Fluorescence In Situ Hybridization (FISH) and Risk Factors for Non-Hodgkin Lymphoma (NHL) Subtypes Defined by t(14;18) Translocations and bcl-2 Expression

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ABSTRACT

CINDY M. CHANG: Fluorescence In Situ Hybridization (FISH) and Risk Factors for Non-Hodgkin Lymphoma (NHL) Subtypes Defined by t(14;18) Translocations and bcl-2 Expression
(Under the direction of Jane C. Schroeder)

In hopes of increasing etiologic specificity of non-Hodgkin lymphoma (NHL), we defined NHL tumors by acquired chromosomal translocations involving the immunoglobulin heavy chain (any *IGH*), t(14;18), t(8;14) and *BCL6* translocations using fluorescence in situ hybridization (FISH) assays of archival paraffin-embedded tumor blocks. Translocations were identified in samples from over 200 unselected NHL cases originally enrolled in the National Cancer Institute's Factors Affecting Rural Men (FARM) study (1981-1984). We re-evaluated reported associations between tobacco exposures and t(14;18)-NHL case-subtypes that were previously defined based on polymerase chain reaction (PCR) assays. We also evaluated bcl-2 protein expression based on immunohistochemistry. t(14;18)-FISH case-subtypes were compared with t(14;18)-PCR case-subtypes by frequency according to histologic subtype and bcl-2 status. Case:control associations were estimated using multivariate polytomous logistic regression for t(14;18)-NHL and factors including tobacco use, family history of hemolymphatic cancer, and hair dye use. The expectation-maximization (EM) algorithm was applied to case:control models to reduce bias due to missing case-subtype data. *BCL6* translocations, t(8;14), and other *IGH* translocations were uncommon in the study population. t(14;18) was identified in 53% of cases, including 39% of diffuse large cell lymphomas (26 of 66 cases) and 81% of follicular lymphomas (35 of 43

cases). FISH assays detected almost twice as many t(14;18)-positive follicular lymphomas as PCR assays (44%) run on the same samples. The majority of cases expressed bcl-2, including 87% of t(14;18)-positive cases and 58% of t(14;18)-negative cases. Adjusting for age, state, and proxy status, t(14;18)-negative NHL was associated with any tobacco use (OR=1.98, 95% CI=1.09-3.59) and cigarette smoking, without evidence of a linear dose-response with increasing pack-years or intensity of smoking. In contrast, tobacco and cigarette use were not clearly associated with t(14;18)-positive NHL or with bcl-2 case-subtypes. Our results support the use of FISH assays of archival samples to identify t(14;18)-NHL case-subtypes. The association between t(14;18)-negative NHL and cigarette smoking was unexpected given previous evidence of associations between smoking and follicular lymphoma (which is largely t(14;18)-positive). Additional molecular characterization of t(14;18)-negative cases may clarify whether the association between tobacco use and t(14;18)-negative NHL was causal versus an artifact of chance or bias.

To my father, Dr. Paul S. Ma (1942-2006)

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LIST OF ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
AT	ataxia-telangiectasia
ATL	adult T-helper cell lymphoma
BCG	Bacillus Calmette-Guerin
BL	Burkitt lymphoma
BLL	Burkitt-like lymphoma
BMI	body mass index
CI	confidence interval
CLL	chronic lymphocytic leukemia
CTL	cytotoxic T cells
DLBCL	diffuse large B-cell lymphoma
DLCL	diffuse large cell lymphoma
DNA	deoxyribonucleic acid
eBL	endemic Burkitt lymphoma
EBV	Epstein-Barr virus
EM	expectation-maximization
FARM	Factors Affecting Rural Men
FISH	fluorescence in situ hybridization
FL	follicular lymphoma
HBB	*hemoglobin beta*
HBV	Hepatitis B virus (HBV)
HCV	Hepatitis C Virus
HL	Hodgkin lymphoma
HLP	hemolymphatic
H. pylori	*Helicobacter pylori*
HTLV-1	Human T-lymphotropic virus-1
IA	Iowa
IARC	International Agency for Research on Cancer
IGH	immunoglobulin heavy chain
IHC	immunohistochemistry
ILSG	International Lymphoma Study Group
IM	infectious mononucleosis
InterLymph	International Lymphoma Epidemiology Consortium
IPSID	immunoproliferative small intestinal disease
Kb	kilo base pair
MALT	Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphatic tissue (MALT)
MBR	Major breakpoint region
mcr	minor breakpoint region
MCL	mantle cell lymphoma
MN	Minnesota
NCI	National Cancer Institute
NHL	non-Hodgkin lymphoma
NSAID	non-steroidal anti-inflammatory

OR	odds ratio
PCNSL	primary central nervous system lymphoma
PCR	polymerase chain reaction
pro-B cells	progenitor B-cells
sBL	sporadic Burkitt lymphoma
SEER	Surveillance, Epidemiology, and End Results
SLL	small lymphocytic lymphoma
REAL	Revised European-American Lymphoma
RNA	ribonucleic acid
TB	tuberculosis
TMA	tissue microarrays
US	United States
UV	ultraviolet
WF	Working Formulation
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

Non-Hodgkin lymphoma (NHL) is a heterogeneous group of lymphomas involving the malignant clonal proliferation of B- (85% of lymphomas) or T-lymphocytes (15%) (1). Sixty to 80% of lymphomas occur in the lymph nodes, while the remaining occur outside of the lymph nodes or extranodally, most commonly in the bone marrow, spleen, liver and gastrointestinal tract.

According to the International Agency for Research on Cancer (IARC), over 300,000 new NHL cases occurred in 2002 with a male age-adjusted incidence of 6.1 cases per 100,000 and a female age-adjusted incidence of 4 cases per 100,000 (2). In the year 2007, an estimated 34,200 new male cases (9.9 cases per 100,000) and 28,990 new female cases (6.4 per 100,000) accounted for 4.4% of all new cases of cancer occurring in the U.S. (3). By geographic region, North America and Europe have the highest NHL incidences, while Asia and Africa have much lower rates (Figure 1.1). From the 1950s to the 1990s, NHL incidence has increased annually by 3-4% in Europe and North America in both sexes and all ages, but has stabilized in both males and females in the last 10 years (Figure 1.2a-b) (4,5). Much of the increase has been shown to be independent of changes in reporting, diagnosis, or classification of NHL (6). Although AIDS-associated NHL may have accounted for part of the rise of NHL in young men during the 1980s, it does not explain the increase in the elderly nor the increasing trends before the AIDS epidemic (6,7). The increase of NHL is not well understood, and risk factors for the majority of cases are still unknown.

Classification of NHL has remained a challenge due to its heterogeneous nature, though histologic subtypes are increasingly more refined with the incorporation of immunophenotypes and other molecular characteristics (8,9). NHL case-subtypes defined by somatic mutations have been suggested to minimize the etiologic heterogeneity of NHL outcomes for epidemiologic research (10,11). Chromosomal translocations occur when segments from two different chromosomes are exchanged. In lymphomas, they often involve the juxtaposition of an enhancer, such as the *IGH* gene promoter on chromosome 14, with an oncogene involved with cell proliferation and differentiation. Overexpression of the oncogene as a result of this fusion is believed to play a role in lymphomagenesis (1,8,10). This study's objective is to assess whether NHL defined by molecular characteristics including *IGH* translocations, t(14;18), t(8;14), *BCL6* translocations, and bcl-2 expression might better address the many questions that remain unanswered in lymphoma etiology.

Figure 1.1. Age-adjusted NHL incidence in males and females across geographic regions, Globocan 2002 (2)

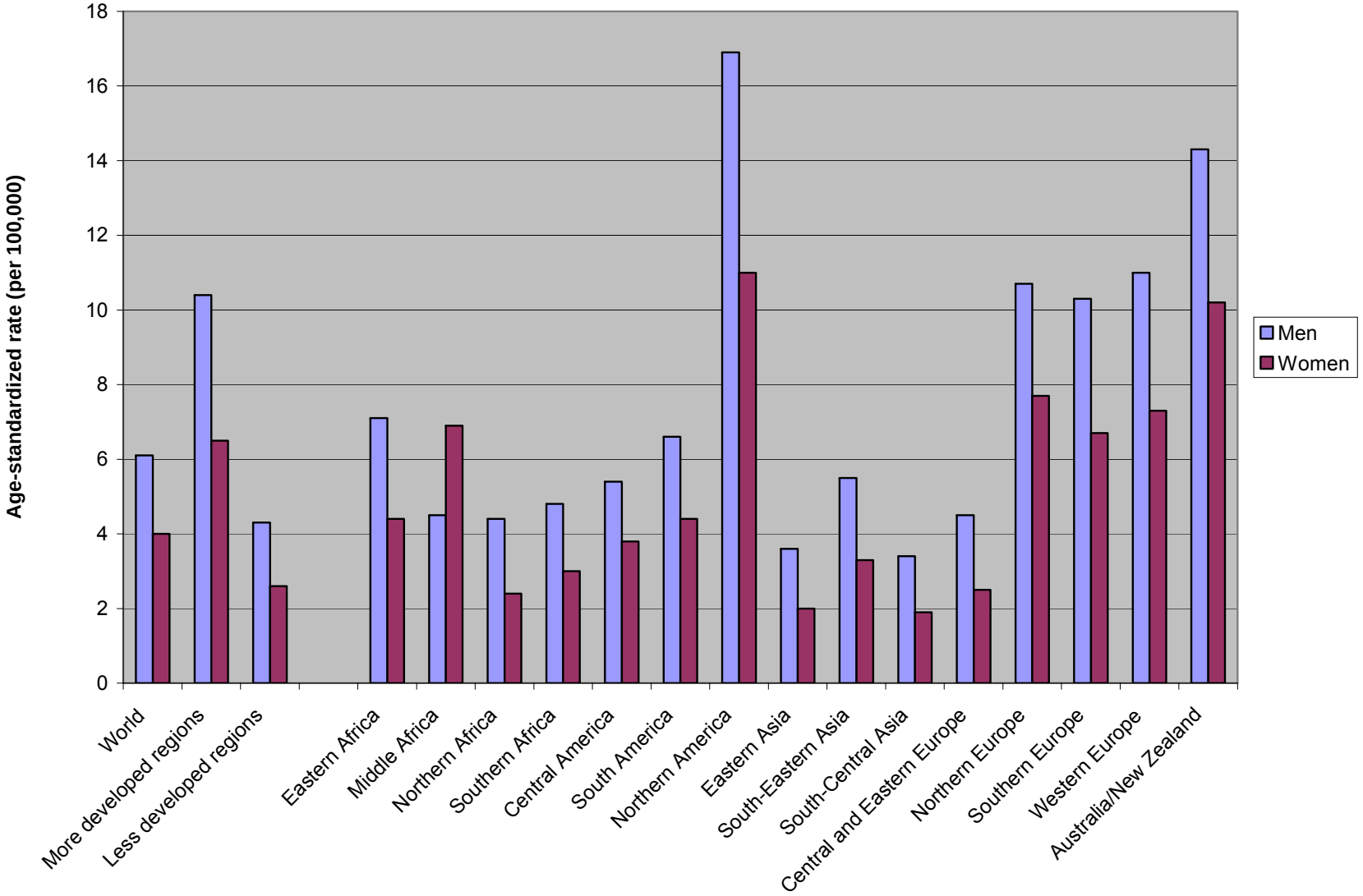


Figure 1.2a. International NHL trends in males around the world between 1958 and 1997 (12)

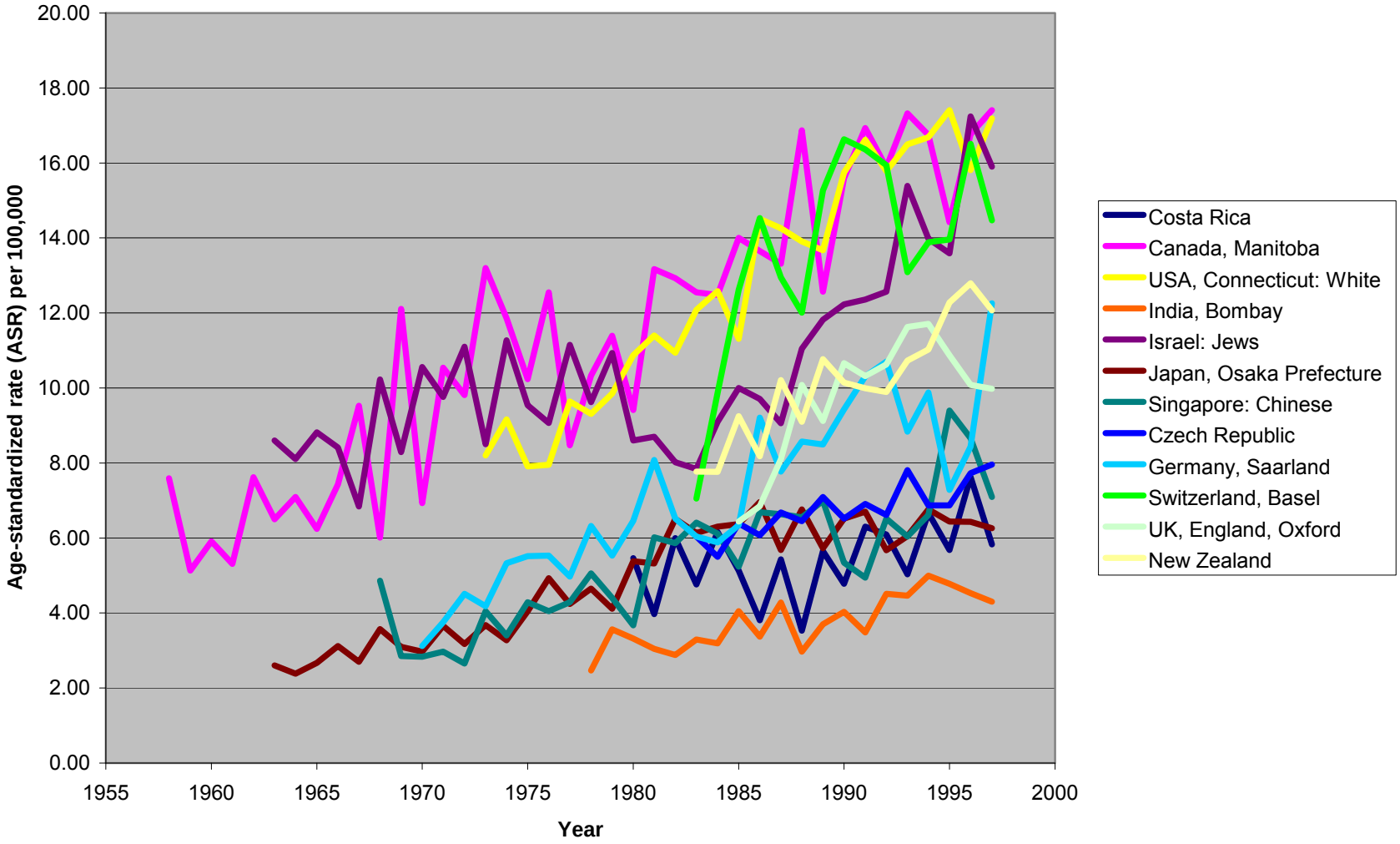
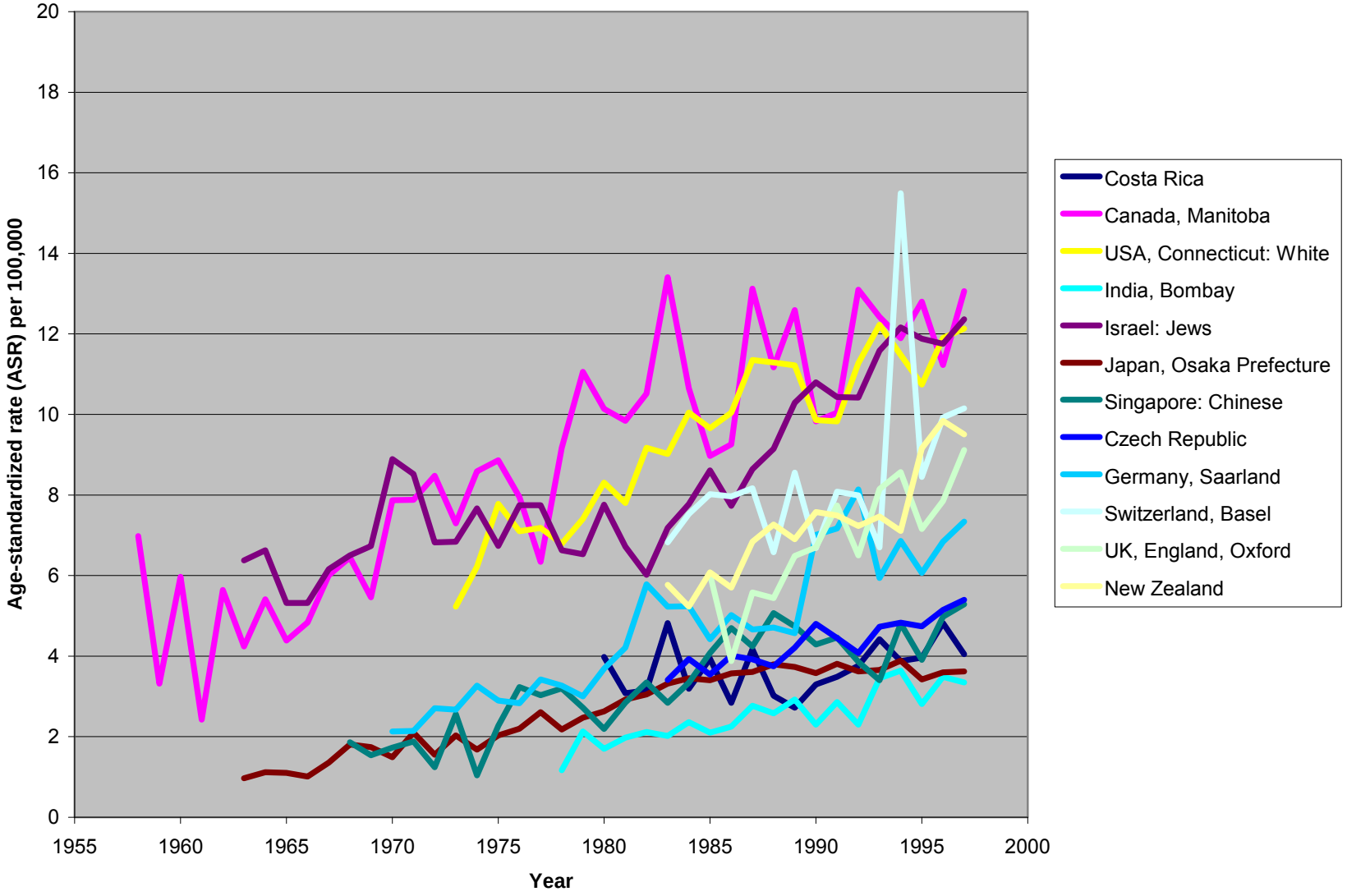


Figure 1.2b. International NHL trends in females around the world between 1958 and 1997 (12)



A. References

1. Magrath IT. 1997. Historical perspective: the evolution of modern concepts of biology and management. In: IT Magrath, ed. The non-Hodgkin lymphomas, 2nd Edition, London: Oxford University Press, 1997: pp. 46-71.

2. Ferlay J, Bray F, Pisani P and Parkin DM. GLOBOCAN 2002: Cancer Incidence, Mortality and Prevalence Worldwide IARC CancerBase No. 5. version 2.0, IARCPress, Lyon, 2004.

3. American Cancer Society. Cancer facts and figures 2007. Atlanta: American Cancer Society, 2007.

4. Devesa SS, Fears T. Non-Hodgkin lymphoma time trends: United States and international data. Cancer Res. 1992 Oct 1;52(19 Suppl):5432s-5440s.

5. Clarke CA and Glaser SL. Changing incidence of non-Hodgkin lymphomas in the United States. Cancer 2002; 94: 2015-23.

6. Holford TR, Zheng T, Mayne ST, McKay LA. Time trends of non-Hodgkin Lymphoma: Are they real? What do they mean? Cancer Res 1992; 52: 5443s-6s.

7. Zheng T, Mayne ST, Boyle P, Holdford TR, Liu WL, Flanner J. Epidemiology of non-Hodgkin lymphoma in Connecticut 1935-1988. Cancer 1992; 70: 840-9.

8. Medeiros LJ, Carr J. Overview of the role of molecular methods in the diagnosis of Malignant lymphomas. Arch Pathol Lab Med. 1999; 123: 1189-1207.

9. Jaffe ES, Harris NL, Stein H, Vardiman JW (editors). Pathology and genetics of tumours of haematopoietic and lymphoid tissues: Lyon. IARCPress, 2001.

10. Jaffe ES, Raffeld M, Medeiros LJ, Stetler-Stevenson M. An overview of the classification of non-Hodgkin lymphomas: an integration of morphological and phenotypical concepts. Cancer Res 1992; 52: 5447s-5452s.

11. Potter M. Pathogenetic mechanisms in B-cell non-Hodgkin lymphomas in humans. Cancer Res 1992; 52: 5522s-5528s.

12. Parkin, D.M., Whelan, S.L., Ferlay, J., and Storm, H. Cancer incidence in five continents, Vol. I to VIII IARC CancerBase No. 7, Lyon, 2005.

CHAPTER 2
CRITICAL REVIEW OF THE LITERATURE

A. Pathologic Classification

By 1982, there were 6 different classification schemes for NHL, including the World Health Organization (WHO), Rappaport, Lukes/Collins, Dorfman, British and the Kiel classification schemes. The National Cancer Institute (NCI) devised the Working Formulation (WF), providing a unified system that could be used to translate among the 6 schemes. The WF separated NHL into 10 morphological categories grouped into 3 broad groups based on low, intermediate, and high grades. Although the WF was quickly adopted as a classification scheme in its own right, it did not distinguish between B and T cell subtypes, grouped biologically unrelated diseases, separated related diseases, and excluded newly recognized lymphomas such as mantle cell lymphomas and marginal zone B-cell lymphomas (1-2).

In 1994, the formulation of the Revised European-American Lymphoma (REAL) scheme by the International Lymphoma Study Group (ILSG) departed from a purely morphologic approach and emphasizes biologic characteristics. REAL subtypes were defined by immunophenotype (B or T-cell origin) with incorporation of stage of differentiation, morphologic features, genotype, etiology, epidemiology, clinical behavior, cytogenetics, and molecular characteristics. In 2001, the new WHO classification was introduced with the addition of new entities (e.g. nodal marginal zone B-cell lymphomas),

refinement of some terminology (e.g. follicular center to follicular lymphoma), and the merging (e.g. Burkitt-like added to Burkitt lymphoma) and splitting of groups (e.g. anaplastic large cell to primary systemic and cutaneous (Table 2.1)) (2).

In spite of the significant progress, the current WHO classification scheme is primarily designed for clinical use rather than for finding NHL risk factors. The suggestion by epidemiologists to divide NHL as finely as possible for reflecting common etiologies is supported by examples of lymphoma subtypes with distinct etiology (i.e. endemic Burkitt lymphoma and EBV, AIDS-associated NHL, etc.) (3-4). Since their discovery in the 1970s, the presence of nonrandom cytogenetic abnormalities has helped explain the causes of altered growth and differentiation of neoplastic cells. The advent of techniques for detecting cytogenetic and molecular genetic abnormalities has provided clearly defined, reproducible diagnostic tests that have the ability to verify the neoplastic nature and lineage of a tumor independent of clinical and histological characteristics (5). Although the new WHO classification scheme incorporates cytogenetic and molecular findings into subtypes, subtyping solely by molecular characteristics may provide further refinements to the current classification scheme.

B. Epidemiologic, Morphologic, Molecular Characteristics of Selected Lymphomas

B.1. Diffuse large B-Cell lymphoma (DLBCL)

Patients having diffuse large B-Cell lymphoma (DLBCL) may present with large tumors in the lymph node or in extranodal sites, most frequently the gastrointestinal tract. DLBCL, believed to encompass a heterogeneous group of lymphomas, accounts for 25-40%

of the NHL cases in most countries (6). Most common in men at middle age and elderly whites, the median age at diagnosis is 70 years, but the age range is broad (4). There are several subtypes of DLBCL including anaplastic large B cell, which accounts for 25% of NHL in children. Morphologically, DLBCL infiltrates the normal architecture of the lymph node or extranodal tissue in a diffuse pattern. The morphologic variants are distinguished by nuclear appearance, the presence of centroblasts, and immunophenotyping (expression of various markers and proteins). DLBCL is characterized by a number of gene rearrangements, including translocations involving the *BCL2* gene (20-30% of cases), *CMYC* gene (6-16%) and *BCL6* gene (10-30%) (7-9).

B.2. Follicular lymphomas (FL)

Follicular lymphoma (FL) is one of the most common non-Hodgkin lymphomas in the U.S. and Europe, accounting for 20-35% of all cases. Incidence of FL is lower in Asia and developing countries (4,6). In a study of Asian migrants, follicular lymphoma incidence was higher in US-born Chinese (2.2 per 100,000) and Japanese (1.0 per 100,000) compared to their Asian-born counterparts (0.29 per 100,000 and 0.39 per 100,000, respectively) (10). Because such a difference is not seen for other NHL subtypes, this observation suggests that environmental factors may play a larger role in follicular lymphoma risk than for other subtypes, further making the case for using subtype-specific analyses for finding risk factors.

FLs are usually found in the lymph nodes, but also occur in the spleen, bone marrow, skin and other areas. Progression to an intermediate-grade DLBCL occurs in 25-60% of patients and often involves a secondary genetic alteration, particularly 9p deletions. Follicular lymphomas occur more frequently in white males and females (17.4% and 22.2%,

respectively) than in black males and females (9.7% and 12.0%, respectively) with an overall median age of 59 years (8,11). The neoplastic cells of FL have a distinctive appearance resembling cells in a normal germinal center of lymphoid follicles, and the neoplastic follicles are closely packed. Two types of cells are found in FL: small to medium-sized cells with cleaved nuclei and large transformed cells with occasionally undented nuclei. Grading is based on the presence of centroblasts with the greater number of centroblasts per field corresponding to a higher grade. t(14;18) is present in 85-100% of cases, *BCL6* rearrangements in 9-14%, and *BCL6* 5' mutations in 40% of cases (7,8,12).

B.3. Small lymphocytic lymphoma/ chronic lymphocytic leukemia (SLL/CLL)

Small lymphocytic lymphoma (SLL) presents in the lymph nodes, while chronic lymphocytic leukemia (CLL) presents in the bone marrow and peripheral blood. SLL/CLL accounts for about 6.7% of non-Hodgkin lymphomas worldwide. Most patients are older than 50 years (median 65 years) and have a male to female ratio of 2:1. Morphologically, the neoplastic cells form a pseudofollicular pattern with larger cells surrounded by smaller cells. The main type of cell is a small lymphocyte with clumped chromatin and a round nucleus. Up to 80% of cases have genetic abnormalities, including t(14;18) in 1-2% of cases, trisomy 12 in 20% of cases, deletions at 13;14 in 50%, deletions in p53, and expression of p53 in other cases (8-9).

B.4. Mantle cell lymphoma (MCL)

Mantle cell lymphoma (MCL) usually presents in lymph nodes, but also appears in the spleen and bone marrow. Gastrointestinal involvement accounts for 30% of patients.

MCL accounts for 3-10% of NHL cases, occurs in older adults (median age 60 years), and has a 2:1 male to female ratio. Mantle cell lymphoma is more frequent in Switzerland (14%) than in other sites (1-8%) (6). Neoplastic cells can have a nodular, diffuse, or mantle zone growth pattern. The cells are small to medium-sized, and the nuclei are slightly irregular. t(11;14), the *IGH* translocation with the *CCND1* gene is found in almost 100% of cases by FISH, and cyclin D1 overexpression detected by immunohistochemistry is found in 67% of MCL (8;13).

B.5. Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphatic tissue (MALT)

Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphatic tissue (MALT) lymphomas present most commonly in the gastrointestinal tract (50% of cases), particularly in the stomach, small intestine, and colon. MALT accounts for 7-8% of all B-cell lymphomas, has a median age at diagnosis of 61 years, and is slightly more common in women than in men (male to female ratio 1:1.2). A subtype of gastric MALT lymphoma called immunoproliferative small intestinal disease (IPSID) occurs frequently in the Middle East and South Africa. Patients with MALT often have a history of autoimmune disorders and chronic inflammatory disorders, including Sjögren syndrome and *Helicobacter pylori* infection. Neoplastic cells usually are small to medium-sized with irregular nuclei, dispersed chromatin, inconspicuous nucleoli and a large amount of pale cytoplasm. Chromosome abnormalities include trisomy 3 in 60% of cases and t(11;18); however, neither t(14;18) nor t(11;14) has been found (8).

