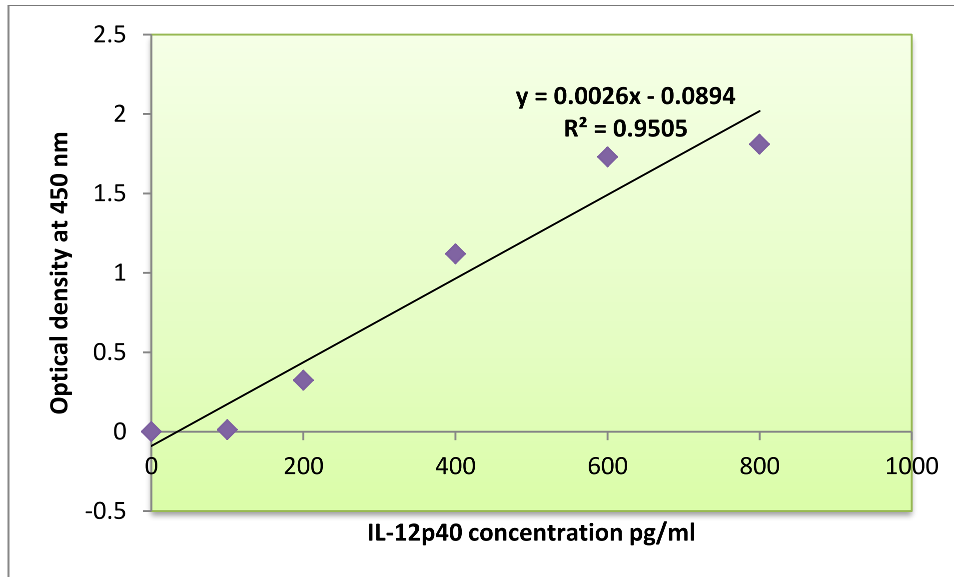


Figure (2-2). Standard curve of IL-12p40.

2.9.5. Cytokines (IL-10 and IL-12p40) Assay Procedure

The following steps were adapted according to the manufacturer's manual:

1- One hundred µl of Standard and serum samples was poured for the first and the rest wells of the plate respectively. The plate was covered with adhesive strip and incubated for 2 hrs at 37℃.

2- The liquid was removed from the wells without washing, and 100 µl of Biotin-antibody was added to each well. The plate was again covered with a new adhesive strip and incubated for 1 hr at 37℃.

3- The content of each well was aspirated and the plate was washed. This process was repeated three times, after which each well was filled with 200 µl of Wash Buffer using multichannel pipette. The plate was left to stand for 2 min, after which the liquid was removed and the plate was inverted against clean filter paper.

4- HRP-avidin (100 µl) was added to each well and the plate was covered with a new adhesive strip and incubated for 1 hr at 37℃.

5- Step 3 (aspiration and washing) was repeated 5 times.

6- Ninety µl of TMP Substrate was added to each well, and the plate was incubated for 30 min at 37℃ protected from light.

7- Stop solution (50 µl) was added to each and the plate was gently taped to ensure thorough mixing.

8- The optical density was determined for each well within 5 min using microplate reader (Diagnostic Automation Inc, USA) set at 450 nm.

9- Optical densities were converted into concentrations using the standard curve.

2.10 Statistical Analysis

Data were expressed as a mean± standard deviation. The Statistical Package for the Social sciences (SPSS, version 14) was used for statistical analysis. Risk association between the genotype and NHL susceptibility was estimated by the calculation of adjusted odd ratio (OR) and 95% confidence intervals (95%CI) using multivariate logistic regression. Chi-square was used for testing the deviation from Hardy-Weinberg equilibrium, distribution of different alleles between patients and control, and to compare serum levels of IL-10 and IL-12p40. A p-value < 0.05was considered statistically significant.

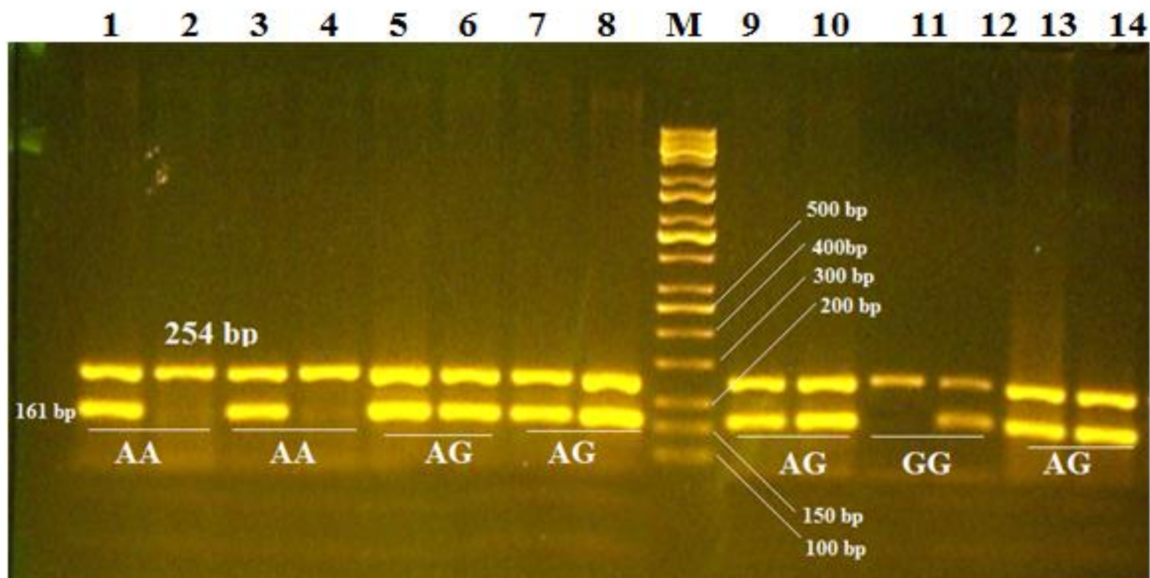
CHAPTER THREE

Results and Discussion

3.1 Allele Specific PCR for detection of IL-10 -1082A/G and IL-12p40 1188A/C

3.1.1 Allele Specific Detection of IL-10-1082A/G

Figures 3-1 and 3-2 show the results of allele specific PCR for the SNP IL-10 -1082A/G in NHLs patients and controls, respectively.



receptor-2 (TLR-2) gene. IL-10 A/G polymorphism of patients' samples run side-by-side and shown in in lane order (1, 3, 5, 7, 9, 11 and 13) for IL-10A alleles and in lanes (2, 4, 6, 8, 10 and 14) for IL-10G alleles.

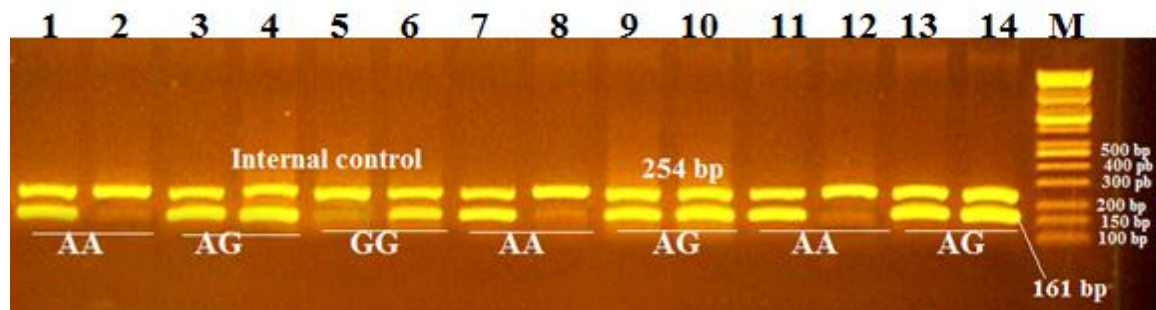


Figure (3-2). Genotyping of IL-10 -1082A/G patterns in control healthy Experimental conditions of PCR, gel electrophoresis and detection are similar to those described in

Figure legend 4-1. IL-10 A/G polymorphism of controls' samples run side-by-side and shown in lanes (1, 3, 5, 7, 9, 11 and 13) for IL-10A alleles and in lanes (2, 4, 6, 8, 10 and 14) for IL-10G alleles.

The SNP IL-10 -1082A/G had three genotypes; AA, AG, and GG. In NHL patients, it was found that these genotypes account for 20 (36.36%), 25 (45.46%), and 10 (18.18 %), respectively, compared to 13 (32.5%), 14 (35%), and 13 (32.5%) respectively, in control group. After the analysis of results there was insignificant differences neither for heterozygous genotype (OR=2.0, 95%CI=0.679-5.892 *P*=0.209) nor for homozygous mutant genotype (OR=2.321, 95%CI=0.810-6.650, *P*=0.117).

Analysis of allele frequencies of this SNP (IL-10 -1082A/C) revealed insignificant differences in the frequency of A allele between NHL patients and controls (59.09% and 50%, respectively). Similar results were obtained for the frequency in G allele of the two groups (40.91% and 50%, respectively, OR=1.444, 95%CI=0.809-2.580, *P>0.05*). Accordingly, there was no significant difference in the distribution of A and G alleles between NHL patients (59.09% and 40.91%, respectively) and controls (50% each) as shown in Table 3-1.

Table (3-1): Genotypes and alleles distribution of SNP IL-10-1082A/G in NHL patients and in control individuals.

Variables	Cases N=55	Control N=40	*P*-value	OR(95%CI)
Genotypes				
AA	20(36.36%)	13 (32.5%)	0.27	1.0
AG	25(45.46%)	14(35%)	0.209	2.0(0.679-5.892)
GG	10(18.18%)	13(32.5%)	0.117	2.321(0.810-6.650)

Alleles			0.214	
A	65(59.09%)	40(50%)		1.0
G	45(40.91%)	40(50%)		1.444 (0.809-2.580)

Abbreviations: N, number; OR, odds ratio; CI, confidence interval, *P*, calculated probability (*P* value).

These results are in consistent with that of Talaat *et al*. (2014) where they found no association between the two SNPs (-1082 and -819) studied in the promoter region of IL-10 gene and DLBCL among Egyptian population. Similarly, these results are in consistent with a recent investigation conducted by Lim *et al*. (2015) who found no association of IL-10 -1082A/G polymorphism with the susceptibility to NHL in three major races of the Malaysian population. It appears that the issue of association between the IL-10 polymorphism and the susceptibility to NHLs are still controversial and not conclusive, where other studies have shown that the frequency of the low-IL-10 producing AA allele at position -1082 was significantly higher in patients with aggressive NHL compared to the healthy controls (Cunningham *et al*., 2003, Bogunia-Kubik *et al*., 2008). In addition, this type of association is even get more complicated by recent findings (Ahmed *et al*., 2014), which have shown a strong link between allele G of this SNP and the high risk of developing NHL in Egyptian subjects. Furthermore, a recent meta-analysis study of 7794 NHL cases and 8584 controls has demonstrated that the significant high risk to NHL was associated with higher distribution of G allele of IL-10 -1082A/G polymorphism (OR= 1.22, 95%CI= 1.08-1.39) (Dai et al.,2014). Thus it seems that multiple factors are involved in the differences among these data about NHL such as race, sample size and probably the statistical method used. Taken all these factors together, if such

an association exists, it is more likely to be linked to the G allele and its effect on the gene expression and production of IL-10. Indeed, genetic factors may relatively determine the high level of IL-10 expression, while other environmental factors like smoking and BMI may influence the disease pathogenesis and outcome (Reuss *et al*., 2002). It has been shown that there is a stronger binding affinity of the -1082A allele to specific transcription factor (PU.1) compared to the G allele. Thus, A allele partially inhibits gene expression (Reuss *et al*. 2002). Although it has been showed that IL-10 expression was less efficient in transformed cell with A allele using monocyte cells. This allele (A) was found to be very weak to exert its effect in whole blood and the SNP may probably explain why only a small fraction of IL-10 produced. Accordingly, it is plausible to say that IL-10 -1082 polymorphism has no significant association with the susceptibility to NHL in Iraqi patients.