B.6. Burkitt/Burkitt-like lymphoma (BL/BLL)

Burkitt lymphoma (BL) is an aggressive B-cell lymphoma that has three main subtypes: endemic BL, occurring in equatorial Africa and presenting in the jaw and other facial bones in 50% of cases; sporadic BL cases presenting more often in the ileo-coecal region (intestinal region); and AIDS-BL presenting in the lymph nodes and the bone marrow. Endemic BL is the most common childhood cancer in Africa, with a peak incidence from ages 4 to 7 years and a male to female ratio 2:1. Sporadic BL accounts for 1-2% of all lymphomas in the Western world and accounts for 30-50% of childhood lymphomas, with a male to female ratio of 2-3:1. Epstein-Barr virus (EBV)-positive BL is associated with low socioeconomic status and EBV infection early in childhood. The EBV genome has been detected in 100% of endemic BL, 25-40% of AIDS-BL, and less than 30% of sporadic BL cases (7,8). "Classical" BL is a morphologic type seen in both endemic and sporadic BL and is characterized by a high proliferation rate with many mitotic figures and a "starry sky" pattern due to the presence of numerous macrophages that have ingested dead tumor cells. Nearly 100% of AIDS-associated BL cases have mutations involving activation of *CMYC* and 50-60% have inactivating mutations of p53 tumor suppressor (14). Approximately 80% of BL cases have rearrangement of the *CMYC* gene on chromosome 8, involving the *IGH* gene (t(8;14)). *CMYC* translocations with genes on the kappa (*IGK*) and lambda (*IGL*) light chain loci occur in 15% and 5% of BL cases, respectively. t(14;18) has been reported to occur in 0-50% of BL cases (15-18).

B.7. Variation in subtypes

In a descriptive study, the proportion of DLBCL in total NHL cases did not vary substantially (25%-36%) across geographic regions, but FL was more common in Omaha, Nebraska, United States, Vancouver, Canada, London, England, and Capetown, South Africa (28-32%), compared to other areas (8-18%) (6). Asian countries, including India and Thailand, are frequently observed to have lower incidences of FL compared to Western countries (19,20). Mantle cell lymphoma was more frequent in Switzerland (14%,) than in other sites (1-8%). Angiocentric nasal T-/NK-cell tumors are present exclusively in Hong Kong (8%) and Lyon, France (2%) (Figure 2.1) (6).

High-grade NHL tripled in males and doubled in females between 1978 and 1995 in the U.S. During this same time period, DLBCL increased for males by 30-41% and females by 19-25% (11). More recently (1992-2001), DLBCL has declined 0.5% per year, primarily in men 25 to 54 years old (11,21). Whereas FL was on the rise between 1963-1982 and 1984-1988 particularly in whites and black men, from 1992 to 2001, FL remained stable overall, but increased 1.8% per year among the elderly (4,11,21). From 1992 to 2001, incidence of marginal zone, mantle cell, and BL rose between 4% and 10% per year during this period; CLL/SLL decreased 2-3% per year in men and 9% in Asian females (21). The different patterns and trends of NHL by subtype and by population are likely to reflect different etiologic and host factors, further supporting the need to define subtypes as specifically as possible.

C. Chromosomal translocations involved in NHL

The generation of antibody diversity requires a number of recombination events from which chromosomal translocations are believed to arise, though the mechanism is not known. Supporting this hypothesis are the observations that many translocations involve genes that play a role in normal recombination events including the J segments of the immunoglobulin heavy chain (*IGH*) gene (9).

C.1. Generation of antibody diversity

Immunoglobulins (Ig) are the antigen-binding proteins located on the membrane of B-cells and secreted by plasma cells. These proteins are made up of two long polypeptide chains (heavy chains) and two short chains (light chains) that form a "Y" linked by disulfide bonds (Figure 2.2). Functioning as antibodies, they play an important role in adaptive immunity responsible for providing lifelong immunity following disease or vaccination. Lymphocytes bearing these antigen receptors carry a unique and single specificity. In other words, they recognize only one protein (or other residue) that is usually just one small part of a larger molecule. The interactions that bind the receptor to antigen involve a combination of noncovalent forces including hydrogen bonds and electrostatic forces. Once the antigen is recognized and bound, the immune response is elicited and other cells are recruited to destroy the antigen. Because each antibody carries a unique specificity, diversity is needed to enable the body's immune system to fight off a wide range of invading foreign antigens (22-23).

Progenitor B-cells (pro-B cells) interact with cells in the bone marrow environment in order to undergo heavy chain gene rearrangements (Figure 2.3). Three separate gene

segments, named V, D, and J, that encode the variable region undergo rearrangement and assemble to form a complete variable region sequence. Productive VDJ joining results in expression of the immunoglobulin heavy chain protein in a precursor (pre-B) cell, while nonproductive rearrangements result in cell death. Once the pre-B receptor is assembled from the heavy chain protein and a surrogate light chain protein, small pre-B cells arise and light-chain rearrangements begin. Light-chain rearrangements of the kappa (*IGK*) and lambda (*IGL*) light chain genes are similar to heavy-chain rearrangements except they occur on two different chromosomes and involve joining V and J segments (instead of V, D, J), and repeated segments of unused V and J allow for several successive attempts at productive rearrangement. This organization allows the rescue of a pre-B cell if the initial attempt at productive rearrangement fails (22,23).

The assembled light-chain protein and heavy chain protein form a complete immunoglobulin M (IgM**),** one of the 5 main classes of antibodies. Expression of the IgM on the cell membrane defines the B cell as an immature B cell. Of the 50 million B-cells produced per day, only 5 million (10%) leave the bone marrow. The other 90% are cells with non-productive rearrangements or cells that express autoantibodies that undergo apoptosis (programmed cell death). The antigen-dependent phase starts once mature B cells leave the bone marrow and circulate in the body between the blood and peripheral lymphoid tissues. Mature B cells that recognize and bind to an antigen become activated, then proliferate and differentiate into memory B cells and plasma cells. Circulating naïve B cells that are not activated within a few weeks undergo apoptosis (22,23).

After a B cell is activated, additional diversity is generated when the B cell undergoes somatic hypermutation which introduces a high rate of point mutations, deletions, insertions

into the variable region of rearranged heavy and light-chain genes. B cells with antibodies that bind better to the antigen after this process are preferentially selected to mature into antibody-secreting plasma cells. Further somatic recombination involving the C region allows isotype or class switching (from IgM to IgG, IgA or IgE) without changing antigen specificity (23).

C.2. Chromosomal translocations

During VDJ rearrangement and heavy chain isotype switching, DNA is broken and rejoined to bring distant Ig gene elements together. This process may result in the exchange of genetic material, known as chromosomal translocations. Because DNA rearrangement is restricted to specific stages of B-cell development, translocations are hypothesized to arise during the B cell's pro-B phase, pre-B phase and antigen-activated phase (24). *IGH* translocations involve the exchange of DNA fragments from the *IGH* gene on chromosome 14 with DNA from another chromosome (Figure 2.4). How these non-Ig genes become partners with the *IGH* gene may be a result of the non-Ig gene and the Ig gene rearrangements being transcribed at the same time and the non-Ig gene being in close proximity to the rearranging complex. Genes that are juxtaposed with the *IGH* gene on 14q32 may become transcriptionally deregulated by the *IGH* promoter.

C.2.1. t(14;18)

The most common chromosomal translocation in NHL is t(14;18) which is found in 85-100% of follicular lymphomas and 20-30% of diffuse large B-cell lymphomas (Table 2.2). Almost all breakpoints within the *IGH* gene cluster on the J_h segment, while on the *BCL2* gene 50% of breaks are in the major breakpoint region (MBR) and another 25% fall within the minor breakpoint region (mcr) (9). t(14;18) is believed to be an early step in lymphomagenesis, in part because non-neoplastic t(14;18)-positive lymphocytes are also detected at low levels in 50-80% of healthy individuals (23,24). Furthermore, the observation that the breakpoint on chromosome 14 occurs at the J region adjacent to heptamer-nonomer signal sequences (needed for Ig gene rearrangement) suggests t(14;18) arises close to the time of Ig gene rearrangement. The juxtaposition of the *BCL2* oncogene on 18q21 with the *IGH* gene on 14q32 results in the overexpression of antiapoptic bcl-2 protein. Consequently, the B-cell escapes cell death and becomes immortalized, potentially undergoing additional "hits" leading to malignant transformation. Additionally, bcl-2 promotes cell-cycle arrest, essentially retarding entry into the cell cycle. The anti-apoptotic and cell-cycle arrest functions of bcl-2 may explain the indolent nature of follicular lymphoma (25).

C.2.2. *BCL6* translocations

The second most common lymphoma-associated translocation involves the *BCL6* gene on 3q27 which may be exchanged with a number of partner chromosome loci. *BCL6* rearrangements occur in 30-40% of diffuse large B-cell lymphomas, 20% of AIDS-associated diffuse large B-cell lymphoma and 5-14% of follicular lymphomas (Table 2.2) (26). The

mechanism of how bcl-6 overexpression leads to transformation is not known (9). However, *BCL6* encodes a zinc-finger transcription factor implicated in germinal center B cell differentiation, antibody affinity maturation and T helper cell mediated responses. The protein is normally expressed by germinal center B cells, but down-regulated in postgerminal center formation. The dysregulation of bcl-6 due to translocation is believed to play a role in the malignant transformation of germinal center-derived B cells (25).

C.2.3. t(8;14)

The *CMYC* gene resides on 8q24. The function of the c-myc protein is not well understood, but it has been observed to undergo a burst of transcription when a cell transitions from a resting state to a presynthetic state (G0 to G1), committing the cell to a program of DNA synthesis and mitosis. Overexpression of c-myc may keep the B cell in a proliferative cycling mode (24). t(8;14) occurs in 80-85% of BL cases (Table 2.2). Additionally, 65% of BL have demonstrated point mutations in the *C-MYC* gene (25). Endemic BL occurs primarily in young children in Africa and is associated with EBV. Sporadic BL occurs much less frequently in the US and is not EBV-associated.

The locus where the piece of DNA carrying the *CMYC* gene breaks off of chromosome 8 differs between endemic BL and sporadic BL. Chromosome 8 breakpoints for endemic BL generally occur upstream of the *CMYC* gene, while they occur within or close to *CMYC* in sporadic BL. A study of the molecular epidemiology of BL in different regions of the world found that African tumors had breakpoints far from the 5' end of chromosome 8, while US tumors had breakpoints occurring at the first exon of chromosome 8. South American tumors suggested a third type of BL with a breakpoint immediate to the

5' end of chromosome 8 (26,27). Breakpoints in chromosome 14 also varied between endemic and sporadic BL: while breakpoints occurred in the J or D regions of the *IGH* gene in eBL, they occurred in the *IGH* switch C regions for sBL. Because of these breakpoint locations, eBL is likely to arise in the pro-B cell before DJ joining, while sBL may arise later in development during *IGH* class switching (28). The geographical variation in the occurrence of BL and chromosomal breakpoint patterns suggest the role of environmental factors in the formation of t(8;14) and development of BL.

D. bcl-2 Expression

bcl-2 is a 25 kilodalton protein that enables B-cells to survive in absence of growth factors (29,30). Localized within the cell cytoplasm, bcl-2 is quiescent in resting B cells, upregulated in proliferating B cells but is down regulated in differentiating B cells (29,31). When bcl-2 is inappropriately expressed in resting B cells, their survival is prolonged, increasing chances of acquiring secondary genetic changes that could progress to lymphoma. Although t(14;18) translocation is an established mechanism leading to bcl-2 expression, studies show that many bcl-2-positive lymphomas are t(14;18)-negative, suggesting that alternative mechanisms may also cause bcl-2 expression (30). A recent study detected bcl-2 in 87% of 45 lymphoma cases that were t(14;18)-negative among an unselected sample of lymphomas. Additionally, they found no association between histologic subtype and level of bcl-2 expression (31).

Among alternative deregulating mechanisms for bcl-2 that have been proposed, gene amplification of *BCL2* has been explored. A study found a correlation between bcl-2 gene amplification and bcl-2 expression with 6 out of 7 DLBCL cases with extra bcl-2 signals also

having bcl-2 expression. However, 20% of the cases with strong bcl-2 expression had neither bcl-2 amplification nor t(14;18), leaving the possibility of other mechanisms (32). Viral infections including EBV and cytomegalovirus, hypomethylation of the 5' end of the bcl-2 gene, and cytokine effects have all been proposed to cause bcl-2 expression (31).

The new WHO classification scheme for defining NHL subtypes is highly evolved, encompassing a wide variety of features including morphologic, immunophenotypic, cytogenetic as well as etiologic data (e.g. *H. pylori* and MALT). In spite of the advances in classification, the fact that common translocations are associated with several subtypes (i.e. t(14;18) in FL, DLBCL, CLL/SLL, BL) raises the possibility that etiologically similar lymphomas are being separated, while etiologically different lymphomas are being grouped, and as a result, associations with risk factors may be diluted. Defining subtypes by common translocations and other molecular characteristics could provide an alternative way of examining NHL patterns and may provide further refinements to the current classification scheme.

E. Risk Factors for Non-Hodgkin Lymphoma

E.1.Age, race, sex and education

Age is the most established NHL risk factor. Incidence of NHL increases exponentially with age, which may be associated with a weakened immune system and reflect the accumulation of exposures over a long time (Figure 2.5). NHL subtypes occur more frequently in men than women (i.e. FL, SLL/CLL, MCL). Between 1973 and 2002 in the U.S., NHL incidence in white and black males increased at higher rates than for white and black females (Figure 2.6). The highest rates of NHL occur in geographic regions (U.S.,

Canada, Australia/New Zealand, and Europe) that are predominantly Caucasian (see Chapter I). According to the Surveillance, Epidemiology, and End Results (SEER) registry between 1998 and 2002, white males and females had the highest incidence of NHL of all other groups within their respective sexes (Table 2.3). The sex and race differences are hypothesized to be related to biological differences and exposure differences (i.e. occupation) (33,34). NHL has been positively associated with income and years of education (35), but studies have been conflicting (36).

E.2. Immune suppression

Immune suppression, especially in AIDS and transplant patients, is strongly associated with NHL. Both solid organ and stem cell transplants are associated with NHL, though it varies by type of transplant (37,38). A Swedish cohort study found that patients receiving organ transplants other than kidney, including heart, lung, liver and pancreas, have a risk of NHL eight times that of kidney transplant recipients (RR=8.4, 95% CI=4.3-16) (37). Uncontrolled proliferation of EBV infected B-cells resulting from medically induced immune suppression may explain the etiology in many cases of post-transplant lymphomas. Chronic antigenic stimulation by the transplanted organ may also contribute to pathogenesis given that the primary lymphoma occurs at the site of the transplant in many cases (39,40).

In a cohort of AIDS patients, the risk of NHL in 366,034 patients 4 to 27 months after developing AIDS was 73 times the expected risk in the general population (RR=72.8, 95% CI=70.4-75.3) (41). Factors associated with NHL in AIDS patients include duration of immune suppression and degree of B-cell stimulation due to HIV and high serum globulin (4). AIDS-related NHL is a heterogeneous group of malignancies, consisting primarily of

Burkitt lymphomas (BL) (50%), diffuse large B-cell lymphoma with centroblastic or immunoblastic features (35%), and lymphomas of the central nervous system (15%). EBV association with NHL also varies among the subtypes, with EBV-positive neoplastic cells present in essentially all central nervous system (CNS) lymphomas and at least half of other AIDS-associated NHL subtypes. Similar to medically-induced immune suppression for transplant patients, HIV-related immune suppression promotes development of EBV-infected and immortalized B-cell clones that are susceptible to further genetic damage leading to malignant transformation (42). The variation in subtypes for both post-transplant and AIDS-associated lymphomas is likely to reflect different pathogenetic mechanisms, and underscores the value of treating these subtypes as distinct diseases rather than as one entity.

E.3. Genetic factors

Lymphomas account for approximately 50% of the tumors that develop in people with primary immune deficiencies due to inherited genetic defects. The risk of NHL in Wiskott-Aldrich syndrome and Ataxia-telangiectasia (AT) patients is at least 15 times or higher than the baseline risk in people without these conditions (43). Defective immune regulation after B- and T-cell activation has been observed to foster a favorable environment for lymphoma development in these patients. As with AIDS and organ transplant patients, EBV is believed to play an important role in the development of NHL in patients with primary immune deficiencies (44).

Families with a history of multiple cases of NHL more frequently have immune function abnormalities such as ataxia-telangiectasia and X-linked recessive immune deficiency disorder than families without history of NHL (45). Clinical studies have found

depressed levels of immunoglobulins and cutaneous anergy, impaired in vitro lymphocytic responses to phytohemagglutinin, and other markers of immune dysfunction in individuals with family history of hemolymphatic (HLP) cancer (44). The association between NHL and family history of hematopoeitic cancer is consistent with a role of early environmental exposures or an association with inherited genetic variation such as X-linked or recessive genes. In the Scandinavian Lymphoma Etiology (SCALE) study, a population-based case-control study, having a first-degree relative diagnosed with NHL was positively associated with any NHL (OR=2.2, 95% CI=1.4-3.5) (46). Among Iowa and Minnesota farmers, having a sibling with a hemolymphatic cancer was associated with NHL (OR=2.7, 95% CI=1.5-5.1) and some subtypes including FL (OR=3.2, 95% CI=1.3-7.6), DLCL (OR=2.3, 95% CI=0.9-5.7) and SLL (OR=5.2, 95% CI=1.9-13.6) (47). The InterLymph study group found an increased risk of NHL (10,211 cases) associated with individuals who report having a first-degree relative with NHL, Hodgkin lymphoma (HL), or leukemia. Having a brother with NHL was strongly associated with NHL (OR=2.8, 95% CI=1.6-4.8) which suggests that shared environmental and genetic factors among male relatives may be important (48).

E.4. Infections

E.4.1. Epstein-Barr Virus

Epstein-Barr virus (EBV), a member of the herpes virus family, is a prevalent infection in over 95% of adults worldwide that is transmitted person to person through the saliva or breast milk. In developing countries infants are often infected in the first year of life. In developed countries, primary infection usually is delayed to young adulthood (49-51). The clinical manifestation of EBV infection depends primarily on age at infection.

Young children (under 5 years) usually experience no symptoms when infected. For adolescents and young adults, some infections produce symptoms similar to those of a cold or other mild viral illness, while about half develop infectious mononucleosis (IM) (52). EBV establishes a lifelong asymptomatic infection, usually in a latent or nonreplicating form in resting memory B cells. Infected B cells that continue to proliferate would normally be destroyed by cytotoxic T cells (CTL) with specificity for B cells expressing EBV antigens, but in the immune deficient host, inadequate CTL response may lead to uncontrolled proliferation of EBV-infected B cells (53,54).

EBV DNA has been found in almost 100% of endemic BL, 50% of AIDS-associated BL cases, and 20% of sporadic BL cases (51). The widespread presence of EBV in the world's population and its unchanging prevalence suggest that EBV plays a necessary but not sufficient role in lymphomagenesis. In equatorial Africa, children infected with EBV may be susceptible to BL due to immune suppressive and chronic antigen stimulating effects of malaria, in combination with the B cell transforming properties of *Euphorbia tirucalli* plants commonly used for medicinal purposes (54). A major clue supporting the role of environmental factors is the variation of t(8;14) breakpoint patterns in chromosome 8 that correspond with BL subtypes in different regions of the world. It is conceivable that each environment or geographic region would offer a different set of cofactors to act with EBV to directly or indirectly produce a certain type of t(8;14) (49-51).

E.4.2. Human T-lymphotropic virus-1 (HTLV-1)

Human T-lymphotropic virus-1 (HTLV-1) infection is endemic in Japan, the Carribean, central Africa, parts of South America, Melanesia, Papua New Guinea, and the

Solomon Islands, with about 15-20 million carriers worldwide (55). HTLV-1 was identified as a cause of adult T-helper cell lymphoma (ATL) in 1978. In some cases, HTLV-1 infection stimulates polyclonal expansion of infected T-helper cells. Infected cells escaping immune surveillance and undergoing monoclonal expansion may be at increased risk of progression to malignancy. The lifetime risk of developing ATL among adult HTLV-1 carriers in southwest Japan (where HTLV-1 seroprevalence is 16.2% in adults) is 6.6% in men and 2.1% in women. However, because of its limited geographical distribution, low transmission, and stable presence, HTLV-1 is not believed to contribute to the increased incidence of NHL worldwide (51,56).

E.4.3. *H. pylori*

Helicobacter pylori is a gastric pathogen that causes chronic gastritis. It also is believed to play a role in some gastric lymphomas, specifically MALT (mucous-associated lymphoid tissue) lymphoma. Chronic gastric inflammation has been shown to lead to the accumulation of lymphocytes in the submucosa, and *H. pylori* infection helps establish an environment in which cell proliferation and oxidative damage of epithelial and lymphopoetic cells may occur (4). Strong associations between *H. pylori* seropositivity and gastric lymphoma have been found, with a risk in *H. pylori*-positive individuals that is up to 6 times as high as the risk in the general population. Additionally, clinical studies have reported that antibiotic treatment of *H. pylori* leads to regression of early lymphoma lesions in 60-92% cases, possibly due to decreased chronic inflammation and cell proliferation (4,51,57).

E.4.4. Hepatitis C Virus (HCV)

Hepatits C virus (HCV) causes liver disease and is involved in several extrahepatic disorders. HCV prevalence ranges from 1-2% in most Western countries to more than 10% in Egypt, Italy, South Korea and parts of Japan. The geographical variation in HCV may explain the discrepancies found in a recent review of US and international studies in which HCV-positive individuals had 2 to 4 times the risk of NHL of HCV-negative individuals (58). An Italian hospital-based study of 400 NHL cases and 396 controls found that HCV was strongly associated with B-NHL (OR = 3.1, 95% CI=1.8-5.2) and many NHL subtypes (59). A SEER study of 813 cases and 684 population-controls found that HCV was associated with NHL overall (OR=1.89, 95% CI=1.00-4.00) and with follicular lymphoma (OR=2.46, 95% CI=1.01-5.81) (60).

The role of HCV in lymphomagenesis is likely to be indirect because HCV is an RNA virus and it cannot integrate into host cell DNA. Possible mechanisms are chronic antigenic stimulation of B-cells by HCV, or neoplastic transformation secondary to the activation of antiapoptotic pathways within HCV-infected B-cells (60). A "triple association" for HCV, autoimmune disease and NHL has been observed in at least one clinical study, suggesting the two may act as cofactors in lymphomagenesis (61). Antiviral treatment in patients having both HCV and t(14;18)-positive cells reportedly eliminated the presence of t(14;18) in the peripheral blood in 50% (15/30) of those responding to therapy, while t(14;18) persisted in nonresponders and the controls (62).

E.4.5 Hepatitis B virus (HBV)

Hepatitis B virus (HBV) is responsible for many cases of chronic hepatitis, cirrhosis, and liver cancer in Asia and Africa. A South Korea case-control study found a relative

increase in B-cell NHL in HBV-positive compared with HBV-negative individuals (OR=3.30, 95% CI=1.69-6.45). Possible mechanisms include a direct immune suppressive effect of HBV infection, chronic antigenic stimulation, and a causal effect of some other unmeasured virus associated with HBV due to similar routes of transmission (63).

E.5. Medical factors

E.5.1. Vaccination

In at least two studies, Bacillus Calmette-Guerin (BCG), a tuberculosis (TB) vaccine derived from *Mycobacterium bovis* widely used in high-prevalence countries, has been weakly associated with an increased risk of NHL (64-66). Because BCG is primarily administered to children in developing countries outside the US, factors associated with living abroad may confound the association between increased risk of BCG vaccination and NHL.

Many vaccinations have been inversely associated with NHL (64,66-67). NHL has been inversely associated with vaccines for cholera, yellow fever, and tetanus (66,67). A Bay Area population-based case-control study found inverse associations between various vaccines and NHL subtypes: hepatitis and DLBCL (OR=0.70, 95% CI=0.50-0.98), diphtheria-pertussis-tetanus (DPT) and diffuse mixed small cleaved and large-cell (OR=0.44, 95% CI=0.26-0.74), and measles and diffuse small cleaved-cell (OR=0.52, 95% CI=0.26-1.0). Additionally, five or more vaccinations were inversely associated with DLBCL (OR=0.65, 95% CI=0.52-0.82) (64). The association between vaccine history and NHL is unclear and may be due to other unmeasured factors.

E.5.2. Blood Transfusions

Transfusion may increase NHL risk by causing immune suppression, transmission of viruses, or engraftment of lymphocytes from a donor with subclinical NHL (68,69). In spite of strict regulation by the Food and Drug Administration (FDA) and other organizations, blood transfusions still pose risks, such as allergic reactions, and bacterial and viral infections (52). Although initial studies suggested an elevated risk of NHL in patients receiving blood transfusions (68,70), recent studies have found no association (69,71). Inconsistent findings across studies may be due to patient populations differing in their reasons for blood transfusions and in their treatment regimens, and other unaccounted viruses (e.g. HCV); recent negative findings may also be a result of improved screening (69).

E.5.3. Infectious Diseases

Reported history of infectious diseases associated with NHL in some studies include scarlet fever (71,72), tuberculosis, kidney infections and chronic bronchitis (61,62,67,68), history of infectious mononucleosis (67,72,74), malaria (67,74), measles (72), and recent history of herpes zoster (67,73), but results have been inconclusive because of imprecise and inconsistent findings.

E.5.4. Autoimmune and chronic inflammatory disorders

Rheumatoid arthritis, primary Sjögren syndrome, systemic lupus erythematosus, and celiac disease are all autoimmune diseases characterized by B-cell proliferation and autoantibody production and have been consistently linked with NHL (52,71,75). In a Swedish population-based case-control study, history of autoimmune conditions was

associated with NHL overall: rheumatoid arthritis (OR=1.5, 95% CI=1.1-1.9), primary Sjögren syndrome (OR=6.1, 95% CI= 1.4-27), systemic lupus erythematosus (OR=4.6, 95% CI=1.0-22), and celiac disease (OR=2.1, 95% CI=1.0-4.8) (75). Among Connecticut women, a history of autoimmune disorders was associated with NHL overall (OR=2.2, 95% CI=1.3-3.7), FL (OR=3.1, 95% CI=1.5-6.5), and DLBCL (OR=2.1, 95% CI=1.0-4.3) (71).

E.5.5. Chronic disease

Anemia has been weakly associated with NHL in at least 2 studies (66,71). Anemia, caused by nutrient deficiencies, blood loss, chronic disease, and medical treatments, may be linked to NHL due to its underlying causes, associated treatments, or stimulation of the bone marrow due to blood loss.

A history of cancer is also positively associated with NHL (66,76). People reporting a history of cancer might all have underlying immune dysfunction that would predispose them to NHL. Alternatively, history of cancer may act as a marker for an unmeasured exposure associated with NHL, including chemo- or radiation therapy, or high doses of UV radiation among whites who develop skin cancer.

E.5.6. Allergic conditions

In a Los Angeles study, allergies to nuts and berries, allergy to insect bites, eczema were inversely associated with NHL (66). A Bay Area study reported associations between plant allergies and NHL subtypes including SLL (OR=0.57, 95% CI=0.36-0.92) and DLBCL (OR=0.72, 95% CI=0.55-0.94). Allergies to animal dander was also inversely associated

with DLBCL (OR=0.40, 95% CI=0.21-0.73). One possible explanation is that allergies indicate a hyperactive immune system (64).

E.5.7. Common medications

The exponential growth in antibiotic production between the 1940s and 1970s paralleled the rapid increase in NHL incidence. Sulfonamides and other antibiotics have been associated with NHL, but their effects are difficult to separate from the effects of underlying infections, chronic inflammation, impaired immune function or smoking. Similarly, cimetidine and other histamine-2 receptor antagonists, used to treat ulcers, are inconsistently associated with NHL and may be markers for chronic underlying *H. pylori* infection (64,77-78).

Among a cohort of Iowa women NHL was associated with aspirin use alone (OR=2.31, 95% CI=1.04-5.15), NSAID use alone (OR=3.39, 95% CI=1.38-8.32) and combined NSAID and aspirin use (OR=2.82, 95% CI=1.24-6.39). However, other studies including both men and women have found an inverse association between NHL and NSAIDs (64,71). NSAIDs have been inversely associated with other cancers including colorectal. NSAIDs-mediated inhibition of Cox-2, which normally promotes cell growth and differentiation, may induce apoptosis of cancer cells (64). On the other hand, NSAIDs may increase risk of NHL by interfering with NF-κB function, important for developing immune response (79). The association between NHL and NSAIDs may be confounded by their indication, such as rheumatoid arthritis (71,75).

E.6. Environmental / occupational exposure

E.6.1. Occupational exposure

NHL has been associated with occupations in agriculture, forestry, fishing, and construction industries, crop production, metal-working with machinery and equipment, telecommunications, teaching, farming, welding, soldering, building caretakers, clerks and, working in printing and publishing industry, hairdressers, textile workers (80-85). Exposures to pesticides, herbicides, fertilizers, fuels and engine exhausts, organic and inorganic dusts, metals, organic solvents, and magnetic fields may mediate the association between occupation and NHL (80,83,84,86). Organic solvents have been shown to reduce B and T cell function and numbers, possibly playing a role in lymphomagenesis through immune suppression, but their associations with NHL have been inconsistent (33,87).

E.6.2. Pesticides

Compared to the general population, farmers and other farm workers have an elevated cancer incidence in the lips, skin, stomach, prostate, brain and testis (88). The link between farming and NHL is consistent with an intermediate role played by pesticides, chronic antigenic stimulation, or infectious microorganisms. Exposure to pesticides in farmers and the general population has been increasing since the 1940s. Agricultural exposures associated with NHL include 2,4-D, a phenoxyacetic acid herbicide, organophospate pesticides, fumigants methyl bromide, farm animals, type of farming, orchard work, and the use of mineral-, cutting-, or lubricating oils (88-91).

Associations between pesticides and t(14;18) have been observed in two population-based case-control studies. Among Iowa and Minnesota men, t(14;18)-positive NHL (n=68)

was associated with exposure to several insecticides including lindane (OR=2.3, 95% CI=1.3-3.9), dieldrin (OR=3.7, 95% CI=1.9-7.0), and toxaphene (OR=3.0, 95% CI=1.5-6.1) and the fungicide phthalimides (OR=2.9, 95% CI=1.1-7.5), while t(14;18)-negative NHL (n=114) was not associated (92). The Nebraska study of white men and women also found subtype-specific associations between t(14;18)-positive NHL (n=65) and various insecticides, herbicides, and fumigants, but none were associated with t(14;18)-negative NHL (n=107). Longer duration of use of insecticides and herbicides was associated with t(14;18)-positive NHL as well (93). Both studies support the use of t(14;18)-NHL case-subtypes in etiologic research, but need further confirmation.

E.6.3. Sunlight

The hypothesis that sunlight increased the risk of NHL has been explored with mixed results. A Swedish report found lower south residential latitude (increased sunlight exposure) to be weakly associated with NHL in men and women (men, OR=1.21, 95% CI=1.08-1.35 and women, OR=1.26, 95% CI=1.08-1.40), but no association between occupational sun exposure and NHL was found (94). Other studies have found null or inverse associations with sunlight and ultraviolet radiation exposure (34,95-97). The conflicting findings on UV radiation may reflect bias and chance with different exposure and outcome definitions and not adjusting for other confounding factors including amount of time spent outdoors. UV radiation has been shown to cause DNA damage and immune suppression in both humans and animals in lab studies (98). Additionally, UVB (measured from sunlight records) was reported to have a positive dose-response with t(14;18) frequency in peripheral lymphocytes collected from a people during the summer months in the United

Kingdom (high >125 Jm^{-2} vs. low <125 Jm^{-2} UVB deviation), raising the possibility that sunlight plays a role in increasing the number of t(14;18)-positive B-cells (99).

E.7. Lifestyle factors

E.7.1. Hair dye

The weak association between hair dye use and NHL varies with duration, type of color, and NHL subtype (100-105). A recent meta-analysis pooled estimates from 12 cases-control studies and 2 cohort studies and found a moderately elevated risk of NHL associated with any hair dye use (random-effects RR=1.23, 95% CI=1.07-1.42) (105). The use of hair dye has increased dramatically since the 1940s, paralleling the increase of NHL, and laboratory studies have shown that hair dye components are strongly mutagenic after oxidization. Nonetheless, the potential public health significance of hair dyes is believed to be small.

E.7.2. Tobacco use

Most studies on smoking and other tobacco use and NHL have found a weak association that varied across NHL subtypes and type of tobacco product (Table 2.4). In the InterLymph study, ever smoking was weakly associated with NHL (OR=1.07, 95% CI=1.00-1.15). Associations with follicular lymphoma were slightly stronger than those for NHL overall which is supported by a number of studies (106-111). The association between NHL and smokeless tobacco including snuff and chewing tobacco has also been found in men (103,112).

Tobacco smoke contains many carcinogenic compounds that may increase risk of NHL through a direct or indirect effect on lymphocytes. Two studies suggesting that smoking correlates with the number of t(14;18)-positive lymphocytes in the peripheral blood and bone marrow of healthy individuals and patients with non-lymphoid diseases support the reported associations between smoking and follicular lymphomas which are almost always t(14;18)-positive (113-114). Mutagens in tobacco smoke may cause the translocations to occur, leading to immortalized lymphocytes that become more likely to undergo malignant transformation. Smoking could also increase the number of lymphocytes carrying t(14;18) through chronic antigenic stimulation. In the two population-based studies of t(14;18)-NHL, smoking was not positively associated with t(14;18)-positive NHL, but misclassification may have occurred (103,115).

Another pathway through which smoking may increase risk of lymphomas is through immune suppression or impairment of the body's defenses. Smoking is associated with immunologic alterations including alterations in T cell subsets, elevated white blood cell counts and lower proportions of natural killer cells. Natural killer cells are likely to play an important role in anti-tumor activity, and studies suggest that they are impaired in smokers. At least two studies found that natural killer cell count and activity were lower in peripheral blood lymphoid cells of smokers compared with never smokers (116-117).

Evidence of the association between risk of NHL and smoking remains unclear. No positive dose-response has been established, and point estimates for relative risk are often imprecise. The consistent association between follicular lymphoma and smoking warrants further study, especially for NHL molecular subtype-specific associations.

E.7.3. Alcohol intake

The InterLymph study reported inverse associations between drinking and NHL overall (OR=0.83, 95% CI=0.76-0.89), and some subtypes, particularly FL (OR=0.84, 95% CI=0.73-0.97), DLBCL (OR=0.64, 95% CI=0.53=0.77), and BL (OR=0.29, 95% CI=0.13-0.64) (118). Other studies have found no association between drinking and any of the NHL subtypes (119-120). The protective effect of light to moderate alcohol use may be due to immunomodulatory effects that improve cellular and humoral immune responses (118).

Other studies found an inverse association between NHL and wine drinking. A prospective study of post-menopausal women found an inverse association between NHL and red wine, (OR=0.85, 95% CI=0.51-1.40 for 2 or more servings of red wine) and white wine (higher than median intake, OR=0.35, 95% CI=0.13-0.96) (121). In another study, men 32 to 60 years old, drinking less than 1 glass of wine a day had a decreased risk of NHL (OR=0.5, 95% CI=0.2-0.9), though no association was found for beer and spirits (122). Wine drinking may decrease risk of NHL through the effects of resveratrol, a phytoestrogen produced by grapes and reported to have inhibitory effect on antiapoptotic bcl-2 protein (121-122). The use of NHL case-subtypes defined by bcl-2 expression may elucidate this inverse association between wine drinking and NHL.

E.7.4. Dietary factors

A high intake of meat and high intake of saturated fats were associated with NHL in two case-control studies (123-124). The role of dietary meat in lymphomagenesis has been hypothesized to involve the chronic hyperstimulation of the immune system, mutagenic preservatives in processed meats that contain precursors to N-nitroso compounds, and

polycyclic aromatic hydrocarbons produced when meat is fried or grilled (123). Fat has been hypothesized to hyperstimulate the immune system through its effects on cell membrane structure and function, while fibers have been proposed to affect dilution, absorption, and /or breakdown of fat and animal protein in the gut by modifying the gut microflora composition (124). Dairy, eggs and bread have been found to be associated with elevated NHL risk (123-125). Pasta, bread and rice all contain a high amount of sugars and carbohydrates that could trigger insulin secretion in which high levels have been linked to several cancers in past studies. Inconsistent findings may be due to chance associations or reflect differences in fat or cooking among populations.

Similar to other nutrition and cancer studies, NHL is inversely associated with the high consumption of fruits and vegetables including carrots, broccoli, green vegetables, citrus fruits, tomatoes, squash, cabbage, cauliflower, onions, leeks, salad, citrus fruits, vitamin C, multivitamins, and dietary fiber (124-126). Conversely, low intake of vitamin C and carotene was positively associated with NHL in one study, though the association was restricted to those with a family history of cancer (125).

E.7.5. Anthropometric factors

Several studies have supported an association between body mass index (BMI) and NHL overall or subtypes (127-129). However, a large Swedish cohort study of 330,000 construction workers found no association between NHL and high BMI (≥30) in men or women. Results from animal studies suggest that body mass may affect cell proliferation and apoptosis. Impaired immune function and nutritional factors are some other proposed mechanisms (127,130). Diabetics who are often obese may be susceptible to developing

NHL through impairments of their immune system, metabolic alterations and high levels of insulin which stimulate cell growth through the insulin receptor or insulin-like growth factor I receptor. Among a cohort of Iowa women, history of adult-onset diabetes (age-adjusted RR=2.18, 95% CI=1.22-3.90) was positively associated with NHL (76). In a large population-based Canadian case-control study, the highest quartile of total recreational physical activity compared with the lowest quartile was inversely associated with NHL in men and women (OR=0.79, 95% CI=0.59-1.05 and OR=0.59, 95% CI=0.42-0.81, respectively). Possible mechanisms for physical activity are enhanced immune function resulting in higher clearance of tumor cells, improved antioxidant defense systems, increased insulin sensitivity, decreased insulin and insulin-like growth factors (128).

E.8. Summary of risk factors

In summary, the risk factors for NHL represent some common themes, each of which the establishment and growth of a malignant cell is facilitated: 1) an underlying immune suppressive state whether congenital (e.g. AT) or acquired (HIV, transplant recipients) 2) chronic antigenic stimulation due to infection (e.g. *H pylori*) or other agents (e.g. smoking) and 3) possible mutagenic effects of an agent (e.g. smoking, chemicals and viruses) (51). Our inability to identify these risk factors consistently may be due, in part, to grouping outcomes that may be unrelated etiologically. Equipped with molecular biomarkers of early or late events in lymphomagenesis defining the outcome, we may find more risk factors and provide further refinements to the current WHO classification scheme.

Table 2.1. Comparison of 3 recent classification systems for selected NHL subtypes (2,8)

New World Health Organization (WHO) Classification, 2001	Revised European-American Lymphoma (REAL) Classification, 1994	Working Formulation (WF) for NHL, 1981
B-chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL)	B-cell chronic lymphcytic leukaemia	Small lymphocytic, consistent with CLL
Mantle cell lymphoma	Mantle cell lymphoma	Malignant lymphoma, diffuse, small cleaved cell type
Follicular lymphoma	Follicle centre lymphoma, follicular	Follicular small cleaved, mixed, large, or small non-cleaved cell
Extranodal marginal zone B-cell lymphoma of MALT type	Marginal zone B-cell lymphoma	Small lymphocytic, lympplasmacytoid, diffuse small cleaved cell
Nodal marginal zone B-cell lymphoma	Marginal zone B-cell lymphoma	Small lympocytic, plasmacytoid, follicular or diffuse small cleaved cell, follicular or diffuse mixed small and large cell
Splenic marginal zone B-cell lymphoma	Splenic marginal zone B-cell lymphoma	Small lymphocytic lymphoma
Diffuse large B-cell lymphoma	Diffuse large B-cell lymphoma	Diffuse large cell, large cell immunoblastic, diffuse mixed small and large cell
Burkitt/Burkitt-like lymphoma	Burkitt lymphoma; high-grade B-cell lymphoma, Burkitt-like (provisional entity)	Small non-cleaved cell , Burkitt type

Figure 2.1. NHL REAL subtypes by geographic region (6)

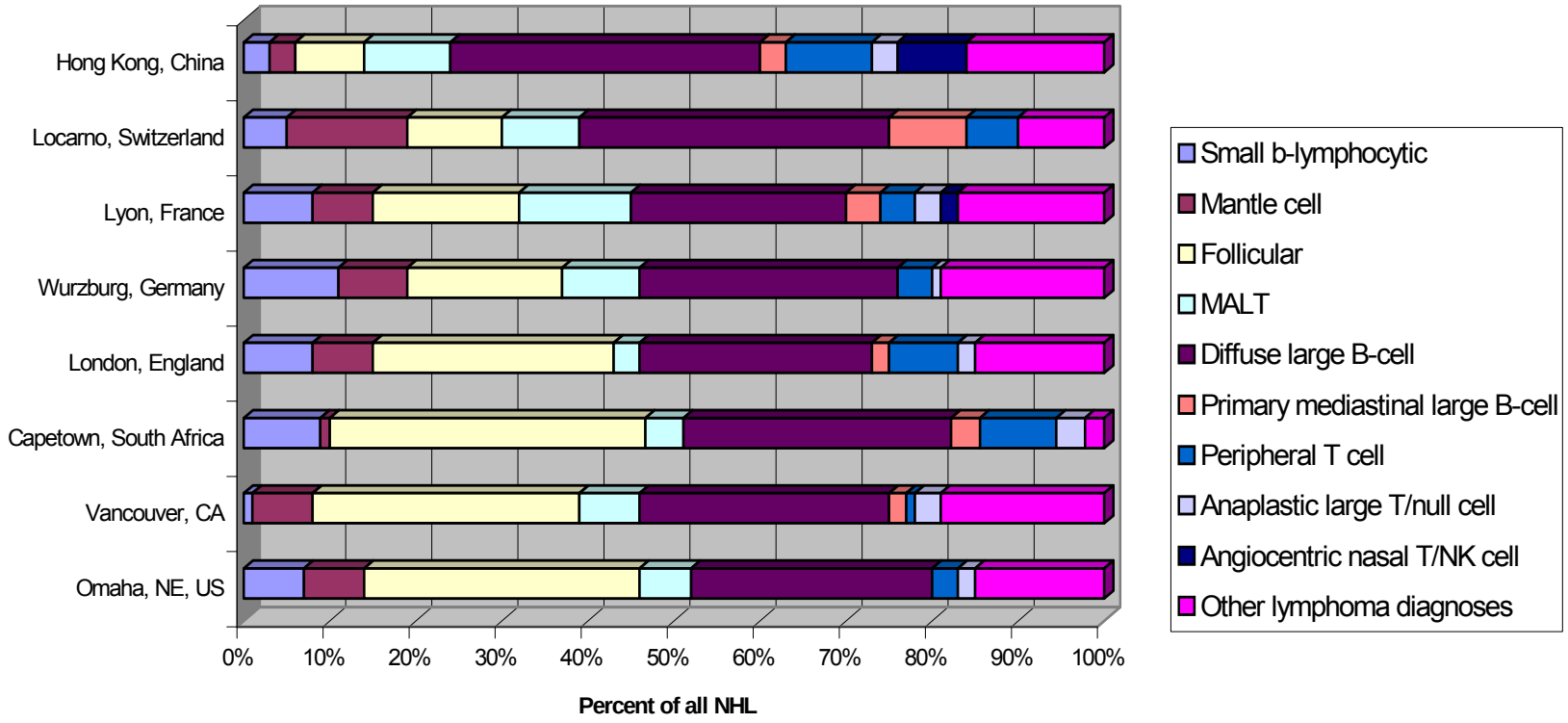


Figure 2.2. Antibody structure and binding sites (23)

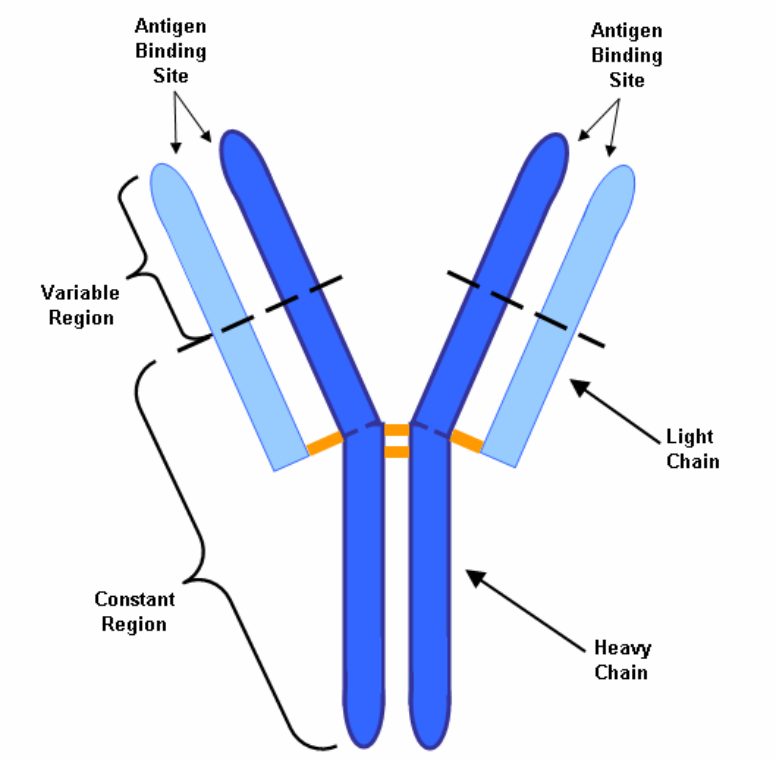


Figure 2.3. B-cell development in the bone marrow and peripheral lymphoid organs (22,23)

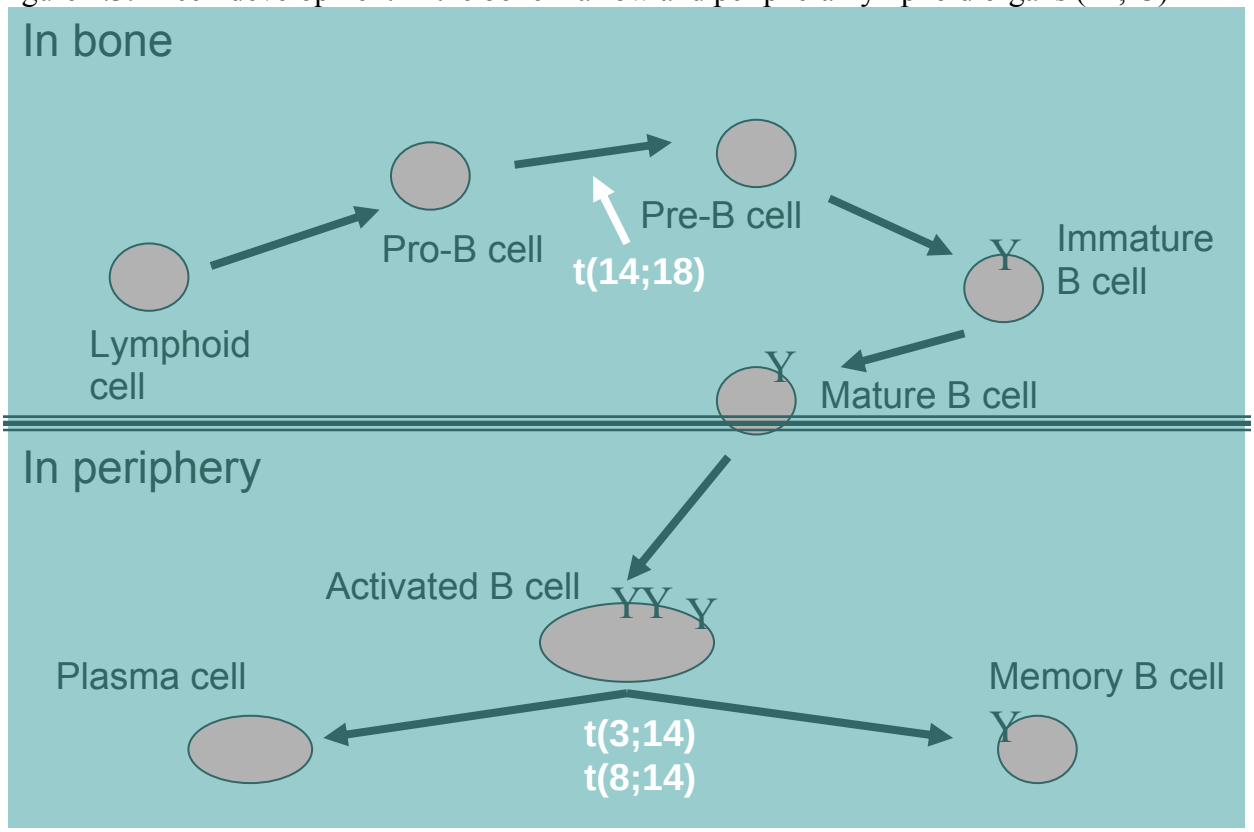


Figure 2.4. Illustration of t(8;14) translocation (adapted from Janeway 2001, 19)

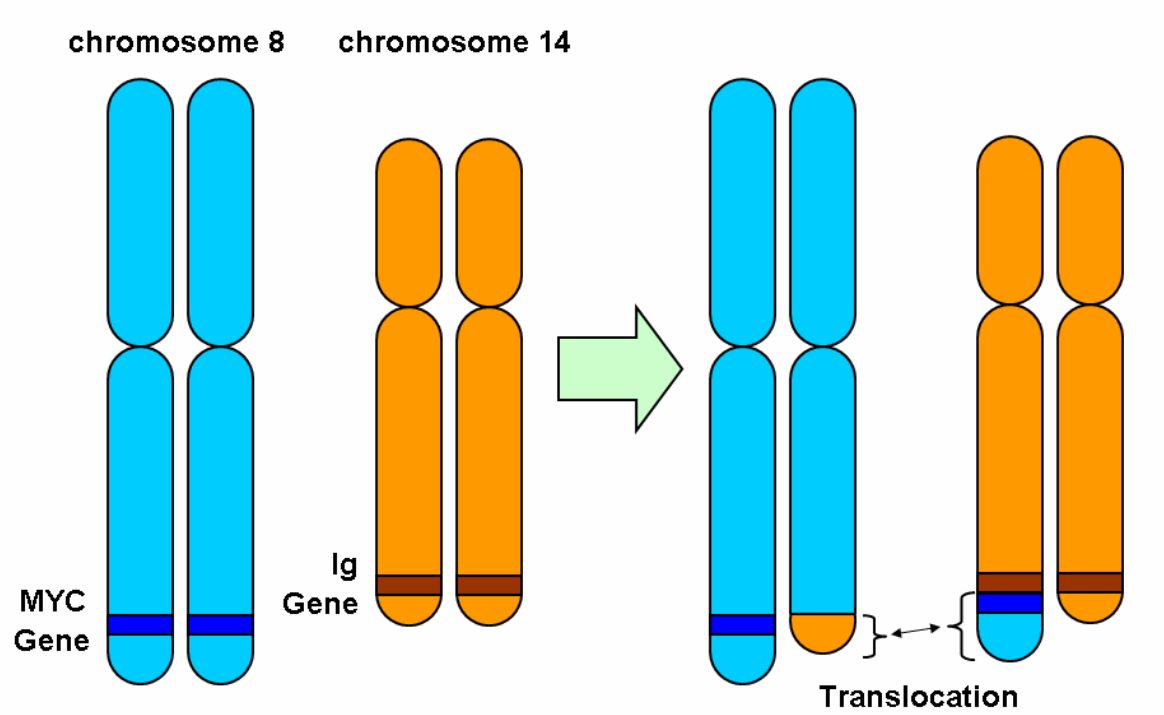


Table 2.2. NHL translocations and associated World Health Organization (WHO) subtypes*

Translocation	NHL Subtype	Frequency
t(14;18)	Follicular Diffuse Large B Cell CLL/SLL	80-90% 20-30% 2%
BCL6 translocations	Diffuse Large B Cell Follicular	27% 5-14%
t(8;14)	Burkitt Diffuse Large B Cell	80-100% 6-16%

*Vega 2003 (26); Medeiros 1999 (28); Bastard 1994 (139); Campbell 2005 (7); Willis 2000 (9)

Figure 2.5. U.S. NHL incidence by age, race, sex (1998-2002) (131)

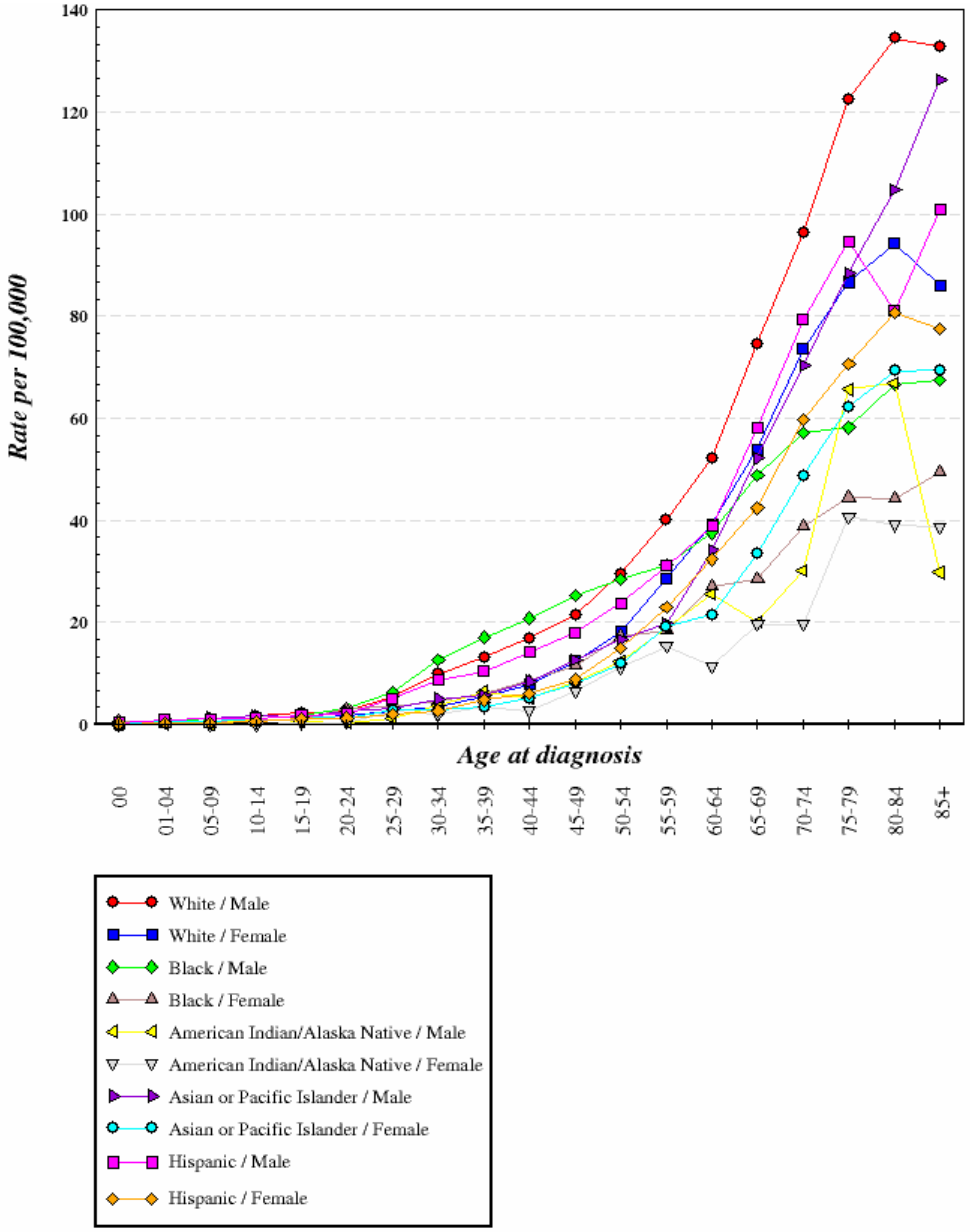


Figure 2.6. Age-adjusted NHL incidence by race and sex, all ages (1973-2002) (131)

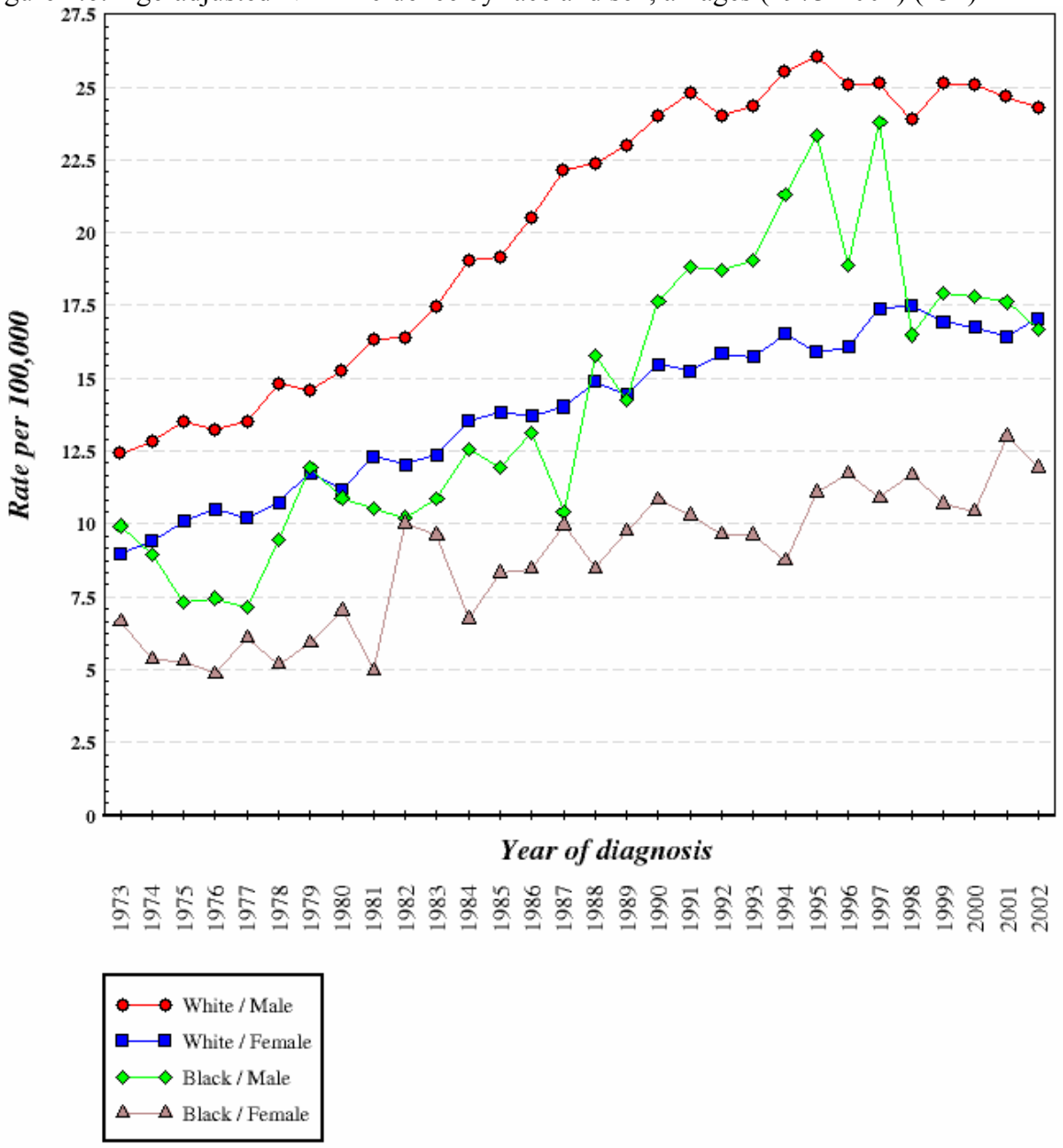


Table 2.3. NHL incidence by race and sex (1998-2002) (131)