3.1.2 Allele specific detection of IL-12p40 1188A/C by PCR

This study was extended to examine the role of IL-12 1188A/C polymorphism in the susceptibility of patients to NHL in comparison to healthy individuals. Figures 3-3 and 3-4 demonstrate clearly the results of allele specific PCR for the SNP of IL-12p40 1188A/C in NHL patients and controls, respectively.

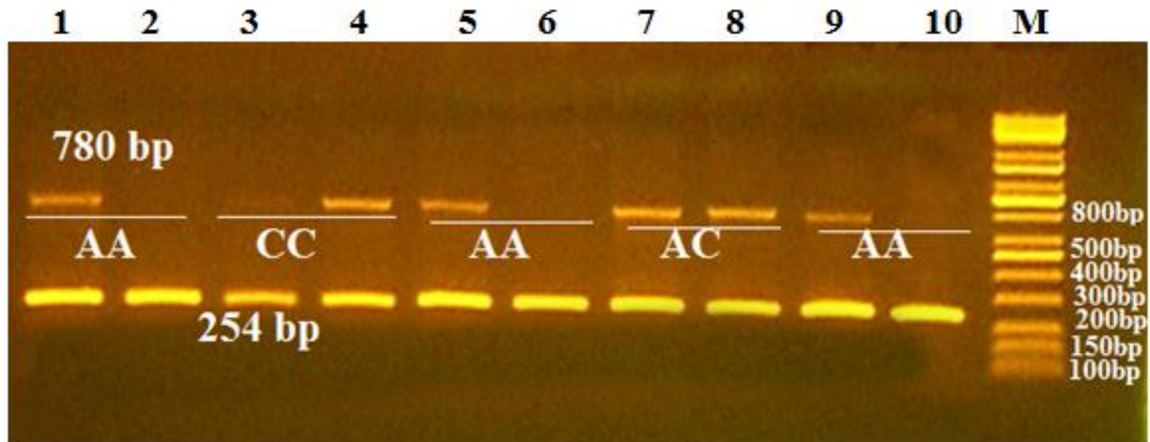


Figure (3-3). Genotyping of IL-12p40 1188A/C allele's distribution in patients with NHL The 780 bp represents the amplification of *IL-12p40* 1188A/C, while the 254 bp represents the amplification of TLR2 gene as an internal control. are 1, 3, 5, 7,9, and for IL-12p40 C alleles are in lanes 2, 4, 6, 8, and 10.

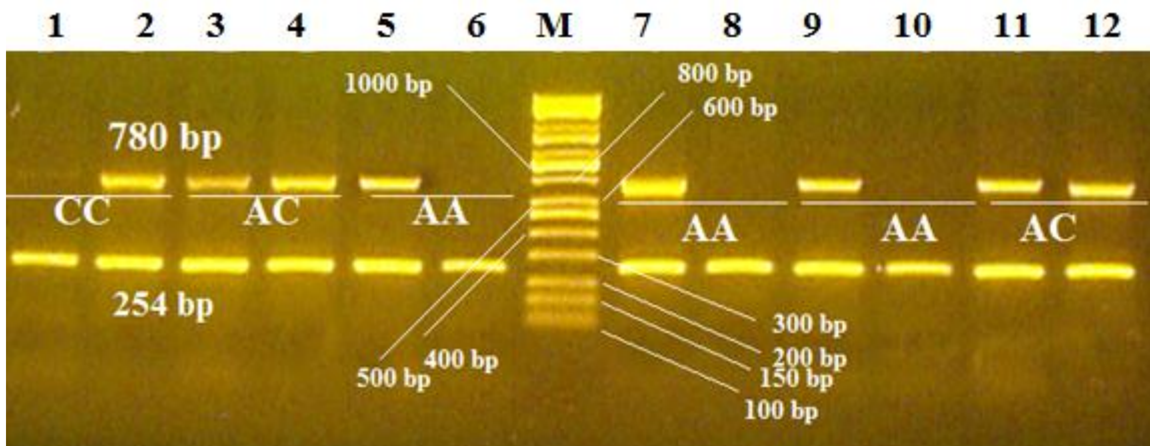


Figure (3-4). Genotyping of IL-12p40 1188A/C allele's distribution in health controls. The 780 bp represents the amplification of *IL-12p40* 1188A/C, while the 254 bp represents the amplification of TLR2 gene as an internal control. Similar to above Figures, the IL-12p40 1188A/C run side-by-side on the gel and the lanes order for IL-12p40 A are 1, 3, 5, 7, 9 and 11 and for IL-12p40 C alleles are in lanes 2, 4, 6, 8, 10, and 12.

Similar to IL-10, IL-12p40 1188A/C has also three genotypes which are AA, AC and CC. Results of this part of the study showed that these genotypes were represented in 32.37%, 38.18% and 29.09%, respectively in NHL

patients, while in control individuals, these genotypes appeared in 52.5%, 37.5% and 10%, respectively. These results demonstrated a significant difference between the patients versus the controls for the homozygous mutant genotype CC (OR= 4.667, 95%CI = 1.319-16.512, *P*= 0.017). At the allelic level, the frequency of C allele in NHL patient was 48.18% compared to 28.75% in controls and this difference was significant (OR= 2.304, 95%CI= 1.250-4.249) (Table 3-2).

Table (3-2). Genotypes and alleles of IL-12p40 1188A/C polymorphism in patients with NHL compared to healthy individuals.

Variables	Cases N=55	Control N=40	*P*-value	OR (95%CI)
Genotypes				
AA	18 (32.73%)	21 (52.5%)	0.056	1.0
AC	21 (38.18%)	15 (37.5%)	0.293	1.633 (0.655-4.074)
CC	16 (29.09%)	4 (10%)	0.017	4.667 (1.319-16.512)
Alleles			0.007	
A	57 (51.82%)	57 (71.25%)		1.0
C	53 (48.18%)	23 (28.75%)		2.304 (1.250-4.249)

Abbreviations: N, number; OR, odds ratio; CI, confidence interval, *P*, calculated probability (*P* value).