Race/Ethnicity	Men (cases per 100,000)	Women (cases per 100,000)
All Races	23.2	15.8
White	24.4	16.7
Black	17.6	11.6
Asian/Pacific Islander	16.8	11.5
American Indian/Alaska Native	10.2	7.5
Hispanic	18.8	13.4

Table 2.4. Selected studies on smoking and NHL

Study	Design	Population	Exposure categories	Estimate	Comments
Chiu 2007 (115)	Population-based case-control	Nebraska men and women, cases=172, controls=1,655	Ever/former/current smoking, cigar smoking, chewing tobacco use, age began smoking, duration, intensity, pack-years	Men: all null associations for both t(14;18)-NHL case-subtypes; Women: t(14;18)-positive NHL-- all null or inverse associations; t(14;18)-negative NHL—ever smoking OR=1.9, 95% CI=1.1-3.3, all smoking categories associated with t(14;18)-negative NHL	Estimates were adjusted for age, type of respondent, and farming status. Smoking was associated with cigarette smoking in women, but number of exposed cases was small; not much dose-response
Besson 2006 (132)	Population based case-control (7 European countries)	Epilymph: Czech Rep, Finland, France, Germany, Ireland, Italy, Spain cases=1,742, controls=2,465	Smoking status, type of tobacco, duration, intensity, age at start	Ever smoking and NHL: OR=1.06, 95% CI=0.92-1.21; most associations null; FL and current smoking OR=1.35, 95% CI=0.96-1.90	Adjusted for age, sex, education, alcohol monthly consumption, center; no support for smoking and NHL
Morton 2005 (106)	Pooled analysis of 8 pop based and 1 hospital based case-control 1998-2004	InterLymph, cases=6,594, controls=8,892	age began smoking, current smking habits, duration/intensity of smk, pack-years	ever smoking pooled OR=1.07, 95% CI=1.00-1.15; current smoker OR=1.10, 95% CI=1.0-1.2; former smoker OR=1.06, 95% CI=0.98-1.15, 36+ pack-years OR=1.21, 95% CI=1.09-1.34; stronger for FL	Sex, age, race, BMI, family history of NHL in 1st degree relative, history of alcohol consumption and SES were considered as potential confounders; final estimates adjusted for study center, sex, age, and race
Bracci 2005 (112)	Bay area population-based case-control 1988-1995	Cases=1,593; Controls=2,515, HIV-negative participants	Cigarette, pipe, cigar, chewing tobacco, snuff, intensity, duration, former, current smoking	Mostly null for smoking in men and women; snuff or chewing tobacco assoc with NHL in men: OR=4, 95% CI=1.3-12 (3 exposed cases), other tobacco only in men OR=1.5, 95% CI=0.96-2.4	Adjusted for age; no overall association between smoking and NHL; smokeless tobacco use based on small numbers
Stagnaro 2004 (107)	population-based case-control (11 Italian areas)	Italian, 1450 cases, 1779 controls	cigarette brand, date of starting and stopping smoking, # cigarettes smoked, use of filters; pack-years=packs/day x years smoked	blond tobacco, OR=1.4, 1.1-1.7 (NHL); OR=2.1, 1.4-3.2 (follicular)	Dose-response for pack-years was limited to men younger than 52 ($p<0.001$); blond worse than black, mixed intermed risk; agrees with Freedman 1998; stronger effect on high-grade NHL

Table 2.4. Selected studies on smoking and NHL (continued)

Study	Design	Population	Exposure categories	Estimate	Comments
Willet 2004 (119)	population-based case-control	England, age 18-64, 700 cases	ever smoking cigarettes, cigars, or pipe once/day for 6 months, age or year started, amt/day, age or year stopped	no association between smoking and NHL or subtypes; no assoc between alc and NHL or subtypes; no dose-response observed	
Morton 2003 (108)	population-based	Conn. Women; 601 cases and 718 controls	smoker=at least 100 cigarettes; age started, ave # cigarettes/day, years smoked at least 1 cig/day, age stopped	risk of FL increased with 16-33 p-y, OR=1.5, 0.9-2.5; 34 p-y OR=1.8, 1.1-3.2 ptrend=0.05	increased risk only seen for follicular
Besson 2003 (133)	hospital case-control	France; 180 cases, 2 groups of 180 hospital-based controls	smoking status, smoking duration, cigs/day, age smking initiated and termin	for NHL, current smoker (women) OR=2.40, 1.19-4.84; smoking for >31 y, OR=5.04, 1.40-18.12; for FL, ORs elevated but CI wide	differences between genders may be biologic diff; no effect on other subtypes except for FL
Schroeder 2002 (103)	population-based case-control	Iowa and Minnesota men, cases=182, controls=1245	Any tobacco use, type of tobacco product (cigarette, pipe, cigar, chewing tobacco, snuff), cigarettes only/cig and other/other tob only, cig pack-years, intensity, age started smoking	t(14;18)-positive NHL associated with chewing tobacco (OR=1.7, 95% CI=0.9-3.1) and young age at first chew tobacco use (before 18) (OR=2.5, 95% CI=1-6), null association for cigarette smoking; t(14;18)-negative NHL not associated with any tobacco exposures	Estimates were adjusted for age and state. EM algorithm was applied to reduce imprecision and bias.
Stagnaro 2001 (109)	Italian pop-based case-control	Italian, 3357cases, 2391 controls	former smokers stopped at 1 year b/c study, cigarettes/day and years--3 levels	current smk, OR=1.2, 1.0-1.5 (NHL); current for FL, OR=2, 1.3-3.0; >36 years OR=2.3, 1.4-3.8	smoking an RF for NHL with a consistent assoc only for FL in women
Parker 2000 (110)	cohort study	Iowa women, 37,336 women, 200 cases (random sample of Iowa women)	current, former, never; pack-years	former smk and FL OR=1.6, 0.7-3.4; current sk and FL OR=2.3, 1.0-5.0	no assoc between smoking history and NHL overall; or for low/intermed--positive for high grade but small cases; suggestive association for FL

Table 2.4. Selected studies on smoking and NHL (continued)

Study	Design	Population	Exposure categories	Estimate	Comments
Freedman 1998 (134)	pop-based case-control	Vietnam vets, 1190 cases, 2299 controls	packs per day, years smoking, age at start, years since quitting, pack-years; current smoking--men who stopped within 1 year of study	sig linear trends for packs/day and pack-years for NHL and >50 p-y OR=1.41, 1.1-1.8; low grade and >50 pack-years OR=1.65 (1.2-2.5); high-grade OR=1.73, 1.1-2.8	in men starting at younger age, smoked more and for longer period of time; effect was modified by age (heavy smk affected younger age risk)' maybe younger men with NHL are more susceptible
Herrinton 1998 (111)	prospective, 11 year follow-up	Kaiser Perman members, 674 cases of incident NHL	current, past, years of regular cig smk, ave pack/day	smokers at increased risk of FL, former smk RR=1.9, 1.2-2.9, current smkers RR=1.4, 0.9-2.2	smoking history only one point in time, no info on HIV
Zahm 1997 (135)	pooled analysis of 3 pop-based case-control	Nebraska, Kansas, IA, MN--1200 cases	age began smoking,cigarettes/day, age stopped, duration/intensity of smk, pack-years	no association with men, but smoking and NHL associated in women	Brown et al. 1992 and Linet et al. 1992 referenced; no clear gradients; study provides evidence that smoking has little effect on NHL in men
Nelson 1997 (136)	population-based case-control	LA county, 378 cases	never, ever, former, current, cigarettes/day	current smoking in men OR=1.90 (1-3.51); in women OR=0.79 (0.44-1.43); cocaine 9+ times OR=3.25 (1.35-7.85)	history of alcohol and tobacco use not associated with NHL in men; cocaine use sig in stepwise & drug use in last 5 & 10 y; increase in NHL coincides w/ epidemic of recreational drug use in US
Brown 1992 (137)	population-based case-control	white men over 30 from Iowa health registry and MN	tobacco use, first and last year of use, age at first use, amt/day	risk of lymphoma, pipes or cigars, smless tob OR=2.9 (1.4-6.1); cig only OR=1.5 (1.2-2.1); ORs decreased when only using living; cig and other tobacco OR for high-grade=2.5 (1.1-5.9)	association with high grade and unclassified are new findings; 1st to use morph classif; no intensity-response gradients seen with hg or unclass lymphomas; new finding inc risk for users of smokeless tobacco who also use pipes and/or cigars; but based on small #'s
Linet 1992 (138)	cohort study	white males of Lutheran Brotherhood Insurance Soc.; 286,731 PY; 1967-86; 49 NHL deaths	tobacco use, cig/day	used any tobacco risk of NHL elevated, RR=1.9 (0.8-4.5); >1 pack/day RR=3.8 (1.4-10.1)	1st cohort study to show dose-response relationship--smoking only measured once in 1966, so risk may be underestimated

F. References

1. Magrath IT. 1997. Historical perspective: the evolution of modern concepts of biology and management. In: IT Magrath, ed. The non-Hodgkin lymphomas, 2nd Edition, London: Oxford University Press, 1997: pp. 46-71.

2. Chan JKC. The New World Health Organization classification of lymphomas: the past, the present and the future. Hematol Oncol 2001; 19: 129-150.

3. Weisenburger DD. Pathological classification of non-Hodgkin lymphoma for epidemiological studies. Cancer Res 1992; 52: 5456s-5464s.

4. Müller AMS, Ihorst G, Metelsmann R, Engelhardt M. Epidemiology of non-Hodgkin lymphoma (NHL): trends, geographic distribution, and etiology. Am Hematol. 2005; 84: 1-12.

5. Medeiros LJ, Bagg A, Cossman J. Molecular genetic in the diagnosis and classification of lymphoid neoplasms. In: ES Jaffe, ed. Surgical Pathology of the Lymph Nodes and Related Organs, 2nd Edition. Philadelphia: WB Saunders, 1994: pp. 58-97.

6. Anderson JR, Armitage JO, Weisenburger DD. Epidemiology of the non-Hodgkin lymphomas: distribution of the major subtypes differ by geographic locations. Ann Oncol 1998; 9: 717-720.

7. Campbell LJ. Cytogenetics of lymphomas. Pathology 2005; 37(6): 493-407.

8. Jaffe ES, Harris NL, Stein H, Vardiman JW (editors). Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues: Lyon. IARCPress, 2001.

9. Willis TG and Dyer MJS. The role of immunoglobulin translocations in the pathogenesis of b-cell malignancies. Blood 2000; 96(3): 808-822.

10. Herrinton LJ, Goldloft M, Schwartz SM, Weiss NS. The incidence of non-Hodgkin lymphoma and its histologic subtypes in asian migrants to the United States and their descendants. Cancer Causes Control 1996; 7: 224-230.

11. Groves FD, Linet MS, Travis LB, Devesa SS. Cancer surveillance series: non-Hodgkin lymphoma incidence by histologic subtype in the United States from 1978 Through 1995. JNCI 2000; 92(15): 1240-1248.

12. Godon A, Moreau A, Talmant P, Baranger-Papot L, Genevieve F, Milpied N, Zandecki M, Avet-Loiseau H.. Is t(14;18)(q32;q21) a constant finding in follicular lymphoma? An interphase FISH study on 63 patients. Leukemia 2003; 17: 255-259.13. Carbone A. Emerging Pathways in the Development of AIDS-Related Lymphomas. Lancet Oncol 2003; 4: 22-29.

13. Belaud-Rotureau MA, Parrens M, Dubus P, Garroste JC, de Mascarel A, Merlio JP. A comparative analysis of FISH, RT-PCR, PCR, and immunohistochemistry for the diagnosis of mantle cell lymphomas. Mod Pathol. 2002 May;15(5):517-25.

14. Carbone A. Emerging pathways in the development of AIDS-related lymphomas. Lancet Oncol. 2003 Jan;4(1):22-9.

15. Mossafa H, Damotte D, Jenabian A, Delarue R, Vincenneau A, Amouroux I, Jeandel R, Khoury E, Martelli JM, Samson T, Tapia S, Flandrin G, Troussard X. Non-Hodgkin lymphomas with Burkitt-like cells are associated with c-Myc amplification and poor prognosis. Leuk Lymphoma. 2006 Sep;47(9):1885-93.

16. McClure RF, Remstein ED, Macon WR, Dewald GW, Habermann TM, Hoering A, Kurtin PJ.Adult B-cell lymphomas with burkitt-like morphology are phenotypically and genotypically heterogeneous with aggressive clinical behavior. Am J Surg Pathol. 2005 Dec;29(12):1652-60.

17. Braziel RM, Arber DA, Slovak ML, Gulley ML, Spier C, Kjeldsberg C, Unger J, Miller TP, Tubbs R, Leith C, Fisher RI, Grogan TM. The Burkitt-like lymphomas: a Southwest Oncology Group study delineating phenotypic, genotypic, and clinical features. Blood. 2001 Jun 15;97(12):3713-20.

18. Fan YS, Rizkalla K. Comprehensive cytogenetic analysis including multicolor spectral karyotyping and interphase fluorescence in situ hybridization in lymphoma diagnosis: a summary of 154 cases. Cancer Genetics and Cytogenetics 2001; 143: 73-79.

19. Naresh KN, Srinivas V, Soman CS. Distribution of various subtypes of non-Hodgkin lymphoma in India: a Study of 2773 lymphomas using R.E.A.L. and WHO classification. Ann Oncol 2000; 11: S63-S67.

20. Intragumtornchai T, Wannakrairoj P, Chaimongkol B et al. Non-Hodgkin lymphomas in Thailand: a retrospective pathologic and clinical analysis of 1391 cases. Cancer 1996; 78: 1813-9.

21. Morton LM, Wang SS, Devesa SS, Hartge P, Weisenburger DD, Linet MS. Lymphoma incidence patterns by WHO subtype in the United States, 1992-2001. Blood. 2006 Jan 1;107(1):265-76.

22. Kuby J. Immunology. New York: WH Freeman and Company, 1997.

23. Janeway Ca, Travers P, Walport M, Shlomchik M. Immunobiology 5. New York: Garland Publishing, 2001.

24. Potter M. Pathogenetic mechanisms in B-cell non-Hodgkin lymphomas in humans. Cancer Res 1992; 52: 5522s-5528s.

25. Vega F, Orduz R, Medeiros LJ. Chromosomal translocations and their role in the pathogenesis of non-Hodgkin lymphomas. Pathology. 2002 Oct;34(5):397-409.

26. Vega F, Medeiros LJ. Chromosomal Translocations Involved in Non-Hodgkin Lymphomas. Arch Pathol Lab Med. 2003 Sep; 127(9): 1148-60.

27. Gutierrez MI, Bhatia K, Barriga F, Diez B, Muriel FS, de Andreas ML, Epelman S, Risueno C, Magrath IT. Molecular epidemiology of Burkitt lymphoma from South America. Blood. 1992 Jun 15;79(12):3261-6.

28. Medeiros LJ, Carr J. Overview of the role of molecular methods in the diagnosis of malignant lymphomas. Arch Pathol Lab Med. 1999; 123: 1189-1207.

29. Zutter M, Hockenbery D, Silverman GA, Korsmeyer SJ. Immunolocalization of the Bcl-2 protein within hematopoietic neoplasms. Blood. 1991 Aug 15;78(4):1062-8.

30. Pezzella F, Tse AG, Cordell JL, Pulford KA, Gatter KC, Mason DY. Expression of the bcl-2 oncogene protein is not specific for the 14;18 chromosomal translocation. Am J Pathol. 1990 Aug;137(2):225-32 29.

31. Papakonstantinou G, Verbeke C, Hastka J, Bohrer M, Hehlmann R. bcl-2 expression in non-Hodgkin lymphomas is not associated with bcl-2 gene rearrangements. Br J Haematol. 2001 May;113(2):383-90.

32. Kusumoto S, Kobayashi Y, Sekiguchi N, Tanimoto K, Onishi Y, Yokota Y, Watanabe T, Maeshima AM, Ishida T, Inagaki H, Matsuno Y, Ueda R, Tobinai K. Diffuse large B-cell lymphoma with extra bcl-2 gene signals detected by FISH analysis is associated with a "non-germinal center phenotype". Am J Surg Pathol. 2005 Aug;29(8):1067-73.

33. Kato I, Koenig KL, Watanabe-Meserve H, Baptiste MS, Lillquist PP et al. Personal and Occupational Exposure to Organic Solvents and Risk of Non-Hodgkin Lymphoma (NHL) in Women (United States). Cancer Causes Control 2005; 16: 1215-24.

34. Hartge P, Lim U, Freedman DM, Colt JS, Cerhan JR, Cozen W, Severson RK, Davis S. Ultraviolet radiation, dietary vitamin D, and risk of non-Hodgkin lymphoma (United States).Cancer Causes Control. 2006 Oct;17(8):1045-52

35. Cantor KP, Fraumeni JF Jr. Distribution of non-Hodgkin lymphoma in the United States between 1950 and 1975. Cancer Res. 1980 Aug;40(8 Pt 1):2645-52.

36. Alexander DD, Mink PJ, Adami HO, Chang ET, Cole P, Mandel JS, Trichopoulos D. The non-Hodgkin lymphomas: A review of the epidemiologic literature. Int J Cancer. 2007;120 Suppl 12:1-39.

37. Adami J, Gäbel H, Lindelöf B, Ekström K et al. Cancer risk following organ transplantation: a nationwide cohort study in Sweden. Br J Cancer 2003; 89: 1221-7.

38. Baker KS, DeFor TE, Burns LJ, Ramsay KC, Neglia JP, Robison LL. New malignancies after blood or marrow stem-cell transplantation in children and adults: incidence and risk factors. J Clin Oncol 2003; 21: 1352-8.

39. Serraino D, Piselli P, Angeletti C, Scuderi M, Ippolito G, Capobianchi MR. Infection with Epstein-Barr virus and cancer: an epidemiological review. J Biol Regul Homeost Agents. 2005 Jan-Jun;19(1-2):63-70.

40. Opelz G, Döhler B. Lymphomas after solid organ transplantation: a collaborative transplant study report. Am J Transplant 2003; 4: 222-30.

41. Frisch M, Biggar RJ, Engels EA, Goedert JJ; AIDS-Cancer Match Registry Study Group. Association of cancer with AIDS-related immunosuppression in adults. JAMA. 2001 Apr 4;285(13):1736-45

42. Del Maso L and Franceschi S. Epidemiology of non-Hodgkin lymphomas and other haemolymphopoietic neoplasms in people with AIDS. Lancet Oncol 2003; 4: 110-19.

43. Filipovich AH, Mathur A, Kamat D and Shapiro RS. Primary Immunodeficiencies: genetic risk factors for lymphoma. Cancer Res 1992; 52: 5565s-7s.

44. Hoover RN. Lymphoma risks in populations with altered immunity: a search for mechanism. Cancer Res 1992; 52: 5477s-8s.

45. Linet MS, Pottern LM. Familial aggregation of hematopoietic malignancies and risk of non-Hodgkin lymphoma. Cancer Res. 1992 Oct 1;52(19 Suppl):5468s-5473s

46. Chang ET, Smedby KE, Hjalgrim H et al. Family history of hematopoietic malignancy and risk of lymphoma. JNCI 2005; 97(19): 1466-74.

47. Pottern LM, Linet M, Blair A, Dick F, Burmeister LF, Gibson R, Schuman LM, Fraumeni JF Jr. Familial cancers associated with subtypes of leukemia and non-Hodgkin lymphoma. Leuk Res. 1991;15(5): 305-14.

48. Wang SS, Slager SL, Brennan P, Holly EA, De Sanjose S, Bernstein L, Boffetta P, Cerhan JR, Maynadie M, Spinelli JJ, Chiu BC et al. Family history of hematopoietic malignancies and risk of non-Hodgkin lymphoma (NHL): a pooled analysis of 10 211 cases and 11 905 controls from the International Lymphoma Epidemiology Consortium (InterLymph). Blood. 2007 Apr 15;109(8):3479-88. Epub 2006 Dec 21.

49. Hsu JL, Glaser SL. Epstein-Barr virus-associated malignancies: epidemiologic patterns and etiologic implications. Crit Rev Oncol Hematol. 2000; 34(1): 27-53.

50. Mueller NE, Mohar A, Evans A. Viruses other than HIV and non-Hodgkin lymphoma. Cancer Res. 1992;52(19 Suppl):5479s-5481s.

51. Fisher SG and Fisher RI. The Epidemiology of non-Hodgkin lymphoma. Oncogene 2004; 23: 6524-6534.

52. Beers MH, editor. The Merck Manual of Medical Information—Second Home Edition (online version). New Jersey: Merck & Co., Inc, 2006.

53. Thorley-Lawson DA. EBV the prototypical human tumor virus--just how bad is it? J Allergy Clin Immunol. 2005;116(2):251-61.

54. IARC Monographs on the Evaluation of Carcinogenic Risks to Human Epstein-Barr Virus and Kaposi's Sarcoma Herpesvirus/Human Herpesvirus 8 (Vol. 70) 1997.

55. Jarrett RF. Viruses and lymphoma/leukemia. J Pathol 2006; 208: 176-86.

56. Arisawa K, Soda M, Endo S, Kurokawa K, Katamine S, Shimokawa I, Koba T, Takahashi T, Saito H, Doi H, Shirahama S. Evaluation of adult T-cell leukemia/lymphoma incidence and its impact on non-Hodgkin lymphoma incidence in southwestern Japan. Int J Cancer. 2000 Feb 1;85(3):319-24.

57. Wotherspoon AC. Gastric lymphoma of mucosa-associated lymphoid tissue and *helicobacter pylori*. Annu Rev Med. 1998;49:289-99.

58. Negri E, Little D, Boiocchi M, La Vecchia C, Franceschi S. B-cell non-Hodgkin lymphoma and hepatits C virus infection: a systematic review. Int J Cancer 2004; 111: 1-8.

59. Mele A, Pulsoni A, Bianco E, Musto P et al. Hepatitis C virus and B-cell non-Hodgkin lymphomas: an italian mulitcenter case-control study. Blood 2003; 102(3): 996-999.

60. Engels EA, Chatterjee N, Cerhan JR, Davis S, Cozen W, Severson RK, Whitby D, Colt JS, Hartge P. Hepatitis C virus infection and non-Hodgkin lymphoma: Results of the NCI-SEER multi-center case-control study. Int J Cancer. 2004 Aug 10; 111(1):76-80.

61. Ramos-Casals M, Trejo O, Garcia-Carrasco M, Cervera R et al. Triple association between hepatits C virus infection systemic autoimmune diseases, and B cell lymphoma. J Rheumatol 2004; 31: 495-9.

62. Giannelli F, Moscarella S, Giannini C, Caini P, Monti M et al. Effect of antiviral treatment in patients with chronic HCV infection and t(14;18) translocation. Blood 2003; 102: 1196-1201.

63. Kim JH, Bang YJ, Park BJ, Yoo T, Kim CW et al. Hepatitis B virus infection and B-cell non-Hodgkin lymphoma in a hepatitis B endemic area: a case-control study. Jpn J Cancer Res 2002; 93: 471-477.

64. Holly EA, Bracci PM. Population-based study of non-Hodgkin lymphoma, histology, and medical history among human immunodeficiency virus-negative participants in San Francisco. Am J Epidemiol. 2003 Aug 15;158(4):316-27

65. Martin C. The dream of a vaccine against tuberculosis; new vaccines improving or replacing BCG? Eur Respir J. 2005; 26(1): 162-7.

66. Bernstein L and Ross RK. Prior medication use and health history as risk factors for non-Hodgkin lymphoma: preliminary results from a case-control study in Los Angeles county. Cancer Res 1992; 52: 5510s-5515s.

67. Tavani A, La Vecchia C, Franceschi S, Serraino D, Carbone A. Medical history and risk of Hodgkin and non-Hodgkin lymphomas. Eur J Cancer Prev 2000; 9: 59-64.

68. Cerhan JR, Wallace RB, Dick F, Kemp J, Parker AS, Zheng W, et al. Blood Transfusions and risk of non-Hodgkin lymphoma subtypes and chronic lymphocytic leukemia. Cancer Epidemiol Biomarkers Prev. 2001 Apr; 10(4): 361-8.

69. Chow EJ and Holly EA. Blood transfusions as a risk factor for non-Hodgkin lymphoma in the San Fancisco Bay Area: a population-based study. Am J Epid 2002; 155(8): 725-31.

70. Brandt L, Brandt J, Olsson H, Anderson H, Moller T. Blood transfusion as a risk factor for non-Hodgkin lymphoma. Br J Cancer. 1996 May;73(9):1148-51.

71. Zhang Y, Holford TR, Leaderer B, Zahm SH, Boyle, Morton LM, Zhang B, Zou K, Flynn S et al. Prior medical conditions and medication use and risk of non-Hodgkin lymphoma in Connecticut United States women. Cancer Causes Control 2004; 15: 419-428.

72. Montella M, Del Maso L, Crispo A, Talamini R, Bidoli E et al. Do childhood disease affect NHL and HL risk? A case-control study from northern and southern Italy. Leukemia Research 2006 (in press).

73. La Vecchia C, Negri E, Franceschi S. Medical history and the risk of non-Hodgkin lymphomas. Cancer Epidemiol Biomarkers Prev. 1992 Nov-Dec;1(7):533-6

74. Vineis P, Crosignani P, Sacerdote C, Fontana A, Masala G, Miligi L et al. Haematopoietic cancer and medical history: a multicentre case control study. J Epidemiol Community Health 2000; 54: 431-6.

75. Smedby KE, Hjalgrim H, Askling, Chang ET, Gregersen H, Porwit-MacDonald A, Sundström C et al. Autoimmune and chronic inflammatory disorders and risk of non-hodgkin lymphoma by subytype. JNCI 2006; 98(1): 51-60.

76. Cerhan JR, Wallace RB, Folsom AR, Potter JD, Sellers TA, Zheng W, Lutz CT. Medical history risk factors for non-Hodgkin lymphoma in older women. JNCI 1997; 89(4): 314-318.

77. Kato I, Koenig KL, Baptiste MS, Lillquist PP, Frizzera G, Burke JS, Watanabe H, Shore RE. History of antibiotic use and risk of non-Hodgkin lymphoma (NHL). Int J Cancer. 2003 Oct 20;107(1):99-105.

78. Beiderbeck AB, Holly EA, Sturkenboom M, Coebergh JW et al. No Increased Risk of Non-Hodgkin lymphoma with steroids, estrogens and psychtropics (Netherlands). Cancer Causes Control 2003; 14: 639-644.

79. Cerhan JR, Anderson KE, Janney CA, Vachon CM, Witzig TE, Habermann TM. Association of aspirin and other non-steroidal anti-inflammatory drug use with incidence of non-Hodgkin lymphoma. Int J Cancer. 2003 Sep 20;106(5):784-8.

80. Zheng T, Blair A, Zhang Y, Weisenburger DD, Zahm SH. Occupation and risk of non-Hodgkin lymphoma and chronic lymphocytic leukemia. J Occup Environ Med 2002; 44: 469-474.

81. Cano MI, Pollan M. Non-Hodgkin lymphomas and occupation in Sweden. Int Arch Occup Environ Health. 2001 Aug;74(6):443-9.

82. Costantini AS, Miligi L, Kriebel D, Ramazzotti V, Rodella S, Scarpi E, Stagnaro E, Tumino R, Fontana A, Masala G, Vigano C, Vindigni C, Crosignani P, Benvenuti A, Vineis P. A multicenter case-control study in Italy on hematolymphopoietic neoplasms and occupation. Epidemiology. 2001 Jan;12(1):78-87

83. Scherr PA, Hutchison GB, Neiman RS. Non-Hodgkin lymphoma and occupational exposure. Cancer Res. 1992 Oct 1;52(19 Suppl):5503s-5509s.