The SNP IL-12p40 1188A/C is located in the 3'UTR of the coding gene. This region although does not encode for protein, it can influence the amount of translated protein through several mechanisms including the

effect on the stability of mRNA and on the transcriptional/posttranscriptional activity (Matoulkova *et al*., 2012). Therefore, the SNP may influence the gene silencing and may regulate the level of IL-12 mRNA expression (Kaarvatn *et al*., 2012). IL-12 lower levels in healthy individuals was consistently shown to be associated with the AA genotype (Peresi *et al*., 2013). In addition, the SNP IL-12p40 1188A genotype was also shown to be correlated with the reduced levels of IL-12p40 subunit of IL-12. On the contrary, the SNP IL-12p40 1188C genotype was correlated with an increase of production of this subunit (Seegers *et al*., 2002; Wong *et al*., 2012). In this study, the CC genotype was demonstrated to be linked with a higher susceptibility to NHLs. On the other hand, the A allele but not C of IL-12p40 SNP was associated with different cancers among which NHLs (Yang *et al*., 2013). In addition, the discrepancy of the increased production of IL-12 does not mean that IL-12p70 is overproduced rather than the induction involves only the IL-p40 subunit. The homodimers of this subunit antagonizes IL-12p70 activity by binding to the β1 subunit of the IL-12 receptor (Ling *et al*., 1995). Therefore, the increased production of this subunit, in fact, causes reduction in the activity of IL-12 and hence reduces the efficiency of host-mediated response and increases the susceptibility to the malignancy. Another possibility that the effect of IL-12p40 homodimers on the activity of IL-23 as these homodimers have high affinity to IL-23 receptor and may abolish IL-23 effect in immune response (Shimozato *et al*., 2006) which leads to production of IL-17 (Aggarwal *et al*., 2003) which in turns will enhance immunity against NHLs (Lu *et al*., 2016).

3.2. Serum levels of IL-10 and IL-12p40

A randomly selected 48 serum samples from NHL patients and 40 samples from healthy controls were used for measuring the levels of IL-10 and IL-12p40 in patients' and controls' sera by using an ELISA kit.

Figure 3-5 shows the mean serum levels of IL-10 in NHL patients and controls. The levels of IL-10 in the sera from NHL patients were significantly higher (458.31±241.126pg/ml) than that of the control group (323.46±242.344 pg/ml) (t=2.606, P>0.05).

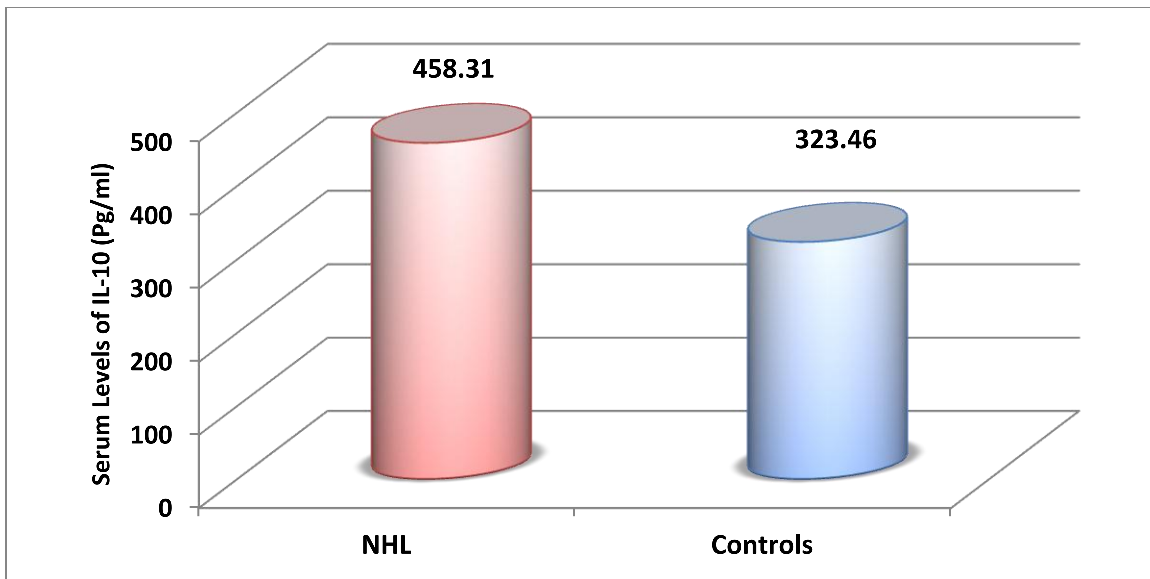


Figure (3-5). IL-10 levels in sera from NHL patients and controls, as measured by an ELISA kit.

Analyses of IL-10 levels among the three genotypes AA, AG and GG in sera from NHL patients revealed that individuals having AG genotype had higher levels of IL-10 (512.03± 205.65pg/ml), followed by GG genotype (489.86±317.55pg/ml) and then AA genotype as being the lowest (381.08±223.14 pg/ml). However, the differences were insignificant among the three genotypes (F=1.54, P=0.225) (Figure 3-6).