84. Pearce N, Bethwaite P.Cancer Res. Increasing incidence of non-Hodgkin lymphoma: occupational and environmental factors. 1992 Oct 1;52(19 Suppl):5496s-5500s.

85. Miligi L, Seniori Costantini A, Crosignani P, Fontana A, Masala G, Nanni O, Ramazzotti V, Rodella S, Stagnaro E, Tumino R, Vigano C, Vindigni C, Vineis P. Occupational, environmental, and life-style factors associated with the risk of hematolymphopoietic malignancies in women. Am J Ind Med. 1999 Jul;36(1):60-9.

86. Band PR, Le ND, Fang R, Gallagher R. Identification of occupational cancer risks in British Columbia: a population-based case-control study of 769 cases of non-Hodgkin lymphoma analyzed by histopathology subtypes. J Occup Environ Med. 2004 May;46(5):479-89.

87. Lamm SH, Engel A, Byrd DM. Non-Hodgkin lymphoma and benzene exposure: a systematic literature review. Chemico-Biological Interactions 2005; 153-154: 231-237.

88. Keller-Byrne JE, Khuder Sa, Schaub Ea, Mcafee O. A Meta-Analysis of Non-Hodgkin Lymphoma among Farmers in the Central United States. Am J of Indus Medicine 1997; 31: 442-4.

89. Melbye M and Trichopoulos D. Non-Hodgkin Lymphomas. In: Adami H, Hunter D, Trichopoulos, editors. Textbook of Cancer Epidemiology. Oxford: University Press, 2002: 535-555.

90. Pearce NE, Sheppard RA, Smith AH, Teague CA. Non-Hodgkin Lymphoma and Farming: an Expanded Case-Control Study. Int J Cancer. 1987;39(2):155-61.

91. Mao Y, Hu J, Ugnat AM, White K. Non-Hodgkin lymphoma and occupational exposure to chemicals in canada. Annals of Oncology 2000; 11(suppl. 1): S69-S73.

92. Schroeder JC, Olshan AF, Baric R, Dent GA, Weinberg CR, Yount B, Cerhan JR et al. Agricultural Risk Factors for t(14;18) Subtypes of Non-Hodgkin Lymphoma. Epidemiology 2001; 12: 701-9.

93. Chiu BC, Dave BJ, Blair A, Gapstur SM, Zahm SH, Weisenburger DD. Agricultural pesticide use and risk of t(14;18)-defined subtypes of non-Hodgkin lymphoma. Blood. 2006 Aug 15;108(4):1363-9. Epub 2006 Apr 18.

94. Adami J, Gridley F, Nyren O, Dosemeci M, Linet M, Glimelius B, Ekbom A, Zahm SH. Sunlight and non-Hodgkin lymphoma: a population-based cohort study in Sweden. Int J Cancer 1999; 80: 641-5.

95. Hartge P, Devesa SS, Grauman D, Fears TR, Fraumeni JF. Non-Hodgkin Lymphoma and Sunlight. JNCI 1996; 88(5): 298-300.

96. Hu S, Ma F, Collado-Mesa F, Kirsner RS. Ultraviolet radiation and incidence of non-Hodgkin lymphoma among hispanics in the United States. Cancer Epidemiol Biomarkers Prev 2004; 13: 59-64.

97. Hughes AM, Armstrong BK, Vajdic CM, Turner J, Grulich AE et al. Sun Exposure May Protect against Non-Hodgkin Lymphoma: a Case-Control Study. Int J Cancer 2004; 112: 865-871.

98. Byrne SN and Halliday GM. B Cells Activated in Lymph Nodes in Response to Ultraviolet irradiation or by interleukin-10 inhibit dendritic cell induction of immunity. J Invest Dermatol 2005; 124: 570-578.

99. Bentham G, Wolfreys AM, Liu Y, Cortopassi G, Green MH, Arlett CF, Cole J. Frequencies of Hprt(-) Mutations and bcl-2 translocations in circulating human lymphocytes are correlated with United Kingdom sunlight records. Mutagenesis. 1999;14(6):527-32.

100. Holly EA, Lele C, Bracci PM. Hair-color products and risk for non-Hodgkin lymphoma: a population-based study in the san francisco bay area. Am J Public Health 1998; 88: 1767-73.

101. Zhang Y, Holford TR, Leaderer B, Boyle P, Zahm SH, Flynn S et al. Hair-coloring product use and risk of non-Hodgkin lymphoma: a population case-control study in Connecticut. Am J Epid 2004; 159(2): 148-154.

102. Tavani A, Negri E, Franceschi S, Talamini R, Serraino D and La Vecchia C. Hair dye use and risk of lymphoid neoplasms and soft tissue sarcomas. Int J Cancer 2005; 113: 629-631.

103. Schroeder JC, Olshan AF, Baric R, Dent GA, Weinberg CR, Yount B, Cerhan JR et al. A case-control study of tobacco use and other non-occupational risk factors for t(14;18) subtypes of non-Hodgkin lymphoma. Cancer Causes Control 2002; 13: 159-168.

104. Cantor KP, Blair A, Everett G, VanLier S, Burmeister L, Dick FR, Gibson RW, Schuman L. Hair dye use and risk of leukemia and lymphoma. Am J Public Health. 1988; 78(5): 570-1.

105. Takkouche B, Etminan M, Montes-Martinez A. Personal Use of Hair Dyes and Risk of Cancer. JAMA 2005; 293 (20): 2516-2525.

106. Morton LM, Hartge P, Holford TR, Holly EA, Chiu BC, Vineis P, Stagnaro E, Willett EV, Franceschi S, La Vecchia C, Hughes AM, Cozen W, Davis S, Severson RK, Bernstein L, Mayne ST, Dee FR, Cerhan JR, Zheng T. Cigarette smoking and risk of non-Hodgkin lymphoma: a pooled analysis from The International Lymphoma Epidemiology Consortium (InterLymph). Cancer Epidemiol Biomarkers Prev. 2005; 14(4):925-33.

107. Stagnaro E, Tumino R, Parodi S, Crosignani P, Fontana A, Masala G, Miligi L, Nanni O, Ramazzotti V, Rodella S, Senoiri Constantini A, Vigano C, Vindigni C, Vineis P. Non-Hodgkin lymphoma and type of tobacco smoke. Cancer Epidemiol Biomarkers Prev. 2004; 13(3): 431-7.

108. Morton LM, Holford TR, Leaderer B, Boyle P, Zahm SH, Zhang Y, Flynn S, Tallini G, Zhang B, Owens PH, Zheng T. Cigarette smoking and risk of non-hodgkin lymphoma subtypes among women. Br J Cancer. 2003; 89(11): 2087-92.

109. Stagnaro E, Ramazzotti V, Crosignani P, Fontana A, Masala G, Miligi L, Nanni O, Neri M, Rodella S, Costantini AS, Tumino R, Vigano C, Vindigni C, Vineis P. Smoking and hematolymphopoietic malignancies. Cancer Causes Control. 2001; 12(4): 325-34.

110. Parker AS, Cerhan JR, Dick F, Kemp J, Habermann TM, Wallace RB, Sellers TA, Folsom AR. Smoking and risk of non-hodgkin lymphoma subtypes in a cohort of older women. leuk lymphoma. 2000; 37(3-4): 341-9.

111. Herrinton LJ, Friedman GD. Cigarette smoking and risk of non-Hodgkin lymphoma subtypes. Cancer Epidemiol Biomarkers Prev. 1998;7(1):25-8.

112. Bracci PM, Holly EA. Tobacco use and non-Hodgkin lymphoma: results from a population-based case-control study in the San Francisco Bay area, California. Cancer Causes Control. 2005; 16(4): 333-46.

113. Bell DA, Liu Y, Cortopassi GA. Occurrence of bcl-2 oncogene translocation with increased frequency in the peripheral blood of heavy smokers. JNCI 1995; 87(3): 223-4.

114. Rauzy O, Galoin S, Chale JJ, Adoue D, Albarede JL, Delsol G, al Saati T. Detection of t(14;18) carrying cells in bone marrow and peripheral blood from patients affected by non-lymphoid diseases.Mol Pathol. 1998 Dec;51(6):333-8.

115. Chiu BC, Dave BJ, Blair A, Gapstur SM, Chmiel JS, Fought AJ, Zahm SH, Weisenburger DD. Cigarette smoking, familial hematopoietic cancer, hair dye use, and risk of t(14;18)-defined subtypes of non-Hodgkin lymphoma. Am J Epidemiol. 2007 Mar 15;165(6):652-9. Epub 2007 Jan 4.

116. Tollerud DJ, Clark JW, Brown LM, Neuland CN, Mann DL, Pankiw-Trost LK, Blattner WA, Hoover RN. Association of cigarette smoking with decreased numbers of circulating natural killer cells. Am Rev Respir Dis 1989; 139: 194-198.

117. Phillips B, Marshall E, Brown S, Thompson JS. Effect of smoking on human natural killer cell activity. Cancer 1985; 56: 2789-2792.

118. Morton LM, Zheng T, Holford TR, Holly EA, Chiu BC, Costantini AS, Stagnaro E, Willett EV, Dal Maso L, Serraino D, Chang ET, Cozen W, Davis S, Severson RK, Bernstein L, Mayne ST, Dee FR, Cerhan JR, Hartge P; InterLymph Consortium. Alcohol consumption and risk of non-Hodgkin lymphoma: a pooled analysis. Lancet Oncol. 2005;6(7):469-76.

119. Willett EV, Smith AG, Dovey GJ, Morgan GJ, Parker J, Roman E. Tobacco and alcohol consumption and the risk of non-Hodgkin lymphoma. Cancer Causes Control 2004; 15: 771-780.

120. Chang ET, Smedby KE, Zhang SM, Hjalgrim H, Melbye M, Ost A, Wolk A, Adami HO, Glimelius B. Alcohol intake and risk of non-Hodgkin lymphoma in men and women. Cancer Causes Control. 2004; 15(10): 1067-76.

121. Chiu BH, Cerhan JR, Gapstur SM, Sellers TA, Zheng W, Lutz CT, Wallace RB, Potter JD. Alcohol consumption and non-Hodgkin lymphoma in a cohort of older women. 1999; 80(9): 1476-1482.

122. Briggs NC, Levine RS, Bobo LD, Haliburton WP; Brann EA, Hennekens CH. Wine drinking and risk of non-Hodgkin lymphoma among men in the United States: a population-based case-control study. Am J Epid 2002; 156: 454-462.

123. Purdue MP, Bassani DG, Klar NS, Sloan M, Kreiger N, the Canadian Cancer Registries Epidemiology Research Group. Dietary factors and risk of non-Hodgkin lymphoma by

histologic subtype: a case-control analysis. Cancer Epidemiol Biomarkers Prev 2004; 13(10): 1665-76.

124. Zheng T, Holford TR, Leaderer B, Zhang Y, Zahm SH, Flynn S, Tallini G et al. Diet and nutrient intakes and risk of non-Hodgkin lymphoma in Connecticut women. Am J Epidemiol 2004; 159(5): 454-466.

125. Ward MH, Zahm SH, Weisenburger DD, Gridley G, Cantor KP, Saal RC, Blair A. dietary factors and non-Hodgkin lymphoma in Nebraska (United States). Cancer Causes Control 1994; 5: 422-432.

126. Talamini R, Polesel J, Montella M, Dal Maso L, Crovatto M, Crispo A et al. Food groups and risk of non-Hodgkin lymphoma: a multicenter, case-control study in Italy. Int J Cancer 2005 (in press).

127. Chang ET, Hjalgrim H, Smedby KE, Åkerman M, Tan E et al. Body mass index and risk of malignant lymphoma in Scandinavian men and women. JNCI 2005; 97: 210-218.

128. Pan SY, Mao Y, Ugnat A, the Canadian cancer registries epidemiology research group. Am J Epidemiol 2005; 162: 1162-1173.

129. Willett EV, Skibola CF, Adamson P, Skibola DR, Morgan GJ, Smith MT, Roman E. Non-Hodgkin lymphoma, obesity and energy homeostasis polymorphisms. Br J Cancer. 2005 Oct 3;93(7):811-6.

130. Fernberg P, Odenbro Å, Bellocco R, Boffeta P, Pawitan Y, Adami J. Tobacco use, body mass index and the risk of malignant lymphomas—a nationwide cohort study in sweden. Int J Cancer 2006; 118: 2298-2302.

131. Surveillance, Epidemiology, and End Results (SEER) Program (www.seer.cancer.gov) SEER*Stat Database: Incidence - SEER 9 Regs Public-Use, Nov 2004 Sub (1973-2002), National Cancer Institute, DCCPS, Surveillance Research Program, Cancer Statistics Branch, released April 2005, based on the November 2004 submission.

132. Besson H, Brennan P, Becker N, Nieters A, De Sanjose S, Font R, Maynadie M, Foretova L, Cocco PL, Staines A, Vornanen M, Boffetta P. Tobacco smoking, alcohol drinking and non-Hodgkin lymphoma: a European multicenter case-control study (Epilymph). Int J Cancer. 2006 Aug 15;119(4):901-8.

133. Besson H, Renaudier P, Merrill RM, Coiffier B, Sebban C, Fabry J, Trepo C, Sasco AJ. Smoking and non-Hodgkin lymphoma: a case-control study in the Rhone-Alpes region of France. Cancer Causes Control. 2003 May;14(4):381-9.

134. Freedman DS, Tolbert PE, Coates R, Brann EA, Kjeldsberg CR. Relation of cigarette smoking to non-Hodgkin lymphoma among middle-aged men.. Am J Epidemiol. 1998 Nov 1;148(9):833-41.

135. Zahm SH, Weisenburger DD, Holmes FF, Cantor KP, Blair A. Tobacco and non-Hodgkin lymphoma: combined analysis of three case-control studies (United States). Cancer Causes Control. 1997 Mar;8(2):159-66.

136. Nelson RA, Levine AM, Marks G, Bernstein L. Alcohol, tobacco and recreational drug use and the risk of non-Hodgkin lymphoma. Br J Cancer. 1997;76(11):1532-7.

137. Brown LM, Everett GD, Gibson R, Burmeister LF, Schuman LM, Blair A. Smoking and risk of non-Hodgkin lymphoma and multiple myeloma. Cancer Causes Control. 1992 Jan;3(1):49-55.

138. Linet MS, McLaughlin JK, Hsing AW, Wacholder S, Co Chien HT, Schuman LM, Bjelke E, Blot WJ. Is cigarette smoking a risk factor for non-Hodgkin lymphoma or multiple myeloma? Results from the Lutheran Brotherhood Cohort Study. Leuk Res. 1992 Jun-Jul;16(6-7):621-4.

139. Bastard C. LAZ3 rearrangements in non-Hodgkin lymphoma: correlation with histology, immunophenotype, karyotype, and clinical outcome in 217 patients. Blood 1994; 83(9): 2423-2427.

CHAPTER 3

STATEMENT OF SPECIFIC AIMS

A. Specific Aims

The current study extends a previous study of risk factors for t(14;18)-non-Hodgkin lymphoma (NHL) case-subtypes based on polymerase chain reaction (PCR) assays (J. Schroeder, PI) by running fluorescence in situ hybridization (FISH) assays to detect common translocations in over 200 NHL cases originally enrolled in the National Cancer Institute's (NCI) Factors Affecting Rural Men (FARM) population-based case-control study (A. Blair, PI) (1,2).

A.1. Utility of FISH in a Case-Control Study

A.1.1. Primary Aim

To use and assess commercially available FISH assays to identify any *IGH*, t(14;18), t(8;14), and any *BCL6* translocations, in archival paraffin tumor sections from FARM study cases and to compare the detection of t(14;18) by FISH and PCR previously run on the same samples (1).

A.1.2. Secondary Aim

To use bcl-2 immunostaining to identify bcl-2 expression in archival paraffin tumor sections from FARM study cases and describe the distribution of bcl-2 status across translocations and histological classification subtypes.

A.2. Risk Factors for NHL Defined by t(14;18) and bcl-2 Expression

A.2.1. Primary Aim

To determine whether data support NHL molecular subtype-specific associations with putative risk factors including tobacco products (by type, intensity, and duration of use), family history of hemolymphatic (HLP) cancer in first and second-degree relatives, any hair dye use, and alcohol consumption when NHL subtypes are defined based on t(14;18) and bcl-2 expression.

A.2.2. Secondary Aim

To re-evaluate associations previously assessed using a PCR method believed to be less accurate than FISH.

B. Hypothesis

This study is designed to evaluate the following hypotheses:

1. FISH assays will be a practical and effective means of classifying archival samples into molecular subtypes for epidemiologic research.

2. The incidence of NHL molecular subtypes defined by t(14; 18) translocations and bcl-2 expression will vary by tobacco use and other risk factors.

C. Rationale

NHL case-subtypes defined by somatic mutations have been suggested to minimize the etiologic heterogeneity of NHL outcomes for epidemiologic research (3,4). The previous

analysis of t(14;18)-NHL based on PCR found subtype-specific associations between family history and t(14;18)-negative NHL, but did not find a clear association between smoking and t(14;18)-NHL case-subtypes (1). Misclassification of cases may have occurred. Although PCR is believed to be a reasonably specific method for detecting t(14;18) in archival tumor samples, assays will fail to detect translocations with *BCL2* breakpoints that occur outside of regions bounded by the PCR primers used (approximately 15-25% of translocations depending on the specific assay) (5).

In contrast, fluorescence in situ hybridization (FISH) assays can detect translocations with more widely dispersed breakpoints than PCR. The sensitivity of fluorescence in situ hybridization (FISH) has been shown to be higher than that of polymerase chain reaction (PCR) for detecting t(14;18)-positive non-Hodgkin lymphomas (NHL) (6-12). Therefore, we re-evaluated associations estimated for tobacco use and other factors among study participants using FISH assays to determine the t(14;18) status of archival tumor samples previously classified based on PCR.

In addition, we estimate associations with lymphoma subtypes defined by bcl-2 expression (based on immunohistochemistry), since expression may be increased through mechanisms other than t(14;18) (13). To our knowledge, this study is the first to incorporate both t(14;18) translocations and bcl-2 expression in defining NHL case-subtypes in a population-based case-control study.

D. References

1. Schroeder JC, Olshan AF, Baric R, Dent GA, Weinberg CR, Yount B, Cerhan JR, Lynch CF, Schuman LM, Tolbert PE, Rothman N, Cantor KP, Blair A. A case-control study of tobacco use and other non-occupational risk factors for t(14;18) subtypes of non-Hodgkin lymphoma (United States). Cancer Causes Control. 2002 Mar;13(2):159-68.

2. Blair A, Linos A, Stewart PA, Burmeister LF, Gibson R, Everett G, Schuman L, Cantor KP. Evaluation of risks for non-Hodgkin lymphoma by occupation and industry exposures from a case-control study. Am J Industrial Med 1993; 23: 301-312.

3. Jaffe ES, Raffeld M, Medeiros LJ, Stetler-Stevenson M. An Overview of the Classification of non-Hodgkin lymphomas: an integration of morphological and phenotypical concepts. Cancer Res 1992; 52: 5447s-5452s.

4. Potter M. Pathogenetic Mechanisms in B-cell non-Hodgkin lymphomas in Humans. Cancer Res 1992; 52: 5522s-5528s.

5. Vega F, Medeiros LJ. Chromosomal translocations involved in non-Hodgkin lymphomas. Arch Pathol Lab Med. 2003 Sep;127(9):1148-60.

6. Poetsch M, Weber-Matthiesen K, Plendl HJ, Grote W, Schlegelberger B. Detection of the t(14;18) chromosomal translocation by interphase cytogenetics with yeast-artificial-chromosome probes in follicular lymphoma and nonneoplastic lymphoproliferation. J Clin Oncol. 1996 Mar;14(3):963-9.

7. Barrans SL, Evans PA, O'Connor SJ, Owen RG, Morgan GJ, Jack AS.The detection of t(14;18) in archival lymph nodes: development of a fluorescence in situ hybridization (FISH)-based method and evaluation by comparison with polymerase chain reaction. J Mol Diagn. 2003 Aug;5(3):168-75.

8. Godon A, Moreau A, Talmant P, Baranger-Papot L, Genevieve F, Milpied N, Zandecki M, Avet-Loiseau H.. Is t(14;18)(q32;q21) a constant finding in follicular lymphoma? An interphase FISH study on 63 patients. Leukemia 2003; 17: 255-259.

9. Einerson RR, Kurtin PJ, Dayharsh GA, Kimlinger TK, Remstein ED. FISH is superior to PCR in detecting t(14;18)(q32;q21)-IgH/bcl-2 in follicular lymphoma using paraffin-embedded tissue samples. Am J Clin Pathol. 2005 Sep;124(3):421-9.

10. Belaud-Rotureau MA, Parrens M, Carrere N, Turmo M, Ferrer J, de Mascarel A, Dubus P, Merlio JP. Interphase fluorescence in situ hybridization is more sensitive than BIOMED-2 polymerase chain reaction protocol in detecting IGH-BCL2 rearrangement in both fixed and frozen lymph node with follicular lymphoma. Hum Pathol. 2007 Feb;38(2):365-72. Epub 2006 Nov 28.

11. Streubel B, Scheucher B, Valencak J, Huber D, Petzelbauer P, Trautinger F, Weihsengruber F, Mannhalter C, Cerroni L, Chott A. Molecular cytogenetic evidence of t(14;18)(IGH;BCL2) in a substantial proportion of primary cutaneous follicle center lymphomas. Am J Surg Pathol. 2006 Apr;30(4):529-36.

12. Medeiros LJ, Carr J. Overview of the role of molecular methods in the diagnosis of malignant lymphomas. Arch Pathol Lab Med. 1999 Dec;123(12):1189-207.

13. Papakonstantinou G, Verbeke C, Hastka J, Bohrer M, Hehlmann R. bcl-2 expression in non-Hodgkin lymphomas is not associated with bcl-2 gene rearrangements. Br J Haematol. 2001 May;113(2):383-90.

CHAPTER 4

METHODS

A. Overview of Methods

The first goal is to determine whether the detection of translocations by FISH compared with PCR, for t(14;18), is advantageous for epidemiologic studies. The second goal is to evaluate whether associations between risk factors and NHL differ according to the t(14;18) and bcl-2 status of the tumor.

B. Design

B.1. Subject Identification

B.1.1. FARM Study Population

The cases and controls for the current study were originally enrolled in the FARM study (A. Blair, PI), a large population-based study of incident, pathologically confirmed hemolymphatic cancer cases in Iowa and Minnesota men, was conducted by the National Cancer Institute to evaluate associations between agricultural exposures and hemolymphatic cancers including NHL. Population-based cases diagnosed in 1980-1983 were white rural men aged 30 or older identified through Iowa and Minnesota state registries. Controls were 1,245 white males aged 30 or older without a history of hemolymphatic cancer that were frequency-matched to cases by age (within five-year groups), state, and vital status. Detailed information on agricultural exposures, other occupational exposures, medical history, smoking, and other potential risk factors for NHL were obtained through in-person structured

interviews of cases, controls, or next-of-kin (1981-1984). The FARM study population is described in greater detail in published results and Chapter 5 (1-6).

B.1.2. t(14;18)-PCR Study

In hopes of increasing etiologic specificity, an alternative method of NHL classification based on t(14;18) was proposed for FARM study cases (J Schroeder, P.I.). In 1997, tumor blocks for 248 (40%) of the original 622 FARM study cases were retrieved and sectioned. Translocation status of each case was determined by performing polymerase chain reaction (PCR) assays on DNA extracted from each archival paraffin-embedded tumor block. Primers used for each reaction included a J_h consensus primer on chromosome 14, the MBR1 primer corresponding to the BCL2 major breakpoint region (MBR) on chromosome 18, and the MBR2 primer 360 base pairs upstream of MBR1. A 175-base pair segment of the human *hemoglobin beta* (*HBB*) gene was amplified as an internal positive control to confirm that the DNA extracted from each sample was adequate for PCR amplification of t(14;18). Amplified DNA was isolated, denatured, and separated by gel electrophoresis. The t(14;18)-PCR study is described in greater detail in Chapter 5 and published results (1-6).

B.1.3. Case-Subtype Ascertainment Based on FISH and IHC

For the current study, commercially available FISH *IGH*, t(14;18), *BCL6*, and t(8;14) assays were run on 5-micron archival paraffin-embedded tumor sections from FARM study cases. bcl-2 immunostaining was performed on paraffin sections from 229 FARM study cases using a mouse monoclonal antibody (clone 124; Dako) corresponding to bcl-2 amino acids 41-54 (16). FISH and bcl-2 immunostaining methods and results including a FISH-PCR comparison are described in greater detail in Chapter 5.

B.2. Methods

B.2.1. Classification of Exposure

Tobacco use and use of specific tobacco products (cigarettes, cigars, pipe tobacco, chewing tobacco, snuff) were classified according to ever/never use (daily use for at least 3 months). In addition, cigarette smoking was categorized according to average intensity of use (0, 1-10, 11-20, >20 cigarettes per day), age of first use (18 or less, over age 18), and pack-years (0, >0-20, >20-40, >40). We also estimated associations between NHL case-subtypes and family history of hemolymphatic cancer diagnoses in any first-degree blood relatives including parents, children, and siblings or in any second-degree relatives including aunts/uncles, grandparents/children, and nieces/nephews, any hair dye use (ever use of hair dye at least once a month for at least one year or occupational exposure on any job held for 1 or more years), and weekly alcohol use (ever drank beer, wine or hard liquor weekly for at least one year). Occupation as farmer (ever worked on a farm for 6 continuous months or longer since the age 18), education (<=12 years, >12 years), marital status (married/widowed, divorced/separated, never married), state (living in Minnesota versus Iowa (reference category)), proxy status (whether next-of-kin was interviewed in place of a deceased participant), and age (restricted quadratic splines) were also evaluated as covariates.

B.2.2. Classification of Outcomes

The main outcomes were t(14;18)-positive and –negative NHL case-subtypes based on FISH and bcl-2-positive and –negative NHL case-subtypes based on IHC. The joint distribution of t(14;18) and bcl-2 (t(14;18)-positive and bcl-2-positive cases, t(14;18)-positive and bcl-2-negative cases, t(14;18)-negative and bcl-2-positive cases, and t(14;18)-

negative and bcl-2-negative cases. For risk factor analysis, NHL cases were not subtyped based on other *IGH*, *BCL6*, and t(8;14) translocations due to their small numbers, but their distribution is decribed in Chapter 5.

B.3. Data Analysis

B.3.1. Univarite Analysis

The distributions of exposures and covariates among the outcomes were assessed through univariate analysis. Selected characteristics such as education, marital status, age, family history and smoking were compared among t(14;18) case-subtypes classified based on FISH versus PCR. t(14;18)-positive cases identified using FISH were more likely than PCR-t(14;18)-positive cases to be between 55 and 65 years of age (21 of 81 cases (25.9%) and 12 of 68 cases (17.7%), respectively); and were less likely to report ever use of alcohol as least once a week (for at least one year) (36 of 81 cases (44.4%) and 38 of 68 cases (55.9%), respectively) (Table 4.1). Otherwise, characteristics were comparable between case-subtypes defined by the two assays.

B.3.2. Case-Case Analyses

Case-only (case-case) analyses were performed using unconditional logistic regression models adjusted for matching factors (see below) to determine whether estimates were comparable between molecular subtypes, including t(14;18)-positive vs. –negative case-subtype comparisons and bcl-2 positive vs. -negative case-subtype comparisons. Case-only comparisons among joint t(14;18) and bcl-2 case-subtypes were also estimated using

polytomous logistic regression with t(14;18)-negative and bcl-2-positive cases as a common referent group.

B.3.3. Multivariate Modeling

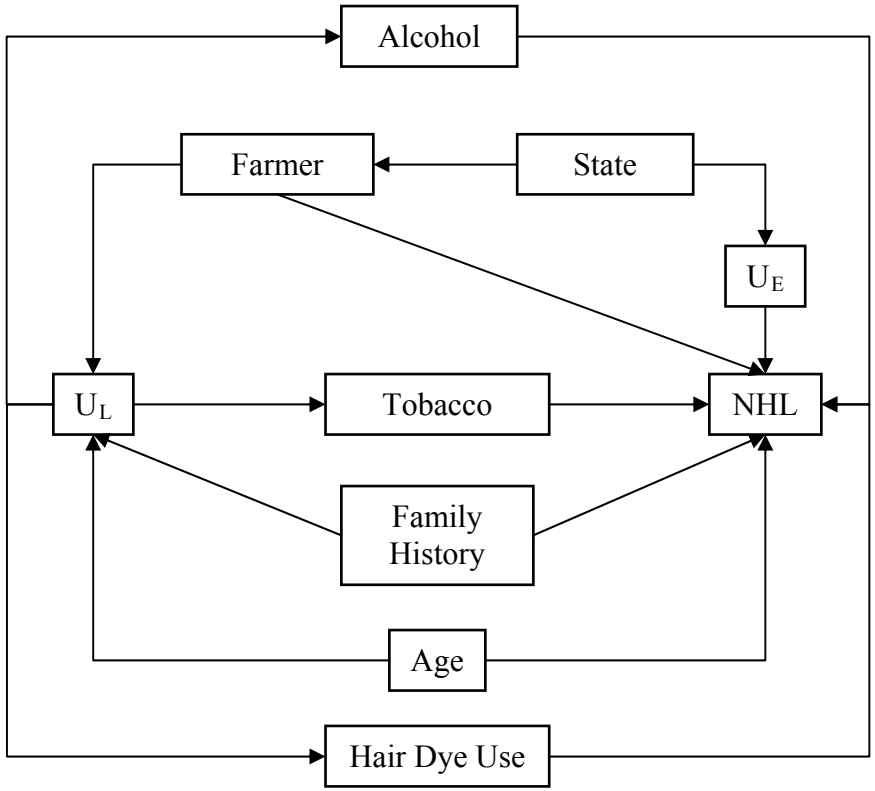
Polytomous logistic regression was used to estimate case-subtype: control associations. All models included the matching factors, state (living in Minnesota versus Iowa (reference category)), proxy status (whether next-of-kin was interviewed in place of a deceased participant), and age (restricted quadratic splines). For each main exposure evaluated, the covariates selected were factors associated with NHL in past studies (e.g. alcohol use (21), family history of hemolymphatic (HLP) cancer (22) and farming (23) as well as demographic factors (age (24) and education (24,25)). For the full logistic model of each main exposure evaluated, potential confounders were selected based on being a common cause of both the exposure and disease using conceptual models (26) (Figures 4.1-4.4). Using a backwards elimination approach, no confounding was evident based on a 0.15 or greater change in the beta coefficient of the main exposure variable when the potential confounder was removed from the model; therefore, final models included the matching factors only. Details of how missing case-subtypes were handled are further discussed in Chapter 6.

Table 4.1. Characteristics of controls and t(14;18)-non-Hodgkin lymphoma (NHL) case-subtypes based on FISH versus PCR, Iowa and Minnesota, 1981-1983

	Controls		FISH				PCR			
			t(14;18)-positive (n=81)		t(14;18)-negative (n=73)		t(14;18)-positive (n=68)		t(14;18)-negative (n=114)	
Exposure	N	%	N	%	N	%	N	%	N	%
Farmer										
Yes	698	56.1	52	64.2	40	54.8	44	64.7	65	57.0
No	547	43.9	29	35.8	33	45.2	24	35.3	49	43.0
State										
Iowa	603	48.4	51	63.0	39	53.4	41	60.3	64	56.1
Minnesota	642	51.6	30	37.0	34	46.6	27	39.7	50	43.9
Age										
30-55	252	20.3	20	24.7	16	22.2	18	26.5	27	23.9
>55-65	283	22.8	21	25.9	18	25.0	12	17.7	26	23.0
>65-75	352	28.3	23	28.4	22	30.6	21	30.9	33	29.2
>75	357	28.7	17	21.0	16	22.2	17	25.0	27	23.9
Respondent status										
Self	826	66.4	63	77.8	51	69.9	53	77.9	85	74.6
Proxy	419	33.7	18	22.2	22	30.1	15	22.1	29	25.4
Any hair dye										
Yes	58	4.7	8	9.9	12	16.4	9	13.2	17	14.9
No	1187	95.3	73	90.1	61	83.6	59	86.8	97	85.1
Education										
= 12 years	878	70.8	60	74.1	49	67.1	53	77.9	82	71.9
>12 years	363	29.3	21	25.9	24	32.9	15	22.1	32	28.1
Drank alcohol weekly										
Yes	686	55.5	36	44.4	41	56.9	38	55.9	60	53.1
No	551	44.5	45	55.6	31	43.1	30	44.1	53	46.9
Family History of HLP										
Yes	51	4.2	3	3.9	6	8.2	4	6.0	11	9.7
No	1169	95.8	75	96.2	67	91.8	63	94.0	102	90.3
Marital status										
Married or widowed	1128	90.6	74	91.4	63	86.3	55	80.9	105	92.1
Divorced or separated	47	3.8	3	3.7	7	9.6	6	8.8	5	4.4
Never married	70	5.6	4	4.9	3	4.1	7	10.3	4	3.5

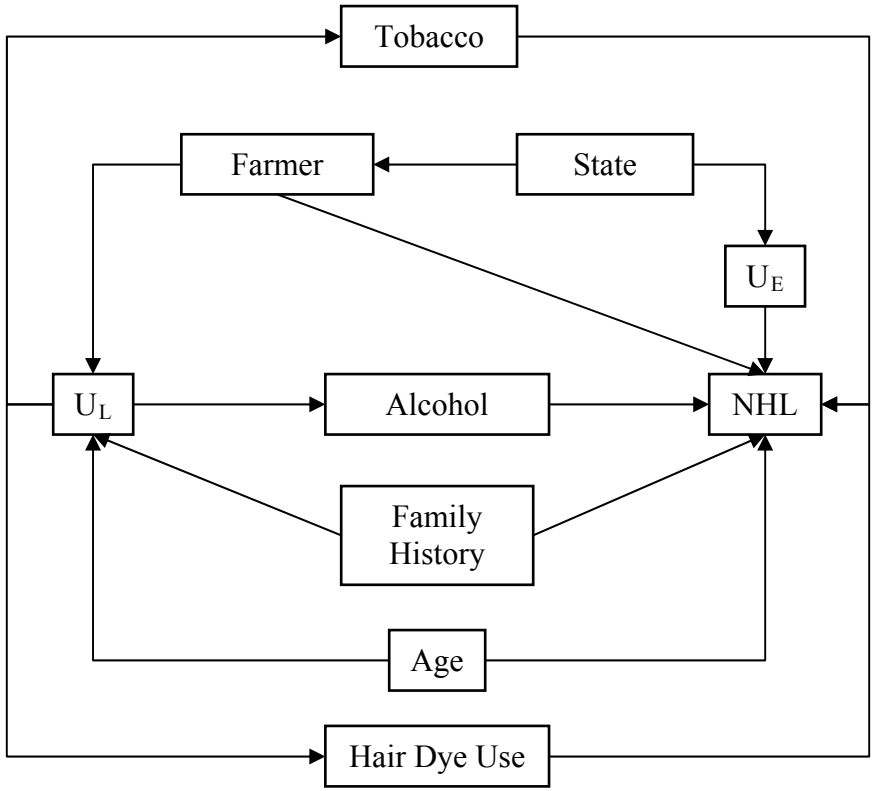
Percents are the proportion of cases or controls in each exposure category out of the total number of case-subtypes or controls

Figure 4.1. Conceptual model for the relation of tobacco use and covariates with NHL case-subtypes



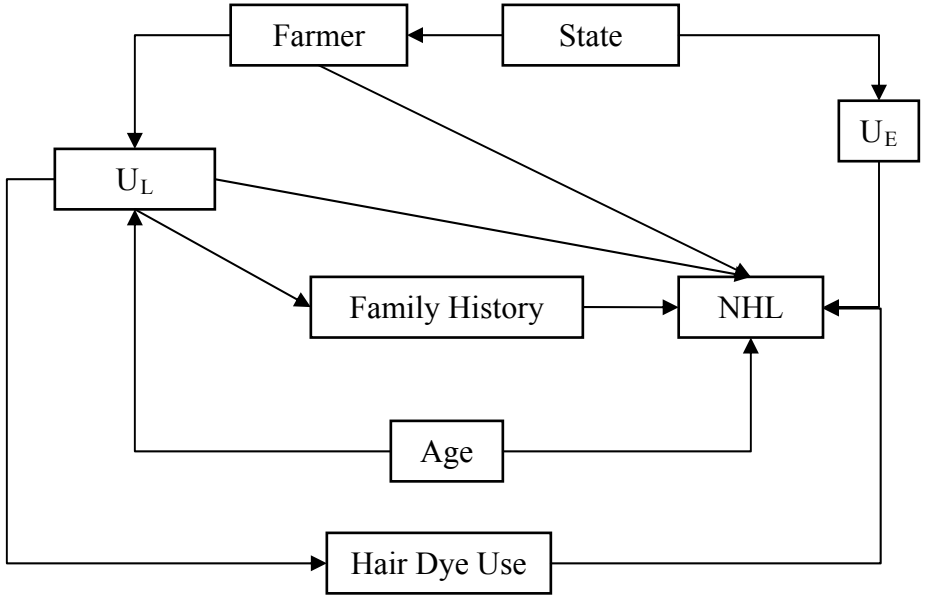
U_L= Unmeasured lifestyle/religion
U_E= Unmeasured environmental factors

Figure 4.2. Conceptual model for the relation of alcohol consumption and covariates with NHL case-subtypes



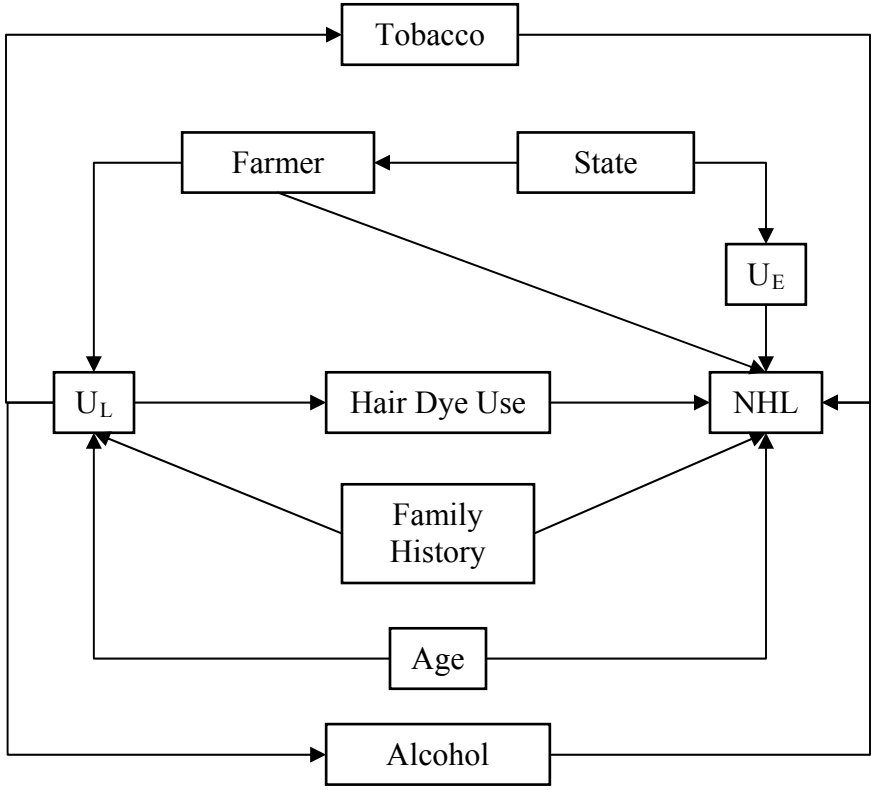
U_L= Unmeasured lifestyle/religion
U_E= Unmeasured environmental factors

Figure 4.3. Conceptual model for the relation of family history of hemolymphatic cancer in 1st or 2nd degree relatives and covariates with NHL case-subtypes



U_L= Unmeasured shared lifestyle/environment/genetic factors
U_E= Unmeasured environmental factors

Figure 4.4. Conceptual model for the relation of hair dye use and covariates with NHL case-subtypes



U_L= Unmeasured lifestyle/religion
U_E= Unmeasured environmental factors

C. References

1. Blair A, Linos A, Stewart PA, Burmeister LF, Gibson R, Everett G, Schuman L, Cantor KP. Evaluation of risks for non-Hodgkin lymphoma by occupation and industry exposures from a case-control study. Am J Industrial Med 1993; 23: 301-312.

2. Brown LM, Blair A, Gibson R, Everett GD, Cantor KP, Schuman LM, Burmeister LF, Van Lier SF, Dick F. Pesticide exposures and other agricultural risk factors for leukemia among men in Iowa and Minnesota. Cancer Res. 1990 Oct 15;50(20):6585-91.

3. Cantor KP, Blair A, Everett G, VanLier S, Burmeister L, Dick FR, Gibson RW, Schuman L. Hair Dye Use and Risk of Leukemia and Lymphoma. Am J Public Health. 1988; 78(5): 570-1.

4. Pottern LM, Linet M, Blair A, Dick F, Burmeister LF, Gibson R, Schuman LM, Fraumeni JF Jr. Familial cancers associated with subtypes of leukemia and non-Hodgkin lymphoma. Leuk Res. 1991;15(5): 305-14.

5. Linos A, Blair A, Gibson RW, Everett G, Van Lier S, Cantor KP, Schuman L, Burmeister L. Leukemia and non-Hodgkin lymphoma and residential proximity to industrial plants. Arch Environ Health. 1991;46(2):70-4.

6. Cantor KP, Blair A, Everett G, Gibson R, Burmeister LF, Brown LM, Schuman L, Dick FR. Pesticides and other agricultural risk factors for non-Hodgkin lymphoma among men in Iowa and Minnesota. Cancer Res. 1992;52(9):2447-55.

7. Herrinton LJ. Epidemiology of the Revised European-American Lymphoma classification subtypes. Epidemiol Rev. 1998; 20(2): 187-203.

8. Schroeder JC. 1999. Agricultural and non-occupational risk factors for t(14;18) non-Hodgkin lymphoma [dissertation]. Chapel Hill (NC): University of North Carolina. 157 p.

9. Schroeder JC, Olshan AF, Baric R, Dent GA, Weinberg CR, Yount B, Cerhan JR et al. Agricultural risk factors for t(14;18) subtypes of non-Hodgkin lymphoma. Epidemiology 2001; 12: 701-9.

10. Schroeder JC, Olshan AF, Baric R, Dent GA, Weinberg CR, Yount B, Cerhan JR et al. A case-control study of tobacco use and other non-occupational risk factors for t(14;18) subtypes of non-Hodgkin lymphoma. Cancer Causes Control 2002; 13: 159-168.

11. Jaffe ES, Harris NL, Stein H, Vardiman JW (editors). Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues: Lyon. IARCPress, 2001.

12. Vysis, Abbott Molecular Inc. (2007) Des Plaines, IL.

13. Vysis. Cellular Genomic Products for Hematopoietic Disorders: DNA Fluorescence in situ Hybridization Probes for Detection of Genetic Aberrations. Downers Grove: Vysis Inc, 2001.

14. Carl Zeiss (2007), Thornwood, NY.

15. Improvision, Inc. (2007) Openlab version 4.0.3, Lexington, MA, U.S.A.

16. Dako Denmark A/S (2007), Copehagen, Denmark.

17. Microsoft Corp. (1992-2001) Microsoft Access 2002, Redmond, WA, U.S.A

18. SAS Institute Inc (2002-2003) SAS version 9.1, Cary, NC.

19. Schroeder JC, Weinberg CR. Use of missing-data methods to correct bias and improve precision in case-control studies in which cases are subtyped but subtype information is incomplete. Am J Epidemiol. 2001 Nov 15;154(10):954-62.

20. StataCorp LP (2007) Stata Statistical Software, 9.0 ed. College Station, TX.

21. Morton LM, Zheng T, Holford TR, Holly EA, Chiu BC, Costantini AS, Stagnaro E, Willett EV, Dal Maso L, Serraino D, Chang ET, Cozen W, Davis S, Severson RK, Bernstein L, Mayne ST, Dee FR, Cerhan JR, Hartge P; InterLymph Consortium. Alcohol consumption and risk of non-Hodgkin lymphoma: a pooled analysis. Lancet Oncol. 2005;6(7):469-76.

22. Wang SS, Slager SL, Brennan P, Holly EA, De Sanjose S, Bernstein L, Boffetta P, Cerhan JR, Maynadie M, Spinelli JJ, Chiu BC et al. Family history of hematopoietic malignancies and risk of non-Hodgkin lymphoma (NHL): a pooled analysis of 10 211 cases and 11 905 controls from the International Lymphoma Epidemiology Consortium (InterLymph). Blood. 2007 Apr 15;109(8):3479-88. Epub 2006 Dec 21.

23. Keller-Byrne JE, Khuder Sa, Schaub Ea, Mcafee O. A meta-analysis of non-Hodgkin lymphoma among farmers in the central United States. Am J of Indus Medicine 1997; 31: 442-4.

24. Alexander DD, Mink PJ, Adami HO, Chang ET, Cole P, Mandel JS, Trichopoulos D. The non-Hodgkin lymphomas: A review of the epidemiologic literature. Int J Cancer. 2007;120 Suppl 12:1-39.

25. Cantor KP, Fraumeni JF Jr. Distribution of non-Hodgkin lymphoma in the United States between 1950 and 1975. Cancer Res. 1980 Aug;40(8 Pt 1):2645-52.

26. Hernan MA, Hernandez-Diaz S, Werler MM, Mitchell AA. Causal knowledge as a prerequisite for confounding evaluation: an application to birth defects epidemiology. Am J Epidemiol. 2002 Jan 15;155(2):176-84.

CHAPTER 5

NON-HODGKIN LYMPHOMA (NHL) SUBTYPES DEFINED BY COMMON TRANSLOCATIONS: UTILITY OF FLUORESCENCE IN SITU HYBRIDIZATION (FISH) IN A CASE-CONTROL STUDY

Extending a previous analysis on non-Hodgkin lymphoma (NHL) defined by t(14;18) translocations based on polymerase chain reaction (PCR), we used fluorescence in situ hybridization (FISH) to identify any *IGH*, t(14;18), t(8;14), and *BCL6* translocations in paraffin-embedded tumor sections from over 200 unselected NHL cases originally enrolled in the National Cancer Institute's (NCI) Factors Affecting Rural Men (FARM) study (1981-1984). Additionally, we evaluated bcl-2 protein expression based on immunohistochemistry. *BCL6* translocations, t(8;14) and other *IGH* translocations were uncommon in the study population. t(14;18) was identified in 39% of diffuse large cell lymphomas (26 of 66 cases) and 81% of follicular lymphomas (35 of 43 cases). FISH assays detected almost twice as many t(14;18)-positive follicular lymphomas as PCR assays (44%) run on the same samples. The majority of cases expressed bcl-2, including 87% of t(14;18)-positive cases and 58% of t(14;18)-negative cases. Consistent with expectations, FISH appeared to be more sensitive than PCR for detecting t(14;18). The value of the FISH assays to subtype cases according to *BCL6* and t(8;14) was limited in this study due to the small numbers of positive cases. Study findings support the use of FISH assays to detect t(14;18) in archival tumor samples for population-based studies of NHL subtypes.

B. Introduction

Classification of lymphomas according to common somatic mutations has been suggested to reduce the etiologic heterogeneity of lymphoma outcomes for epidemiologic research (1-2). Chromosomal translocations associated with lymphomas often juxtapose the *IGH* promoter on chromosome 14 with an oncogene whose subsequent overexpression contributes to lymphomagenesis (1, 3-4). t(14;18) translocations involving *IGH* and *BCL2* at 18q21 cause overexpression of the anti-apoptotic bcl-2 protein, while t(8;14) translocations disrupt the normal regulation of the *CMYC* transcription factor located at 8q24. Translocations involving *BCL6* at 3q27 and *IGH* or other partner genes result in overexpression of bcl-6 protein, which may contribute to malignant transformation in germinal center-derived B cells.

Two recent population-based case-control studies reported evidence of subtype-specific associations between potential risk factors and non-Hodgkin lymphoma (NHL) subtypes defined by the presence or absence of t(14;18) translocations. One used polymerase chain reaction (PCR) assays to detect t(14;18) in archival tumor samples from a subset of participants in the Factors Affecting Rural Men (FARM) study (5-8), while the other used fluorescence in situ hybridization (FISH) assays to identify t(14;18) in archival samples from a population-based case-control study of men and women in Nebraska (9).

We extended the previous analysis of FARM study samples by using FISH assays to identify tumors with any *IGH* translocations, t(8;14) translocations and *BCL6* translocations. In addition, we used FISH to identify t(14;18) translocations in samples that were previously classified based on PCR and evaluated bcl-2 protein expression based on immunohistochemistry. In this report, we describe the frequency of these common

translocations (overall and according to histologic subtype) in archival paraffin-embedded lymphoma samples from the population-based FARM study, compare PCR and FISH assay results for t(14;18), and discuss the utility of FISH-based molecular subtyping for epidemiologic research.

C. Patients, materials, and methods

C.1. Study population

The FARM study (A. Blair, PI), a large population-based study of incident, pathologically confirmed hemolymphatic cancer cases in Iowa and Minnesota men, was conducted by the National Cancer Institute (NCI) to evaluate associations between agricultural exposures and hemolymphatic cancers including NHL. The study population and methods were previously described in detail (5,6). Briefly, cases diagnosed in 1980-1983 among white men aged 30 or older were identified through hospital and pathology laboratory records (in Minnesota) and the Iowa State Health Registry. The study was restricted to white men from areas other than metropolitan Minnesota in order to increase the proportion with agricultural exposures, the primary focus of the study.

Eighty-nine percent (694) of 780 ascertained NHL cases were enrolled, and the diagnosis of NHL was confirmed for 622 cases by a pathology review panel (5,6). NHL cases were limited to solid tumors; therefore lymphocytic leukemias were not included in the NHL case subgroup. Controls were 1,245 white males aged 30 or older without a history of hemolymphatic cancer that were frequency-matched to cases by age (within five-year groups), state, and vital status. Detailed information on agricultural exposures, other occupational exposures, medical history, smoking, and other potential risk factors for NHL

was obtained through in-person structured interviews of cases, controls, or the next-of-kin of deceased cases and controls (6, 10-13).

C.2. t(14;18)-PCR study

In 1997, archival paraffin-embedded tumor blocks were requested and successfully retrieved for 248 FARM study NHL cases (40% of all NHL cases in the original study) (7,8). Over half of the tumor blocks that were not retrieved had been destroyed or lost; the remainder were held at institutions that declined to participate in the t(14;18)-NHL study. Up to 16 5-micron sections and 16 10-micron sections were cut from each archival block. A single pathologist reviewed the first, middle, and last sections to confirm the presence of tumor tissue and assign histological subtypes according to the Revised European American Lymphoma classification scheme (REAL) (14). Five-micron sections and slides were processed with diethyl procarbonate (DEPC) to prevent DNAse or RNAse from interfering with future assays (15).

t(14;18)-PCR assays were performed on DNA extracted from 10-micron sections cut from archival tumor blocks (7,8). Each reaction used a consensus primer corresponding to the J segment of the *IGH* gene on chromosome 14 (5'-ACCTGAGGAGACGGTGAGC-3') and a second primer corresponding to the *BCL2* major breakpoint region (MBR) on chromosome 18 (MBR1: 5'-GAGAGTTGCTTTACGTG-GCCTG-3'). Negative samples were subjected to a second PCR reaction with the *IGH* consensus primer and a second *BCL2* primer 360 base pairs upstream of the MBR primer (MBR2: 5'-CGCTTGACTCCTTTACGTGCTG-3'). Amplified DNA was isolated, denatured, and separated by gel electrophoresis, and transferred to a nylon membrane using a Southern blot

procedure. Amplification was confirmed using radiolabeled probes that hybridized to DNA adjacent to each *BCL2* primer site. A 175-base pair segment of the human *hemoglobin beta* (*HBB*) gene was amplified as an internal positive control to confirm that the DNA extracted from each sample was adequate for PCR amplification of t(14;18). Sixty-six samples that were t(14;18)-negative and *HBB*-negative were classified as inadequate for PCR. Amplification products from 20 of the 68 t(14;18)-positive samples were sequenced to confirm that unique translocation breakpoints were amplified from each sample.

C.3. t(14;18)-FISH study

For the current study, commercially available FISH *IGH*, t(14;18), *BCL6*, and t(8;14) assays were run on 5-micron sections archived from FARM study tumor blocks. All sections were stored at 4°C prior to use. Histologic subtypes were updated according to a modified version of the WHO classification scheme based on morphology only (16). Cases that would be classified as chronic lymphocytic leukemias/small lymphocytic lymphomas (CLL/SLL) according to the WHO scheme are referred to as small lymphocytic lymphomas (SLL) since study cases were limited to solid tumors only. The majority of cases classified as diffuse large cell lymphomas (DLCL) were probably diffuse large B cell lymphomas (DLBCL); however, B-cell status was not confirmed. Cases classified as Burkitt lymphomas (BL) include both Burkitt and Burkitt-like lymphomas.

Samples were assayed using commercially available FISH assay kits (Vysis, 17), run according to the manufacturer's instructions with minor modifications (as described below). Translocations involving *IGH* on chromosome 14 were identified using the LSI® *IGH* Dual Color, Break Apart Rearrangement Probe which includes a 900 kilobase pair (kb)

SpectrumGreen labeled probe that hybridizes to the entire *IGH* variable (V) region and a 250 kb SpectrumOrange labeled probe that hybridizes to the 3' flanking region (Figure 5.1A). In a normal nucleus, the expected signal pattern is two orange/green (yellow) fusion signals (Figure 5.2A). In translocation-positive cells, separation of one pair of probes produces one orange and one green signal in addition to the fusion signal associated with the normal copy of *IGH* (Figure 5.2B).

The LSI® *BCL6* Dual Color, Break Apart Rearrangement assay was used to detect *BCL6* translocations. The assay includes a 300 kb SpectrumOrange probe and a 600 kb SpectrumGreen probe that hybridize to the flanking regions of the *BCL6* gene, (Figure 5.1B) so that probe separation in translocation-positive cells produces one orange signal, one green signal and one fusion signal (18). t(14;18) translocations were identified using the LSI® *IGH*/*BCL2* Dual Color, Dual Fusion Translocation Probe which includes a 1.5 mega base pair (Mb) SpectrumGreen probe that spans the entire *IGH* locus plus 300 kb centromerically, and a 750 kb SpectrumOrange probe that covers *BCL2* plus 250 kb beyond each end of the gene (Figure 5.1C). The expected signal pattern in a normal nucleus is two orange and two green signals (Figure 5.2C). In translocation-positive cells, the juxtaposition of an orange and green probe produces one fusion signal in addition to the separate orange and green signals associated with the normal copies of *IGH* and *BCL2* (Figure 5.2D). The LSI® *IGH/MYC*, CEP 8 Tri-color, Dual Fusion Translocation assay for t(8;14) includes a 1.5 Mb SpectrumGreen *IGH* probe and an 821 kb SpectrumOrange probe that covers the entire *CMYC* gene and plus several hundred kilobase pairs beyond both ends of the gene (Figure 5.1D). A set of green and orange probes is juxtaposed in translocation-positive cells to

produce one fusion signal in addition to the separate orange and green signals associated with the normal copies of *IGH* and *CMYC* (18).

We assayed all cases with at least two 5-micron sections available (N = 230) for any *IGH* translocation. To increase efficiency, subsequent FISH assays were performed only on samples that were of adequate quality to successfully classify them as positive or negative based on the *IGH* assay (n=167). Deparaffinization and pretreatment of archival 5-micron sections was performed using the Vysis® VP2000™ Processor (17). Next, a probe mixture containing a 7:1:2 ratio of buffer, fluorescent probe, and distilled water was applied to each slide. After a coverslip was placed over the tissue and sealed with rubber cement, the slide was placed in a HYBrite™ machine (17) where denaturation occurred at 75°C for 6 minutes, followed by hybridization at 37°C for 16 to 18 hours.

A rapid wash procedure was performed to remove unbound probe. Rubber cement was removed and slides were soaked for 60 minutes at ambient temperature in a 70 ml of solution of sodium chloride, sodium citrate and non-ionic detergent (2x SSC 0.1 NP40, pH 7-7.5). Next, coverslips were removed and slides were soaked for 2 minutes in 70 ml of 2x SSC 0.1 NP40 heated to 73°C. After slides were air-dried in darkness, 10µl of DAPI II counterstain was applied and a new coverslip was placed over the target area. Newly assayed slides were stored at -20°C until scoring.

To conserve archival samples we followed a sequential FISH procedure that allowed reuse of previously assayed sections (17). Used sections were soaked in ambient temperature 2x SSC 0.1 NP40 for 30 to 60 minutes to remove DAPI counterstain and FISH probes. Next, slides were soaked for one minute in 3 different solutions of ethanol and water (70%, 85%, and 100% ethanol), then air dried. FISH hybridization was performed with the denaturation

step reduced to three minutes. Sections were not routinely checked for residual FISH probe, but several test runs showed that no traces of the old probe remained after the denaturation step. A total of 168 assays were run on previously assayed sections, including 110 that were successfully classified and 58 that failed to produce a definitive result. Twenty-eight of 42 unsuccessful assays that were repeated on a new section (69%) were successfully classified based on the second assay.

C.3.1. FISH Scoring

FISH assays were scored using the 40x and 100x oil objectives on a Zeiss Axioskope 2 (Carl Zeiss, 19) equipped with a triple bandpass filter set (DAPI/Green/Orange V.2, 17). Image capture was done using a color digital Axiocam 412-312 (19), and the software Openlab version 4.0.3 (Improvision, Inc, 20).