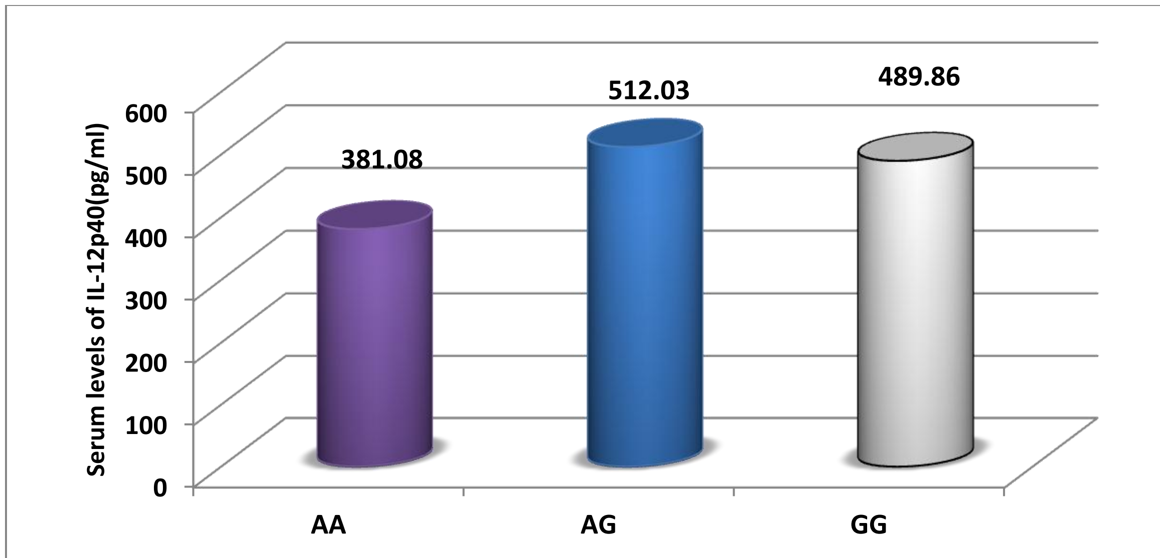


Figure (3-6). The relationship between the levels of IL-10 in sera of individuals and the three genotypes (AA, AG and GG) of the SNP IL-10-1082A/G.

In general, IL-10 levels have been found to be elevated especially in aggressive NHLs and dropped after chemotherapy (Cortes *et al*., 1997; Guney *et al*., 2009; Conroy *et al*., 2013). In this study, although there was an increase of IL-10 level which was associated with G allele, however it was n insignificant. It worth noting that there are other SNPs in the promoter region of the gene which can influence IL-10 production; however, these SNPs are beyond the objectives of the current study and it requires further studies. The elevation of IL-10 could be attributed to different factors associated with NHLs. It has been found that 61% of the malignant cells obtained from NHL patients produce detectable amount of IL-10 protein by using immunohistochemistry and RT-PCR (Voorzanger *et al*., 1996). In addition, all patients that have detectable levels of IL-10 in culture also had elevated serum levels of IL-10 in tumour cells as well as those recruited to the lymphoma microenvironment (Upadhyay *et al*., 2015).

Then, the polymorphism of IL-10 where A allele of IL-10 -1082A/G has shown to bind strongly to transcription factors and may lead to lesser production of IL-10 compared to allele G (Ahmed *et al*., 2014).

To less extend, the involvement of EBV infection, as a risk factor for NHL, with the human IL-10 gene, which has an extensive homology with Bam-HI Cfragment rightward reading frame gene of EBV (viral-IL10) (Vieira *et al*., 1991), may influence the elevation of the total level of IL-10 (Boulland *et al*., 1998). Therefore, a distinguishable detection at the level of gene expression can provide an answer for this synergistic effect.

In regard to IL-12 production in this study, Figure 3-7 shows the mean serum levels of IL-12p40 in NHL patients and controls. NHL patients produced higher levels of IL-12p40 (156.44±54.188 pg/ml) than the control group (107.34±56.957 pg/ml) with a significant difference (t=4.136, *P* >0.001) (Figure 3-7).

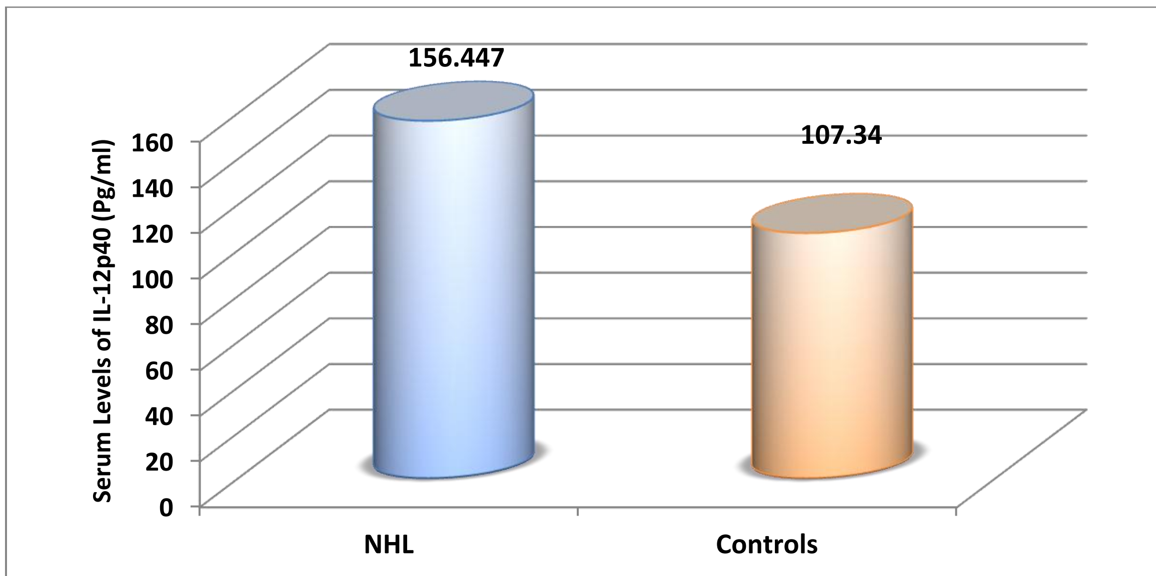


Figure (3-7). Levels of IL-12p40 in the sera of NHL patients and controls as measured by an ELISA kit.

Analysis of the correlation of serum levels of IL-12p40 among the three genotypes (AA, AC and CC) in NHL patients revealed that CC genotype carriers had higher levels of IL-12p40 (119.51± 55.678 pg/ml) than either AC genotype carriers (138.68± 63.102 pg/ml) or AA genotype carriers (155.35± 61.877 pg/ml) with significant differences between CC and AA genotype (*P*>0.05) (Figure 3-8).

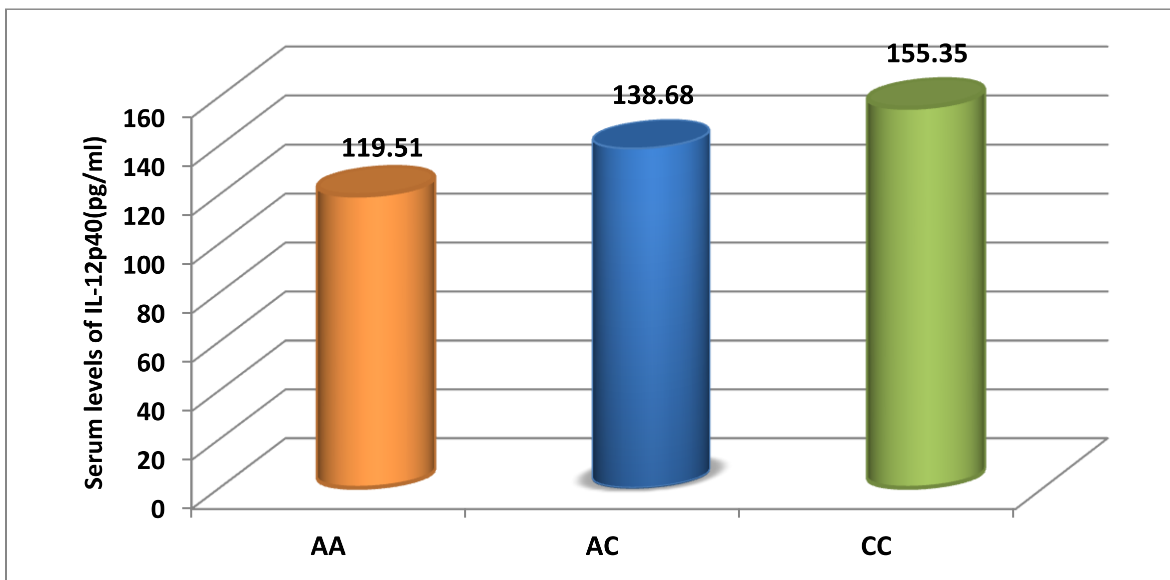


Figure (3-8). The relationship between the levels of IL-12p40 and the three genotypes of the SNP IL-12p40 1188A/C (AA, AC and CC).

The significance of IL-12p40 subunit in CC genotype carriers compared with either AC genotype carriers or AA genotype carriers indicates that the variant IL-12p40 1188C causes an increase in the production of this subunit in an allele-dependent manner. As shown in this study, the increased level of IL-12 was associated with the homozygous mutant genotype (two mutant alleles) and have graded down with the heterozygous genotype (one mutant

allele), to the lowest with the homozygous wild genotype. Whether this elevation can be attributed to the effective role of IL-12 as anti-tumour therapy (Lasek *et al*., 2014) is still not well understood nor conclusive considering the complex nature of other activities of the immune system in cancers and NHLs.

The contradictory findings of IL-12 elevation were reported by a study (Spachacz *et al*., 2003) but using different experimental conditions than this study. The latter have used lymph node tissue microenvironment to assess the total IL-12 production rather than IL-12p40 of this study.

3.3 Risk Factors

Table (3-3) summarizes the association between different risk factors used in this study like age, family history, gender, body mass index (BMI), smoking, residency, and type Ⅱ

diabetes mellitus with the incidence of NHLs. Data obtained from individuals' consent forms were statistically analyzed for all these studied factors and whether these factors had any association with the risks for NHL. In general, all participants have responded that they have never drank alcohol, whereas, there were extreme disparities in occupations that makes nonsense in statistical analysis. Therefore, these latter two factors have been dropped from the analysis.

3.3.1 Age

Since this study's intention was to select control individuals with similar age groups with those of the NHL patients, the effect of age appeared to have

insignificant association with the susceptibility to NHLs (95%CI=0.345-4.104, *P*=0.229).

Table (3-3): Association of risk factors (age, family history, sex, BMI, smoking, residency, and diabetes mellitus) with the incidence of NHLs

Risk Factors	Cases N=55	Control N=40	*P*-value	OR (95% CI)
Mean age in years (SD)	33.45 (4.18)	36.29 (5.22)	0.229	(0.345-4.104)
Family history			0.111	
No	48 (87.27%)	39 (97.5%)		1.0
Yes	7 (12.73%)	1 (2.5%)		5.687 (0.671-48.218)
gender			0.015	
Male	36 (65.45%)	16 (40%)		1.0
Female	19 (34.55%)	24 (60%)		0.352 (0.152-0.817)
Mean BMI (SD)	27.14 (4.27)	23.71 (2.02)	0.028	(1.003- 1.098)
Smoking				
Never	24 (43.63 %)	31 (77.5%)	0.005	1.0
Ex-smokers	16 (29.09 %)	7 (17.5%)	0.005	9.687 (2.018-46.504)
Current smokers	15 (27.28%)	2 (5%)	0.176	3.281 (0.586-18.363)
Residency			0.007	
Urban	50 (90.91%)	27 (67.5%)		1.0
Rural	5 (9.09%)	13 (32.5%)		4.815 (1.551- 14.945)
Diabetes Mellitus			0.317	

Yes	6 (10.91%)	2 (5%)		1.0
No	49 (89.09%)	38 (95%)		2.327 (0.444-12.18)

Abbreviations: BMI, body mass index; CI, confidence interval; N, number; OR, odds ratio; SD, standard deviation.

Generally, the disease is more common in adults than in children and have a steady increase in the incidence from childhood up to the age of 80 years. This pattern may be attributed mainly to the individual's immune status which becomes weaker with older ages. However, certain other risk factors may also influence the incidence of NHL among children, such as exposure to EBV (Sandlund and Onciu, 2014).

3.3.2 Family History

Seven NHL patients (12.73%) have either first or second relatives with NHL compare to 1 (2.5%) of controls. In this study, there was no significant association between the family history and susceptibility to NHL (OR= 5.687, 95%CI= 0.687-48.218, *P*= 0.111). Although it is difficult to draw a conclusive association in this study, previous studies showed a strong familial risk for the most common subtypes of NHL with approximately 2-to-4 folds increased in risk to NHL and FL (Altieri *et al*., 2005; Chatterjee *et al*., 2004).