Criteria for scoring were set before performing FISH assays. For each sample, a minimum of 25 non-overlapping lymphocytes with complete FISH signals were scored; samples with less than 25 readable cells were not classified. A complete FISH signal was defined as one that included the full set of orange, green or fusion signals expected in a positive or negative cell; therefore we did not score lymphocytes with truncated nuclei and incomplete signals, or overlapping cells with multiple signals. In addition, the strength of both the orange and green FISH signals had to be adequate to distinguish separate signals from fusion signals. The appearance and quality of FISH signals varied depending on the source of the tissue, the adequacy and type of sample fixation, and the overall quality of the tissue section. Samples in which the quality of the sample or the strength of the FISH signals were deemed inadequate (i.e. samples having less than 25 cells with adequate signal) to

reliably identify positive cells were classified as unreadable for the assay. Cases were classified as translocation positive if more than 30% of cells showed abnormal signal patterns.

Cells other than lymphocytes, for example, epithelial or connective tissue cells in biopsies from extranodal sites, were not scored. Clonal evolution may result in both positive and negative neoplastic clones within a single tumor, and the distribution and clustering of neoplastic lymphocytes may vary depending on the tumor site and histologic subtype; therefore, each section was examined in its entirety for positive lymphocytes before being classified as translocation negative. Although the proportion of neoplastic versus non-neoplastic lymphocytes within a neoplastic focus may vary, rare lymphocytes with positive FISH signals are unlikely to be representative of the neoplastic clone; consequently, samples with only 1-2 positive lymphocytes were classified as negative. Non-neoplastic cells, which were present in all samples, served as internal negative controls.

IGH, t(14;18), and *BCL6* assays were scored by two investigators (CC and JS). Samples that were initially classified as positive by one reviewer and negative by the other (51 of 579 assays total) were successfully assigned a consensus classification based on a joint review. Sixty-two of the 77 cases that were initially classified as unreadable by one scorer were subsequently classified as positive or negative by both reviews after a joint review; the 15 remaining assays were classified as unreadable. Agreement between scorers was nearly 100% for *BCL6* assays due to the small number of positive cases; therefore, t(8;14) assays for which even fewer positive cases were expected were scored by one investigator only.

C.3.2. Labor and cost

Assays were performed on up to 24 sections at a time. Each run took approximately two days to complete including pretreatment, 16 hours of hybridization, and a rapid wash procedure. On average, it took each scorer about 3 hours to classify 24 slides, with an additional hour for image capture. The cost of materials, including FISH probes and other reagents, was approximately $40 for each section assayed. Using individual sections for more than one assay allowed us to conserve archival sections but increased costs (and labor) by approximately 35%.

C.4. bcl-2 expression

bcl-2 immunostaining was performed on paraffin sections from 229 FARM study cases using a mouse monoclonal antibody (clone 124; Dako) corresponding to bcl-2 amino acids 41-54 (21). Cases were classified as bcl-2 positive if more than 20% of cells showed cytoplasmic staining. Ten samples that were inadequate for scoring were classified as missing for bcl-2 expression. Scoring was performed by two investigators (WY and JS). Independent scores were concordant for 201 of 219 cases (92%). Eighteen cases with discordant results were reviewed and assigned a consensus classification (7 positive, 11 negative for bcl-2).

C.5. Data analysis

FISH data were entered into an Access database (Microsoft Corp., 22). Univariate analyses were carried out using SAS (SAS Institute, 23). The distribution of cases with each type of translocation (*IGH*, *BCL6*, t(14;18) and t(8;14)) was examined according to WHO

histologic subtype. The joint distribution of bcl-2 expression and t(14;18) across WHO case subtypes was also evaluated. In addition, t(14;18)-FISH assay results were compared with t(14;18)-PCR results.

D. Results

D.1. Distribution of Translocations Detected by FISH

One hundred sixty-six of the 230 tumor samples assayed for any *IGH* translocation were successfully classified as positive (n = 94) or negative (n = 72). No additional FISH assays were run on 64 samples that were classified as inadequate for *IGH* scoring.

t(14;18) assays were successful on 154 of 168 cases, including 81 (53%) that were t(14;18)-positive. The *BCL6* assay was successful on 158 cases, including 5 that were positive (3%). Only 134 of 161 cases assayed for t(8;14) were adequate for scoring, and of these, only one was t(8;14)-positive. Ten cases that were classified as positive for an *IGH* translocation were negative for t(14;18), *BCL6*, and t(8;14); seven *IGH*-positive cases were missing or unreadable for other translocations.

Eighty-one percent of follicular lymphomas (FL) (35 of 43 FL cases assayed) were t(14;18)-positive (Table 5.1), including one that was also *BCL6*-positive. One FL was *IGH*-positive but negative for t(14;18), t(8;14) and *BCL6*. Over a third of diffuse large cell lymphomas (DLCL) were t(14;18)-positive (26 of 66 cases assayed), including one that was also *BCL6*-positive and one that was also t(8;14)-positive. Three DLCL were *BCL6*-positive only, and 4 were positive for an *IGH* translocation but negative for t(14;18), t(8;14) and *BCL6* translocations. Five of 15 SLL were t(14;18)-positive only, and 3 were *IGH*-positive

only. Six of 12 Burkitt lymphomas (BL) were t(14;18)-positive only, and one was *IGH*-positive only. Contrary to expectations, all BL were t(8;14)-negative.

D.2. bcl-2 Expression

Over 75% of cases assayed for bcl-2 expression by IHC (168 of 219 total cases) were bcl-2 positive, including 62 of 66 FL and 29 of 29 SLL (Table 5.2). bcl-2 expression was more likely to be positive in t(14;18)-positive cases (87% bcl-2 positive) than t(14;18)-negative cases (58% positive).

D.3. t(14;18)-FISH versus t(14;18)-PCR

PCR and FISH assays for t(14;18) were concordant for 71% (92/132) of cases successfully evaluated by both assays, including 39 that were t(14;18)-positive and 53 that were t(14;18)-negative (Table 5.3). Among the discordant cases, 32 were PCR-negative/ FISH-positive (18 FL, 4 DLCL, 94% bcl-2 positive), and 8 cases were PCR-positive/ FISH-negative (2 FL, 3 DLCL, 88% bcl-2 positive). Overall, cases were more likely to be classified as t(14;18)-positive based on FISH (81 of 154 cases, 52%) than PCR (68 of 182 cases, 37%). The success rate (proportion of cases with interpretable results) of PCR assays (182 of 248 cases, 73%) was slightly higher than that of FISH assays (67%), but almost half of the samples that were inadequate for FISH (36 of 75) were also inadequate for PCR.

E. Discussion

In general, FISH assay results were consistent with expectations. t(14;18), the most common translocation in our samples, was identified in 81% of FL and 39% of DLCL. Other

studies of cases diagnosed in the United States (US), Canada, and Europe reported similar frequencies of t(14;18) by FISH in FL (83-100%) (Table 5.4). Several Japanese studies reported lower t(14;18) frequencies (56-64%), while other Asian studies reported frequencies similar to US and other European studies. Thus, variation in t(14;18) frequency in FL may reflect differences in case selection and protocol rather than biological differences among populations (24). The frequencies of t(14;18) by FISH in DLCL reported by other US studies (20-32%) were similar to our estimates, while the frequency reported in other countries varied widely (0-94%) (Table 5.5). The variation of t(14;18) frequency in DLCL may reflect the heterogeneity of this histologic subtype in addition to methodologic factors.

Only 5 study cases were *BCL6*-positive, including 1 FL (2% of FL) and 4 DLCL (6% of DLCL). Previous studies have reported a similarly low occurrence of *BCL6* translocations in FL (0-13%) (25,27,28,29,50,51). Our estimates of *BCL6* translocations in DLCL are within the range reported in recent cytogenetic studies (0-10%) of cases diagnosed in the US and Canada (25-27); however, *BCL6* frequency in DLCL has ranged from 0 to 27% among international studies based on cytogenetics, FISH and other techniques (28-32).

The frequency of t(8;14) in our study cases was lower than expected. Specifically, only one diffuse large cell lymphoma was t(8;14)-positive, while all 10 Burkitt lymphoma (BL) cases run with t(8;14)-FISH were t(8;14)-negative. This is in marked contrast with the majority of previous FISH and cytogenetic studies, which have reported t(8;14) in 75 to 100% of BL cases (33-37). The BL study cases may have had *CMYC* translocations with other partner genes including 2p12 or 22q11 (t(8;22) or t(2;8)) which occur in 15% and 5% of BL/BLL cases (38). In addition, we did not distinguish classical BL cases from Burkitt-

like (BLL) lymphomas, which are less likely to be t(8;14)-positive (19-33% positive based on FISH and cytogenetics) (35,39,40).

Ten cases (1 FL, 4 DLBCL, 3 SLL, 1 BL, and 1 unclassified) that were positive for an *IGH* translocation but negative for t(14;18), t(8;14) and *BCL6* translocations presumably had t(11;14) or other *IGH* translocations that were not assayed for this study. We had initially considered using a hierarchical approach to increase efficiency and decrease costs, such that t(14;18) assays would have been run on *IGH*-positive samples only, and t(8;14) and *BCL6* assays would have been run on t(14;18)-negative samples only. However, we would have missed five t(14;18)-positive cases that were negative based on the more general *IGH* assay, which indicated that the sensitivity of the *IGH* assay was inadequate to justify this approach. In addition, we would not have detected one *BCL6*-positive case (which may have involved a partner gene other than *IGH*), and would have failed to identify three cases with multiple translocations (two t(14;18)- and *BCL6*-positive; one t(14;18)- and t(8;14)-positive) had we run assays using a hierarchical approach.

In our analysis, bcl-2 was expressed by the majority of cases, regardless of t(14;18) status. This is also consistent with previous research, which suggests that bcl-2 expression may be increased in t(14;18)-negative tumors by *BCL2* amplification or hypomethylation, EBV or CMV proteins, and other mechanisms (41,42).

Overall, our results suggest that FISH was more sensitive than our previous PCR assay for detecting t(14;18)-positive tumors, consistent with previous studies (Table 5.6). We expected that up to 25% of true t(14;18)-positive cases would have been false-negative based on PCR (41), which cannot detect translocation breakpoints that occur outside of the regions bounded by PCR primers. Conversely, PCR may be less specific than FISH to detect

t(14;18)–positive lymphomas because of its potential to detect rare non-neoplastic t(14;18)-positive lymphocytes that may be present in biopsy samples (44). This may explain discordant results for some or all the 8 study cases that were FISH-negative but PCR-positive for t(14;18). Sample contamination could also have caused false-positive PCR assays, but precautions were taken to prevent contamination, and sequencing confirmed unique t(14;18) breakpoints in a subset of samples (7).

Although we cannot confirm the sensitivity and specificity of FISH versus PCR in the absence of a "gold standard" cytogenetic assay, we believe that the weight of the evidence supports FISH versus PCR as the method of choice for detecting t(14;18) in archival samples for observational epidemiologic research. FISH also has advantages over karyotyping for observational research. First, it does not require fresh tissue and dividing cells, but can be performed on preserved sections that may be readily available for a majority of cases (including archival paraffin embedded sections over 25 years old, most of which were successfully assayed in our study). Second, it can be performed directly on tumor sections, thus preserving architecture and allowing localization to neoplastic cells (3).

Disadvantages of FISH include the inability to score overlapping or truncated cells, which contributed to our inability to successfully classify approximately 33% of cases. The high cost of commercial FISH probes and the labor and training required to perform and score assays is also a concern, though costs and labor may be reduced through the use of tissue microarrays (TMAs) (9), and automated scoring (45). Our ability to reuse sections for multiple FISH assays allowed us to conserve archival samples, but a number of assays were unsuccessful and had to be repeated on new sections; therefore, we recommend this procedure only if preservation of sections is a priority and the added cost and time required to

repeat assays is acceptable. Another potential disadvantage of FISH over other in situ assays is the loss of fluorescent signals over time, particularly during scoring; however, we did not experience significant problems with this in our study, and contrary to expectations found that signal strength was maintained for several months after assays were initially scored.

In summary, the frequency of common translocations identified using FISH assays in a group of unselected lymphoma cases was generally consistent with expectations, with the possible exception of t(8;14). FISH appeared to be more sensitive to detect t(14;18)-positive tumors than PCR, though we were unable to formally evaluate either assay against a cytogenetic "gold standard." Large-scale epidemiologic studies of lymphoma subtypes should consider the advantages of FISH over other assays and incorporate strategies for minimizing time and resources. Our experience suggests that large numbers of cases would be needed to provide sufficient power to assess risk factors for translocation subtypes other than t(14;18) in an unselected population-based sample of cases. However, the use of FISH assays to classify lymphomas according to t(14;18), in combination with the use of IHC assays to assess bcl-2 expression, may provide a simple and powerful way of discovering new risk factors and etiologic mechanisms for lymphomas.

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Figure 5.1a). Gene regions corresponding to FISH probes used to detect chromosomal translocations. *IGH* gene regions corresponding to LSI *IGH* Dual Color Break Apart probes used to detect *IGH* translocations (regardless of the partner gene) (adapted from Vysis, 18).

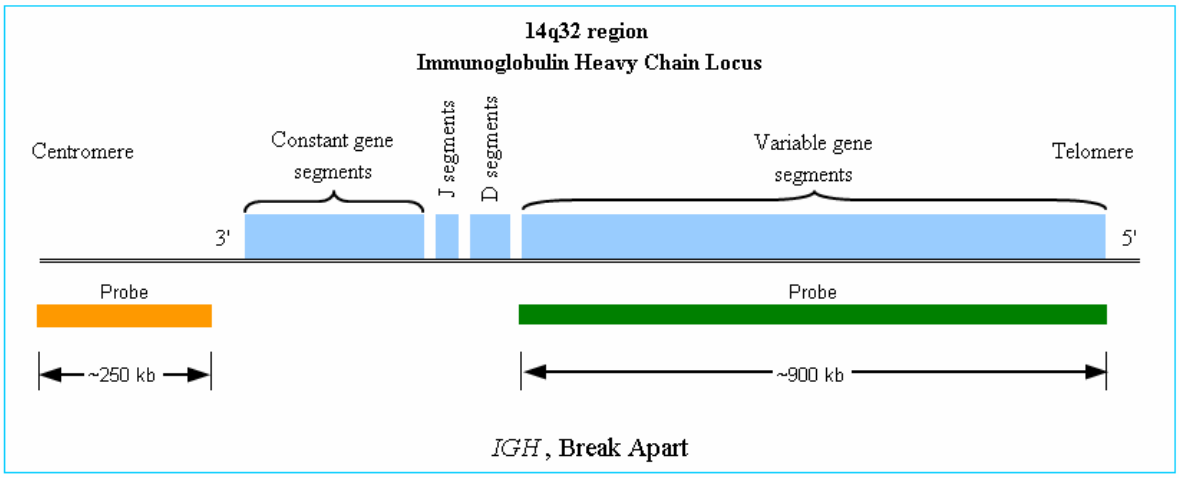

Figure 5.1b). Gene regions corresponding to FISH probes used to detect chromosomal translocations. *BCL6* gene regions corresponding to LSI *BCL6* Dual Color, Break Apart probes used to detect *BCL6* translocations (regardless of the partner gene) (adapted from Vysis, 18).

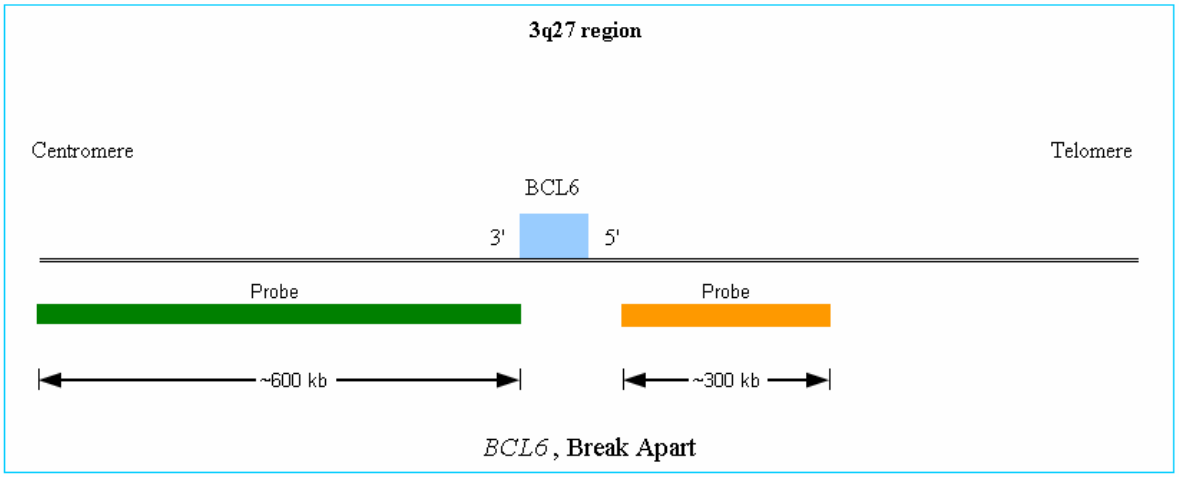

Figure 5.1c). Gene regions corresponding to FISH probes used to detect chromosomal translocations. Regions of *IGH* (upper) and *BCL2* (lower) corresponding to LSI *IGH/BCL2* Dual Color Dual Fusion probes to detect t(14;18). Arrows indicate the location of the J_h consensus primer on chromosome 14, the MBR1 primer corresponding to the *BCL2* major breakpoint region (MBR) on chromosome 18, and the MBR2 primer 360 base pairs upstream of MBR1 (adapted from Vysis, 18).

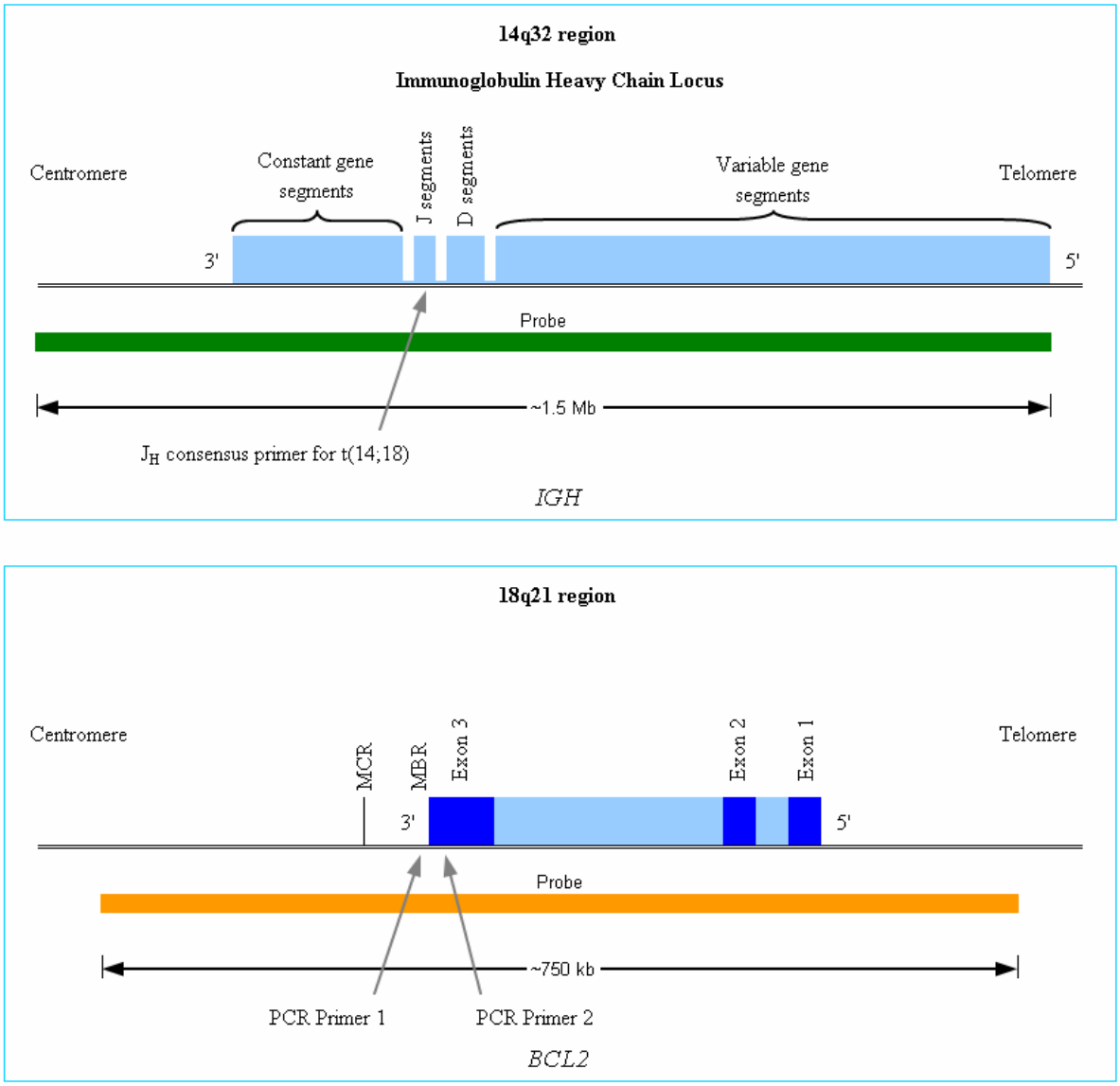

Figure 5.1d). Gene regions corresponding to FISH probes used to detect chromosomal translocations. Regions of *IGH* (upper) and *CMYC* (lower) corresponding to LSI *IGH/MYC*, CEP 8 Tri-color, Dual Fusion probes to detect t(8;14) (adapted from Vysis, 18).

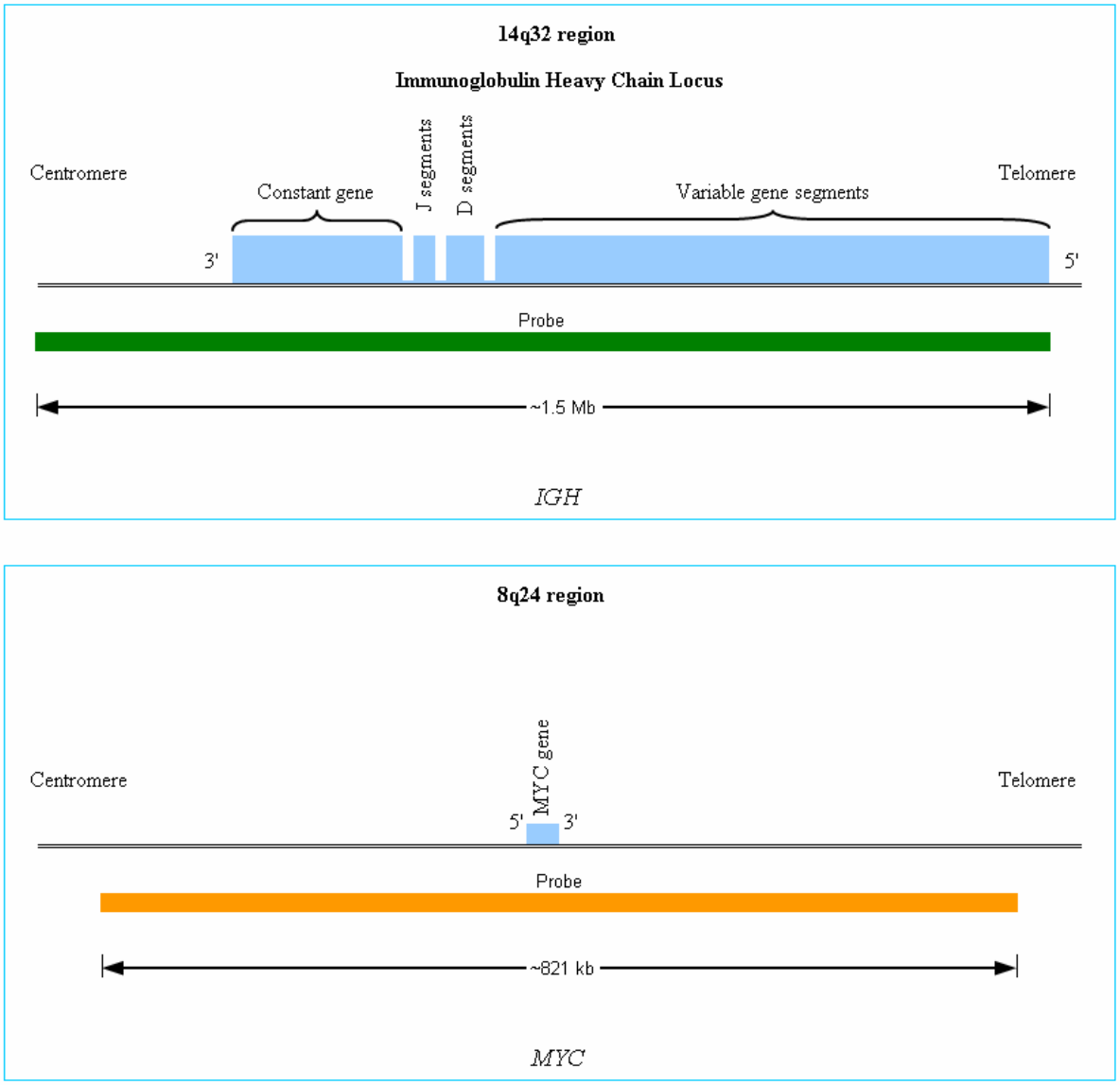

Figure 5.2. Photomicrographs showing signal patterns expected for FISH assays of normal cells and translocation positive cells. a) Vysis LSI *IGH* Dual Color, Break Apart probes for any *IGH* translocation showing the expected signal pattern for a translocation-negative cell (2 fusion signals). b) Vysis LSI *IGH* Dual Color, Break Apart probes for any *IGH* translocation showing the expected signal pattern for an *IGH*-positive cell (1 orange, 1 green, and 1 fusion). c) Vysis LSI *IGH/BCL2* Dual Color, Dual Fusion probes for t(14;18) showing the expected signal pattern for a translocation-negative cell (2 orange, 2 green). d) Vysis LSI *IGH/BCL2* Dual Color, Dual Fusion probes for t(14;18) showing the expected signal pattern for a translocation-positive cell (1 orange, 1 green, and 2 fusion).

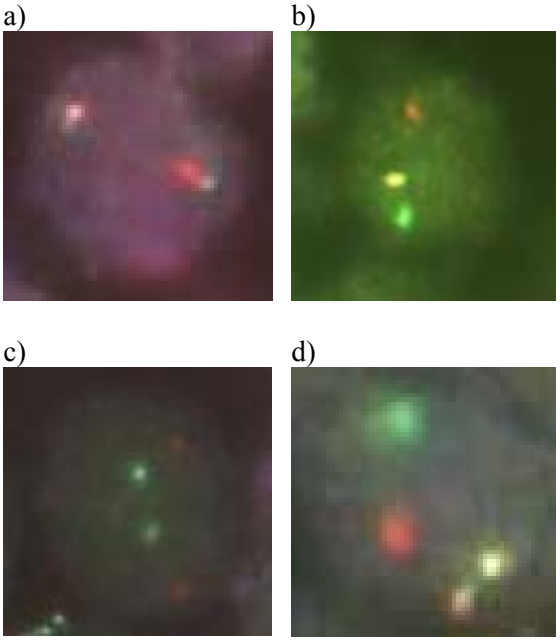

Table 5.1. Frequency of FISH-detected translocations by histologic subtype

Histologic Subtype	Cases Assayed*	Any translocation†		t(14;18)	
	N	N	%	N	%
Follicular	45	38	84	35	81
Diffuse Large Cell	73	37	51	26	39
Small lymphocytic	16	8	50	5	33
Burkitt/Burkitt-like	13	8	62	6	50
Mantel Cell	4	3	75	3	100
Unclassified	10	7	70	6	60

Percents indicate the proportion of cases that were positive for each translocation out of the total number assayed for that translocation. Histologic subtypes were determined using a modified (World Health Organization) WHO classification scheme based on morphology only.
*Numbers of cases within each subtype that were assayed successfully for at least one FISH assay.
†Cases positive for *IGH*, t(14;18), *BCL6*, or t(8;14) translocations based on at least one FISH assay.

Table 5.2. Numbers of positive cases according to histologic subtype, t(14;18) status and bcl-2 expression

Histologic Subtype	t(14;18)-positive, bcl-2-positive	t(14;18)-negative, bcl-2-positive	t(14;18)-positive, bcl-2-negative	t(14;18)-negative, bcl-2-negative	Total
Follicular	32	7	3	1	43
Diffuse Large Cell	18	15	4	23	60
Small lymphocytic	5	10	0	0	15
Burkitt/Burkitt-like	4	2	2	4	12
Lymphoblastic	0	0	0	1	1
Mantel Cell	3	0	0	0	3
Marginal zone	0	1	0	0	1
Unclassified	4	4	1	0	9
Total	66	40	10	29	145

Histologic subtypes were determined using a modified (World Health Organization) WHO classification scheme based on morphology only. t(14;18) status was determined using Vysis LSI *IGH/BCL2* Dual Color, Dual Fusion FISH assays run on archival paraffin-embedded tumor sections. bcl-2 expression was determined using a mouse monoclonal antibody (clone 124; Dako, 21) corresponding to bcl-2 amino acids 41-54 run on paraffin-embedded tumor sections. 17 cases were missing either t(14;18)- or bcl-2-case status.