3.3.3 Gender

The disease was less frequent in females (34.55%) than in males (65.45%) with a significant difference (OR= 0.352, 95%CI= 0.152-0.817, *P*= 0.019),

which means that females have 2.841-fold (1/0.352) less opportunity to have NHL than males. There is almost a general agreement that males are more susceptible than females for this disease with an overall ratio of 2.7:1 for male/female (Burkhardt *et al.*, 2005). Susceptibility to NHL could be attributed to sex hormones (Roemer and Pfreundschuh, 2014). On the other hand, estrogen was shown to inhibit IL-6 secretion which has shown to act as a potent growth factor for intermediate and high grade NHL (Saucedo *et al*., 2002; Rachon *et al*., 2002). On the contrary, those who used contraceptive for more than 5 years have had higher risks to DLBCL and FL (Costas *et al*., 2012).

3.3.4 Body Mass Index (BMI)

The mean BMI, in this study, among NHL patients was 27.14 compared to 23.71 for controls. Results presented as significant association with NHL (95%CI= 1.003-1.098, *P*= 0.028). In both NHL patients and control subjects used in this study, all were within the healthy body mass index; however, the statistical tests revealed a significant difference in susceptibility to NHLs.

3.3.5 Smoking

The smoking status, in this study, was categorized into three categories: never, ex-smokers and current smokers. Among NHL patients, 16 (29.09%) out of 55 were found to be ex-smoker and 15 (27.28%) were current smokers compared to only 7 (17.5%) and 2 (5%), respectively, from the control group. Statistical tests showed a significant association between smoking and NHLs (OR=9.687, 95%CI = 2.018-46.504, *P*= 0.005).

The current study revealed that tobacco smokers (ex-smoker) are at 9.687-fold risk of getting NHL compared to those who have never smoked. Previous studies have indicated that smokers were susceptible to a higher risk for NHL (Stagnaro *et al*., 2004; Schollkopf *et al*., 2005; Diver *et al*., 2014). In contrast, there was no such clear correlation between smoking and NHL (Schroeder *et al*., 2002; Xu *et al*., 2003; Willett *et al*., 2004).

3.3.6 Residency

The vast majority of NHL patients are urban residents (90.91%) compared to 67.5% among the control group with significant difference (OR= 4.815, 95%CI= 1.551-14.945, *P*=0.007). This implies that urban residents (n=50) have 4.8-fold risk to get NHL compared to rural residents (n= 5). Similar results were obtained previously by others (Devesa *et al*., 1992; Muller *et al*., 2005; and Han *et al*., 2010). Many factors could be involved in such association such as exposure to pollutants and/or industrial factors as well as different city life style such as smoking and obesity. On the other hand, pesticides such as DDT and insecticides have been linked with the increased NHL incidence in the country-side living (Brauner *et al*., 2012; Lieberman *et al*., 1998), respectively. Considering such variability in risk factors, the current results although showed a significant association with urban residency but there are only 5 patients were from the rural areas. Therefore, a larger sample size is needed to confirm further such a relationship.

3.3.7 Type 2 Diabetes Mellitus (type-Ⅱ DM)

Six (10.91%) of NHL patients had type-2 DM compared to only 2 (5%) among controls. However, the difference was insignificant (OR=2.317, 95%CI= 0.444-12.18, *P*= 0.317). Due to the sample size that has been

recruited in this study, it is difficult to draw a definite conclusion about the relationship between type 2 diabetes mellitus and NHL in local patients. Therefore, it may worth mentioning that several studies demonstrated a positive association between type 2 DM and NHL (Weiderpass *et al*., 1997; Khan *et al*., 2008; Castillo *et al*., 2012). It is well know that type-2 DM can effectively contribute to NHLs due to many reasons. These reasons may involve its effect on immune dysfunction, the link of insulin and IGF-I with cell proliferation, apoptosis reduction and tumor metastasis and obesity (Frasca *et al*., 2008). All of these factors may contribute to increase the risk and incidence to NHL.

CHAPTER FOUR

Conclusions and Recommendations

4-1 Conclusions

1. Analysis of allele frequencies of IL-10 -1082A/C, for different alleles of the SNP IL-10-1082A/G have no significant effect on the incidence of NHLs.

2. On the other hand, the mean serum levels of IL-10 in NHL patients and controls were significantly higher in patients compared to controls. Although differences among genotypes were not significance, but a pattern of graded level of IL-10 was observed. Patients group with AG genotype presented the highest levels of IL-10, followed by GG and then AA (AG > GG > AA) being the lowest producers.

3. These data suggest that IL-10 -1082A/G polymorphism is not associated with the risk of developing NHL in Iraqi patients. Whereas the elevation of IL-10, in this case, can be attributed to factors other than the SNP itself.

4. The SNP of IL-12p40 1188A/C had three genotypes: AA, AC and CC. Both AC and CC genotypes of the SNP IL-12p40 1188A/C are associated with a significantly increased risk to NHLs among Iraqi patients.

5. Collectively, the mean serum levels of IL-12p40 in NHL patients have a significantly higher levels than the control group. This overproduction of p40 subunit of IL-12 is associated with the CC homozygousity, as being the highest inducer and AA is the lowest (CC > AC > AA) in an allele dependent-manner. This indicates that there is a correlation between the C allele frequency and overproduction of IL-12p40.

6. Additional risk factors such as male gender, increased BMI, smoking, and urban residence are shown to contribute to the risk of NHLs in Iraqi patients.

4-2 Recommendations

In the light of the aforementioned conclusions, the following points are recommended:

1- Conducting a study with a larger sample size to draw solid conclusion about the effect of IL-10 and IL-12 SNPs on NHLs.

2- Conducting larger studies involving different types of SNPs that may be unique for Iraqi populations in order to define the genetic risk factors for different malignancies.

3- Increasing public health awareness about the important risk factors for NHLs through different media and posters.

4- Establishment of Genetic Center for record keeping of all studies in Iraq which are responsible for registration of the results of these studies and providing information for researchers for unification and documentation of all efforts in this field.

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Appendix

Appendix 1

Patients and Healthy Individuals Information Sheet

English	Arabic
Name	الاسم
Age	العمر
Gender	الجنس
Date of Diagnosis Disease Type	تاريخ التشخيص نوع المرض
Family History	تاريخ العائلة
Smoking: Current Smoker / EX-smoker / Not Smoking at all	التدخين: مدخن حالي / مدخن سابق / لا يدخن على الأطلاق
Alcohol Drinking	الكحول
Body Mass Index	معامل كتلة الجسم
Residency	الإقامة
Diabetes Mellitus	إصابة بالسكري
Job	المهنة

I want to donate blood for scientific research purposes to Non Hodgkin Lymphoma Project, therefore I sign below.

Donor Signature

Name of the Consultant:

الزيجة (نسبة الارجحية =4.667، فترة ثقة 95%=1.319-16.512، احتمالية=0.017). وعلى مستوى الأليل فقد كان تكرار الأليل الطافر (C) في مجموعة المرضى (48.18%) أعلى من مجموعة السيطرة (28.75%) وبفرق معنوي عالي (نسبة الارجحية = 2.304، فترة ثقة 95% = 1.25-4.249، احتمالية= 0.007).

أظهرت تقنية ELISA إن لمرضى الأورام اللمفاوية اللاهوجكينية مستويات مصلية من IL-10 و IL-12p40 (458.31±241.126 بيكوغرام/ملم و 156.44±54.188 بيكوغرام /ملم، على التوالي)، أعلى معنوياً من نظيراتها في مجموعة السيطرة (232.13±242.344 بيكوغرام / ملم و 107.34 ±56.957 بيكوغرام/ ملم، على التوالي).

من بين سبعة مخاطر تم دراستها، أظهرت أربعة منها علاقة معنوية مع حدوث الأورام اللمفاوية اللاهوكجينية و هي: الجنس و مؤشر كتلة الجسم والتدخين ومكان الإقامة.

على ضوء نتائج هذه الدراسة يمكن الاستنتاج بأنه ليس للتغاير الجيني IL-10 -1082A/G تأثيراً معنوياً على حدوث الأورام اللمفاوية اللاهوكجينية ولا على المستويات المصلية للسايتوكين IL-10. في حين ان الأليل C للتغاير IL-12p40 1188A/C يمكن أن يعد عامل خطورة للإصابة بهذه الأورام.

الخـــلاصـة

الأورام اللمفاويه لمرض سرطان العقد اللمفاويه نوع "اللاهوجكينية "non-hodgkin's lymphoma هي مجموعة متباينة من أورام الخلايا اللمفاوية الناضجة والتي تتجلى بمدى واسع من المظاهر النسيجية والخلوية. إن هناك العديد من العوامل البيئية والوراثية التي تقترن مع حدوث هذه الأورام، ومع ذلك فان الاسباب الدقيقة لهذه الأورام لا زالت غير معروفة. تهدف الدراسة الحالية الى إستقصاء وجود إرتباط/علاقة بين التغايرات الجينية في موقعين جينيين هما IL-10 -1082A/G و IL-12p40 1188A/C مع حدوث الأورام اللمفاوية اللاهوجكينية في المرضى العراقيين.

جمعت عينات دم كامل و مصل الدم من 55 مريضاً بالأورام اللمفاوية اللاهوجكينية، ومن 40 شخصاً سليماً ظاهرياً ممن ينتمون الى عوائل مختلفة وبأعمار مقاربة لأعمار المرضى ليمثلوا مجموعة السيطرة. بعد إقرار إستمارة الموافقة من قبل لجنة الأخلاقيات العلمية، دُونت المعلومات الآتية: العمر والجنس وحالة التدخي ومؤشر كتلة الجسم والاصابة بالسكري ومكان الإقامة ودرجة القرابة العائلية من المصابين بالاورام اللمفاوية ساللاهوجكينية.

أُستخلص الحامض النووي DNA من عينات الدم الكامل بإستخدام عدة جاهزة (kit)، وأُستخدمت ثلاث مجاميع من البادئات لمضاعفة جينات IL-10و IL-12p40 و TLR2 (كجين سيطرة داخلي) للتنميط الجيني باستخدام تقنية تفاعل سلسلة البلمرة للأليل الخاص Allele-specific PCR). أما كميات السايتوكين المصلية من IL-10 و IL-12p40 في المرضى والاصحاء فقيست بتقنية ELISA.

ظهر التغاير الجيني IL-10-1082A/G بثلاثة أنماط جينية وهي: AA وAG و GG وبنسبة 36.36% و 45.45% و 18.18%، على التوالي، في مجموعة المرضى و 32.5% و 35% و 32.5% ،على التوالي، في مجموعة السيطرة. الاّ أن الفرق لم يكن معنوياً سواء على مستوى النمط الجيني أوعلى مستوى الأليل.

ظهرالتغاير الجيني لـ IL-12p40 1188A/C بثلاثة أنماط جينية هي: AA و AC و CC وبنسة 32.73% و 38.18% و 29.9%، على التوالي، في مجموعة المرضى و 52.5% و 37.5% و 10% في مجموعة السيطرة وبفرق معنوي بالنسبة للنمط الجيني الطافر متماثل

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانة الأحيائية

تأثير التعدد الجيني لإنترليوكينات 10و12p40 على الإصابة بمرض سرطان العقد اللمفاويه نوع اللاهوجكنية

رسالة ماجستير

مقدمة الى مجلس كلية العلوم/ جامعة النهرين كجزء من متطلبات نيل درجة الماجستير علوم/ تقانة احيائية

من قبل

مياسة عبدالله علي
بكالوريوس/ تقنيات التحليلات المرضية (2011)

بإشراف

الأستاذ مساعد د. حسن محمد نايف

جمادى آخر 1437 | 2016 أذار