Table 5.3. Numbers of t(14;18)-positive and t(14 ;18)-negative cases based on FISH versus t(14;18)-PCR

t(14;18)-FISH	t(14;18)-PCR				
	Positive	Negative	Unreadable‡	Not Assayed	Total
Positive	39	32	10	0	81
Negative	8	53	10	2	73
Unreadable†	14	25	37	0	76
Not Assayed	7	4	9	0	20
Total	68	114	66	2	250

t(14;18) status was determined using Vysis LSI *IGH/BCL2* Dual Color, Dual Fusion FISH assays and PCR assays with the J_h consensus primer, MBR1 primer corresponding to the *BCL2* major breakpoint region (MBR), and the MBR2 primer 360 base pairs upstream of MBR1. Assays were run using archival paraffin-embedded tumor sections.

†Unreadable FISH assays could not be scored because of inadequate sample or FISH signal strength.

‡Unreadable PCR assays were negative for the human *hemoglobin beta* (*HBB)* internal control segment and for t(14;18).

Table 5.4. Proportions of follicular lymphoma cases positive for t(14;18) translocations in previous studies of cases classified using FISH assays

Study, year	Location*	Case sample†	Specimen‡	Total cases N	t(14;18) %
Current Study	US	Population-based	Paraffin	43	81
Gong 2003 (46)	US	Hospital-based	FNA	40	85
Chiu 2006§ (9)	US	Population-based	Paraffin	63	66
Richmond 2006 (47)	US	Hospital-based	FNA	35	81
Hirose 2003 (48)	Japan	Convenience	Paraffin	11	64
Matsumoto 2004 (49)	Japan	Hospital-based	Paraffin	50	56
Guo 2005 (50)	China	Convenience	Frozen	147	81
Sekiguchi 2005 (51)	Japan	Hospital-based	Paraffin	47	60
Peh 2004 (52)	Malaysia	Hospital-based	Paraffin	62	74
Fan 2003 (27)	Canada	Hospital-based	Paraffin	24	83
Godon 2003 (53)	France	Convenience	Frozen	63	100
Bernicot 2005 (28)	France	Hospital-based	Metaphase	20	85
Vaandrager‖ 2000 (54)	Netherlands	Convenience	Frozen	40	88

*Country in which study was conducted: US, United States

†Selection of study cases: Population-based, use of state or national registries; Hospital-based, case series; Convenience, use of tissue/cytology files or banks

‡Type of sample on which assay was conducted: Paraffin, paraffin-embedded tumor section; FNA, fine needle aspirate; Frozen, fresh-frozen tumor sample; Metaphase, metaphase spread from fresh tumor specimen; Interphase, interphase nuclei of tumor cells from fresh tumor specimen

§FISH assays run on four representative 0.6-mm cores from each biopsy included on a tissue microarray (TMA).

‖FISH assays performed using yeast-artificial-chromosome (YAC)-DNA probes.

Table 5.5. Proportions of diffuse large cell lymphoma cases positive for t(14;18), *BCL6* and t(8;14) in previous studies of cases classified using FISH assays

Study	Location*	Cases sample†	Specimen‡	Total Cases N	t(14;18) %
Current Study	US	Population-based	Paraffin	66	39
Huang 2002§ (55)	US	Not specified	Paraffin	35	20
Gong 2003 (46)	US	Hospital-based	FNA	17	29
Iqbal 2004 (56)	US	Hospital-based	Paraffin	141	24
Chiu 2006§ (9)	US	Population-based	Paraffin	65	32
Cerretini 2006 (32)	Argentina	Hospital-based	Metaphase	30	30
Hirose 2003 (48)	Japan	Convenience	Paraffin	61	15
Godon 2003 (53)	France	Convenience	Frozen	17	94
Bernicot 2005 (28)	France	Hospital-based	Metaphase	26	19

*Country in which study was conducted: US, United States

†Selection of study cases: Population-based, use of state or national registries; Hospital-based, case series; Convenience, use of tissue/cytology files or banks

‡Type of sample on which assay was conducted: Paraffin, paraffin-embedded tumor section; FNA, fine needle aspirate; Frozen, fresh-frozen tumor sample; Metaphase, metaphase spread from fresh tumor specimen; Interphase, interphase nuclei of tumor cells from fresh tumor specimen.

§FISH assays run on four representative 0.6-mm cores from each biopsy included on a tissue microarray.

Table 5.6. Frequency of t(14;18)-positive follicular lymphomas according to PCR and FISH assays of paraffin-embedded tumor samples

Study	Location	t(14;18) by PCR		t(14;18) by FISH	
		Cases assayed (N)	% positive	Cases assayed (N)	% positive
Einerson 2005 *† (57)	US	14	36	24	100
Barrans 2003 ‡† (58)	UK	20	40	28	93
Godon 2003§‖ (53)	France	33	58	63	100
Godon 2003† (53)	France	NA	NA	39	98
Belaud-Rotureau 2006 ‡† (59)	France	39	64	51	92
Poetsch 1996*¶ (60)	Germany	28	64	28	100

Abbreviations: US, United States; UK, United Kingdom; MBR, major breakpoint region; mcr, minor cluster region

*PCR assays performed using primers for MBR, mcr, and the J_h consensus regions.

†FISH assays performed using Vysis LSI *IGH*/*BCL2* probes.

‡Multiplex PCR assays performed using primers for MBR, 3'MBR, 5'mcr, and the J_h consensus regions.

§PCR assays performed using primers for MBR and J_h consensus regions.

‖FISH assays performed using Cos Ig10/ PAC210c12 probes.

¶FISH assays performed using yeast-artificial-chromosome (YAC)-DNA probes.

G. References

1. Jaffe ES, Raffeld M, Medeiros LJ, Stetler-Stevenson M. An overview of the classification of non-Hodgkin lymphomas: an integration of morphological and phenotypical concepts. Cancer Res 1992; 52: 5447s-5452s.

2. Potter M. Pathogenetic mechanisms in B-cell non-Hodgkin lymphomas in humans. Cancer Res 1992; 52: 5522s-5528s.

3. Medeiros LJ, Carr J. Overview of the role of molecular methods in the diagnosis of malignant lymphomas. Arch Pathol Lab Med. 1999; 123: 1189-1207.

4. Magrath I. Molecular basis of lymphomagenesis. Cancer Res 1992; 52: 5529s-5540s.

5. Brown LM, Blair A, Gibson R, Everett GD, Cantor KP, Schuman LM, Burmeister LF, Van Lier SF, Dick F. Pesticide exposures and other agricultural risk factors for leukemia among men in Iowa and Minnesota. Cancer Res. 1990 Oct 15;50(20):6585-91.

6. Blair A, Linos A, Stewart PA, Burmeister LF, Gibson R, Everett G, Schuman L, Cantor KP. Evaluation of risks for non-Hodgkin lymphoma by occupation and industry exposures from a case-control study. Am J Industrial Med 1993; 23: 301-312.

7. Schroeder JC, Olshan AF, Baric R, Dent GA, Weinberg CR, Yount B, Cerhan JR, Lynch CF, Schuman LM, Tolbert PE, Rothman N, Cantor KP, Blair A. Agricultural risk factors for t(14;18) subtypes of non-Hodgkin lymphoma. Epidemiology 2001; 12: 701-9.

8. Schroeder JC, Olshan AF, Baric R, Dent GA, Weinberg CR, Yount B, Cerhan JR, Lynch CF, Schuman LM, Tolbert PE, Rothman N, Cantor KP, Blair A. A case-control study of tobacco use and other non-occupational risk factors for t(14;18) subtypes of non-Hodgkin lymphoma (United States). Cancer Causes Control. 2002 Mar;13(2):159-68.

9. Chiu BC, Dave BJ, Blair A, Gapstur SM, Zahm SH, Weisenburger DD. Agricultural pesticide use and risk of t(14;18)-defined subtypes of non-Hodgkin lymphoma. Blood. 2006 Aug 15;108(4):1363-9. Epub 2006 Apr 18.

10. Cantor KP, Blair A, Everett G, VanLier S, Burmeister L, Dick FR, Gibson RW, Schuman L. Hair dye use and risk of leukemia and lymphoma. Am J Public Health. 1988; 78(5): 570-1.

11. Cantor KP, Blair A, Everett G, Gibson R, Burmeister LF, Brown LM, Schuman L, Dick FR. Pesticides and other agricultural risk factors for non-Hodgkin lymphoma among men in Iowa and Minnesota. Cancer Res. 1992;52(9):2447-55.

12. Pottern LM, Linet M, Blair A, Dick F, Burmeister LF, Gibson R, Schuman LM, Fraumeni JF Jr. Familial cancers associated with subtypes of leukemia and non-Hodgkin lymphoma. Leuk Res. 1991;15(5):305-14.

13. Linos A, Blair A, Gibson RW, Everett G, Van Lier S, Cantor KP, Schuman L, Burmeister L. Leukemia and non-Hodgkin lymphoma and residential proximity to industrial plants. Arch Environ Health 1991 Sep-Oct;46(5):305.

14. Harris NL, Jaffe ES, Stein H, Banks PM, Chan JK, Cleary ML, Delsol G, De Wolf-Peeters C, Falini B, Gatter KC, et al. A revised European-American classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group. Blood. 1994 Sep 1;84(5):1361-92.

15. Schroeder JC. 1999. Agricultural and non-occupational risk factors for t(14;18) non-Hodgkin lymphoma [dissertation]. Chapel Hill (NC): University of North Carolina. 157 p.

16. Jaffe ES, Harris NL, Stein H, Vardiman JW (editors). Pathology and genetics of tumours of haematopoietic and lymphoid tissues: Lyon. IARCPress, 2001.

17. Vysis, Abbott Molecular Inc. (2007) Des Plaines, IL.

18. Vysis. Cellular genomic products for hematopoietic disorders: DNA fluorescence in situ hybridization probes for detection of genetic aberrations. Downers Grove: Vysis Inc, 2001.

19. Carl Zeiss (2007), Thornwood, NY.

20. Improvision, Inc. (2007) Openlab version 4.0.3, Lexington, MA, U.S.A.

21. Dako Denmark A/S (2007), Copehagen, Denmark.

22. Microsoft Corp. (1992-2001) Microsoft Access 2002, Redmond, WA, U.S.A

23. SAS Institute Inc (2002-2003) SAS version 9.1, Cary, NC.

24. Segel MJ, Paltiel O, Zimran A, Gottschalk-Sabag S, Schibi G, Krichevski S, Ludkovski O, Ben Yehuda D Geographic variance in the frequency of the t(14;18) translocation in follicular lymphoma: an Israeli series compared to the world. Blood Cells Mol Dis. 1998 Mar;24(1):62-72.

25. Cook JR, Shekhter-Levin S, Swerdlow SH. Utility of routine classical cytogenetic studies in the evaluation of suspected lymphomas: results of 279 consecutive lymph node/extranodal tissue biopsies. Am J Clin Pathol. 2004 Jun;121(6):826-35.

26. Cigudosa JC, Parsa NZ, Louie DC, Filippa DA, Jhanwar SC, Johansson B, Mitelman F, Chaganti RS. Cytogenetic analysis of 363 consecutively ascertained diffuse large B-cell lymphomas. Genes Chromosomes Cancer. 1999 Jun;25(2):123-33.

27. Fan YS, Rizkalla K. Comprehensive cytogenetic analysis including multicolor spectral karyotyping and interphase fluorescence in situ hybridization in lymphoma diagnosis: a summary of 154 cases. Cancer Genetics and Cytogenetics 2001; 143: 73-79.

28. Bernicot I, Douet-Guilbert N, Le Bris MJ, Morice P, Abgrall JF et al. Characterization of *IGH* rearrangements in non-Hodgkin B-cell lymphomas by fluorescence in situ hybridization. Anticancer Res 2005; 25(5): 3179-82.

29. Bastard C. LAZ3 rearrangements in non-Hodgkin lymphoma: correlation with histology, immunophenotype, karyotype, and clinical outcome in 217 patients. Blood 1994; 83(9): 2423-2427.

30. Ueda Y, Matsuda F, Misawa S, Taniwaki M. Tumor-specific rearrangements of the immunoglobulin heavy-chain gene in B-cell non-Hodgkin lymphoma detected by in situ hybridization. Blood 1998; 87(1): 292-298.

31. Garcia-Sanz R, Vargas Montero M, Gonzalez Diaz M, del Carmen M, Santos C, Balanzategui Echevarria A, Flores Corral T, Hernandez Martin JM, Caballero Barrigon MD, San Miguel JF. Detection of single and associated lesions of the bcl-1, bcl-2, bcl-6, c-myc, p53 and p16 genes in B-cell non-Hodgkin lymphomas: value of molecular analysis for a better assignment of the histologic subtype. Haematologica. 1998 Mar;83(3):209-16.

32. Cerretini R, Noriega MF, Narbaitz M, Slavutsky I. New chromosome abnormalities and lack of BCL-6 gene rearrangements in Argentinean diffuse large B-cell lymphomas. Eur J Haematol. 2006 Apr;76(4):284-93.

33. Nishida K, Taniwaki M, Misawa S, Abe T. Nonrandom rearrangement of chromosome 14 at band q32.33 in human lymphoid malignancies with mature B-cell phenotype. Cancer Res. 1989 Mar 1;49(5):1275-81.

34. Mossafa H, Damotte D, Jenabian A, Delarue R, Vincenneau A, Amouroux I, Jeandel R, Khoury E, Martelli JM, Samson T, Tapia S, Flandrin G, Troussard X. Non-Hodgkin lymphomas with Burkitt-like cells are associated with c-Myc amplification and poor prognosis. Leuk Lymphoma. 2006 Sep;47(9):1885-93.

35. Pienkowska-Grela B, Witkowska A, Grygalewicz B, Rymkiewicz G, Rygier J, Woroniecka R, Walewski J. Frequent aberrations of chromosome 8 in aggressive B-cell non-Hodgkin lymphoma. Cancer Genet Cytogenet. 2005 Jan 15;156(2):114-21.

36. Braziel RM, Arber DA, Slovak ML, Gulley ML, Spier C, Kjeldsberg C, Unger J, Miller TP, Tubbs R, Leith C, Fisher RI, Grogan TM. The Burkitt-like lymphomas: a Southwest Oncology Group study delineating phenotypic, genotypic, and clinical features. Blood. 2001 Jun 15;97(12):3713-20.

37. Yano T, van Krieken JH, Magrath IT, Longo DL, Jaffe ES, Raffeld M. Histogenetic correlations between subcategories of small noncleaved cell lymphomas. Blood. 1992 Mar 1;79(5):1282-90.

38. Janz S. Myc translocations in B cell and plasma cell neoplasms. DNA Repair (Amst). 2006 Sep 8;5(9-10):1213-24. Epub 2006 Jul 11.

39. McClure RF, Remstein ED, Macon WR, Dewald GW, Habermann TM, Hoering A, Kurtin PJ.Adult B-cell lymphomas with burkitt-like morphology are phenotypically and genotypically heterogeneous with aggressive clinical behavior. Am J Surg Pathol. 2005 Dec;29(12):1652-60.

40. Macpherson N, Lesack D, Klasa R, Horsman D, Connors JM, Barnett M, Gascoyne RD. Small noncleaved, non-Burkitt (Burkit-Like) lymphoma: cytogenetics predict outcome and reflect clinical presentation. J Clin Oncol. 1999 May;17(5):1558-67.

41. Papakonstantinou G, Verbeke C, Hastka J, Bohrer M, Hehlmann R. bcl-2 expression in non-Hodgkin lymphomas is not associated with bcl-2 gene rearrangements. Br J Haematol. 2001 May;113(2):383-90.

42. Kusumoto S, Kobayashi Y, Sekiguchi N, Tanimoto K, Onishi Y, Yokota Y, Watanabe T, Maeshima AM, Ishida T, Inagaki H, Matsuno Y, Ueda R, Tobinai K. Diffuse large B-cell lymphoma with extra bcl-2 gene signals detected by FISH analysis is associated with a "non-germinal center phenotype."Am J Surg Pathol. 2005 Aug;29(8):1067-73.

43. Vega F, Medeiros LJ. Chromosomal translocations involved in non-Hodgkin lymphomas. Arch Pathol Lab Med. 2003 Sep; 127(9): 1148-60.

44. Spagnolo DV, Ellis DW, Juneja S, Leong AS, Miliauskas J, Norris DL, Turner J. The role of molecular studies in lymphoma diagnosis: a review. Pathology. 2004 Feb;36(1):19-44.

45. Reichard KK, Hall BK, Corn A, Foucar MK, Hozier J. Automated analysis of fluorescence in situ hybridization on fixed, paraffin-embedded whole tissue sections in B-cell lymphoma. Mod Pathol. 2006 Aug;19(8):1027-33. Epub 2006 May 5.

46. Gong Y, Caraway N, Gu J, Zaidi T, Fernandez R, Sun X, Huh YO, Katz RL. Evaluation of interphase fluorescence in situ hybridization for the t(14;18)(q32;q21) translocation in the diagnosis of follicular lymphoma on fine-needle aspirates: a comparison with flow cytometry immunophenotyping. Cancer. 2003 Dec 25;99(6):385-93.

47. Richmond J, Bryant R, Trotman W, Beatty B, Lunde J. FISH detection of t(14;18) in follicular lymphoma on Papanicolaou-stained archival cytology slides. Cancer. 2006 Jun 25;108(3):198-204.

48. Hirose Y, Masaki Y, Ozaki M. Fluorescence in situ hybridization detection of chromosome *IGH/BCL2* translocations from paraffin-embedded tissue: evaluation in follicular lymphoma. Int J Hematol 2003; 78: 154-159.

49. Matsumoto Y, Nomura K, Matsumoto S, Ueda K, Nakao M, Nishida K, Sakabe H, Yokota S, Horiike S, Nakamine H, Nakamura S, Taniwaki M. Detection of t(14;18) in follicular lymphoma by dual-color fluorescence in situ hybridization on paraffin-embedded tissue sections. Cancer Genetics and Cytogenetics 2004; 150: 22-26.

50. Guo Y, Karube K, Kawano R, Yamaguchi T, Suzumiya J, Huang GS, Ohshima K. Low-grade follicular lymphoma with t(14;18) presents a homogeneous disease entity otherwise the rest comprises minor groups of heterogeneous disease entities with Bcl2 amplification, Bcl6 translocation or other gene aberrances. Leukemia. 2005 Jun;19(6):1058-63.

51. Sekiguchi N, Kobayashi Y, Yokota Y, Kusumoto S, Tanimoto K, Watanabe T, Matsuno Y, Tobinai K. Follicular lymphoma subgrouping by fluorescence in situ hybridization analysis. Cancer Sci 2005; 96(2): 77-82.

52. Peh SC, Shaminie J, Tai YC, Tan J, Gan SS. The pattern and frequency of t(14;18) translocation and immunophenotype in Asian follicular lymphoma. Histopathology 2004 Nov;45(5):501-10.

53. Godon A, Moreau A, Talmant P, Baranger-Papot L, Genevieve F, Milpied N, Zandecki M, Avet-Loiseau H.. Is t(14;18)(q32;q21) a constant finding in follicular lymphoma? An interphase FISH study on 63 patients. Leukemia 2003; 17: 255-259.

54. Vaandrager JW, Schuuring E, Raap T, Philippo K, Kleiverda K, Kluin P. Interphase FISH detection of BCL2 rearrangement in follicular lymphoma using breakpoint-flanking probes. Genes Chromosomes Cancer. 2000 Jan;27(1):85-94.

55. Huang JZ, Sanger WG, Greiner TC et al. The t(14;18) defines a unique subset of diffuse large B-cell lymphoma with a germinal center B-cell gene expression profile. Blood. 2002 Apr 1;99(7):2285-90.

56. Iqbal J, Sanger WG, Horsman DE, Rosenwald A, et al. BCL2 translocation defines a unique tumor subset within the germinal center B-cell-like diffuse large B-cell lymphoma. Am J Pathol. 2004 Jul;165(1):159-66.

57. Einerson RR, Kurtin PJ, Dayharsh GA, Kimlinger TK, Remstein ED. FISH is superior to PCR in detecting t(14;18)(q32;q21)-IgH/bcl-2 in follicular lymphoma using paraffin-embedded tissue samples. Am J Clin Pathol. 2005 Sep;124(3):421-9.

58. Barrans SL, Evans PA, O'Connor SJ, Owen RG, Morgan GJ, Jack AS. The detection of t(14;18) in archival lymph nodes: development of a fluorescence in situ hybridization (FISH)-based method and evaluation by comparison with polymerase chain reaction. J Mol Diagn. 2003 Aug;5(3):168-75.

59. Belaud-Rotureau MA, Parrens M, Carrere N, Turmo M, Ferrer J, de Mascarel A, Dubus P, Merlio JP. Interphase fluorescence in situ hybridization is more sensitive than BIOMED-2 polymerase chain reaction protocol in detecting IGH-BCL2 rearrangement in both fixed and frozen lymph node with follicular lymphoma. Hum Pathol. 2007 Feb;38(2):365-72. Epub 2006 Nov 28.

60. Poetsch M, Weber-Matthiesen K, Plendl HJ, Grote W, Schlegelberger B. Detection of the t(14;18) chromosomal translocation by interphase cytogenetics with yeast-artificial-chromosome probes in follicular lymphoma and nonneoplastic lymphoproliferation. J Clin Oncol. 1996 Mar;14(3):963-9.

CHAPTER 6

A CASE-CONTROL STUDY OF TOBACCO USE AND NON-HODGKIN LYMPHOMA (NHL) SUBTYPES DEFINED BY t(14;18) TRANSLOCATIONS AND bcl-2 EXPRESSION (UNITED STATES)

We re-evaluated reported associations estimated for tobacco use and other factors among study participants using fluorescence in situ hybridization (FISH) assays to determine the t(14;18) status of archival tumor samples believed to be better classified than previously based on polymerase chain reaction (PCR). Commercial FISH assays were used to detect t(14;18) translocations in samples from unselected NHL cases enrolled in the National Cancer Institute's Factors Affecting Rural Men (FARM) study (1981-1984). In addition, bcl-2 immunostaining was performed on paraffin sections to estimate associations with lymphoma subtypes defined by bcl-2 expression. Polytomous logistic regression models estimated associations between NHL case-subtypes (versus 1,245 population-based controls) and tobacco use, family history of hemolymphatic cancer and hair dye use; expectation-maximization (EM) was applied to the models to reduce bias caused by missing case-subtype data. FISH assays were successful on 154 cases (81 positive). Adjusting for age, state, and proxy status, t(14;18)-negative NHL was associated with any tobacco use (OR=1.98, 95% CI=1.09-3.59), including cigarette use only (OR=2.10, 95% CI=1.12-3.94). t(14;18)-negative NHL was also positively associated with all levels of pack-years and intensity, but without evidence of positive dose-response. Tobacco exposures were not clearly associated with t(14;18)-positive NHL or bcl-2 case-subtypes. The association between t(14;18)-

negative NHL and cigarette smoking was unexpected given previous evidence of associations between smoking and follicular lymphoma (which is largely t(14;18)-positive). Future studies characterizing additional molecular characteristics of t(14;18)-negative NHL may help determine whether the association with smoking may have been causal versus an artifact of chance or bias.

B. Introduction

The International Lymphoma Epidemiology Consortium (InterLymph) pooled analysis of eight case-control studies (6,594 cases and 8,892 controls) reported a weak association between ever smoking and non-Hodgkin lymphoma (NHL) (OR=1.07, 95% CI=1.00-1.15). Associations with follicular lymphoma (n=1,452) were slightly stronger than those for NHL overall, particularly for current smoking (OR=1.31, 95% CI=1.12-1.52) and current heavy smoking (36 or more pack-years) (OR=1.45, 95% CI=1.15-1.82) (1). Several population-based case-control studies also have found stronger associations between cigarette smoking and follicular lymphoma than with NHL overall and other lymphoma subtypes (2-7).

The t(14;18) translocation, which is detected in 85-100% of follicular lymphomas, juxtaposes the *IGH* gene on chromosome 14 with the *BCL2* gene at 18q21 resulting in overexpression of the anti-apoptotic bcl-2 protein (8). t(14;18) is believed to be an early step in lymphomagenesis, in part because non-neoplastic t(14;18)-positive lymphocytes are also detected at low levels in healthy individuals (9) . In addition, it has been reported that numbers of t(14;18)-positive bone marrow and peripheral lymphocytes are increased in smokers compared with non-smokers (10,11). Therefore, it has been suggested that

associations observed between follicular lymphoma and smoking may reflect an effect of smoking on the frequency or prevalence of t(14;18)-positive lymphocytes (12).

A previous analysis in which polymerase chain reaction (PCR) assays were run to detect t(14;18) in archival tumor samples from a subset of Iowa and Minnesota NHL cases in the Factors Affecting Rural Men (FARM) case-control study did not find a clear association between smoking and t(14;18)-NHL case-subtypes, but misclassification of cases may have occurred (12). Although PCR is believed to be a reasonably specific method for detecting t(14;18) in archival tumor samples, assays will fail to detect translocations with *BCL2* breakpoints that occur outside of regions bounded by the PCR primers used (approximately 15-25% of translocations depending on the specific assay) (14).

In contrast, fluorescence in situ hybridization (FISH) assays can detect translocations with more widely dispersed breakpoints than PCR. Studies comparing t(14;18) assays run on the same samples have shown that FISH consistently detects more t(14;18)-positive samples than PCR (15-20). In addition, use of FISH allows in situ localization of t(14;18) to neoplastic lymphocytes, thus decreasing the likelihood of falsely classifying lymphomas as t(14;18)-positive due to amplification of rare non-neoplastic t(14;18)-positive lymphocytes (21-23).

Therefore, we re-evaluated associations between t(14;18)-positive and –negative case subtypes and tobacco use, family history and other factors among FARM study participants after using FISH assays to re-classify the t(14;18) status of archival tumor samples that were previously classified based on PCR (12). In addition, we estimated associations with lymphoma subtypes defined by bcl-2 expression (based on immunohistochemistry) (24).

C. Patients, materials, and methods

C.1. Study population

The FARM study (A. Blair, PI), a large population-based study of incident hemolymphatic cancers among white men from Iowa and Minnesota, was conducted by the National Cancer Institute (NCI) to evaluate associations with agricultural exposures. The study population and methods were previously described in detail (25,26). Briefly, cases diagnosed in 1980-1983 among white men aged 30 or older were identified through hospital and pathology laboratory records (in Minnesota) and the Iowa State Health Registry. The study was restricted to white men from areas other than metropolitan Minnesota in order to increase the proportion with agricultural exposures, the primary focus of the study. Eighty-nine percent (694) of 780 ascertained NHL cases were enrolled, and the diagnosis of NHL was confirmed for 622 cases by a pathology review panel (25,26). NHL cases were limited to solid tumors; therefore lymphocytic leukemias were not included in the NHL case subgroup. Controls were 1,245 white males aged 30 or older without a history of hemolymphatic cancer that were frequency-matched to cases by age (within five-year groups), state, and vital status. Detailed information on agricultural exposures, other occupational exposures, medical history, smoking, and other potential risk factors for NHL was obtained through in-person structured interviews of cases, controls, or next-of-kin (25-30).

C.2. t(14;18)-PCR study

In 1997, archival paraffin-embedded tumor blocks were requested and successfully retrieved for 248 FARM study NHL cases (40% of all NHL cases in the original study) (12).

Over half of the tumor blocks that were not retrieved had been destroyed or lost; the remainder were held at institutions that declined to participate in the t(14;18)-NHL study. Up to 16 5-micron sections and 16 10-micron sections were cut from each archival block. A single pathologist reviewed the first, middle, and last sections to confirm the presence of tumor tissue and assign histological subtypes according to the Revised European American Lymphoma classification scheme (REAL) (31).

t(14;18)-PCR assays were performed on DNA extracted from 10-micron sections cut from archival tumor blocks (12). Each reaction included a consensus primer corresponding to a conserved portion of the J segment of the *IGH* gene on chromosome 14 (5'-ACCTGAGGAGACGGTGAGC-3') and a second primer corresponding to the *BCL2* major breakpoint region (MBR) on chromosome 18 (MBR1: 5'-GAGAGTTGCTTTACGTG-GCCTG-3') which includes 40-60% of *BCL2* breakpoints involved in t(14;18). Translocation-negative samples were subjected to a second PCR reaction with the *IGH* consensus primer and a second *BCL2* primer 360 base pairs upstream of the MBR primer (MBR2: 5'-CGCTTGACTCCTTTACGTGCTG-3'). Amplified DNA was isolated, denatured, and separated by gel electrophoresis, and transferred to a nylon membrane using a Southern blot procedure. Amplification was confirmed using radio-labeled probes that hybridized to DNA adjacent to each *BCL2* primer site. A 175-base pair segment of the *hemoglobin beta* (*HBB*) gene was amplified as an internal positive control to confirm that the DNA extracted from each sample was adequate for PCR amplification of t(14;18). Sixty-six samples that were t(14;18)-negative and *HBB*-negative may have been false-negative because of DNA that was degraded or otherwise inadequate for the assay; therefore, these cases were classified as missing to distinguish them from translocation-negative samples in which *HBB*

amplification was successful. Amplification products from 20 of the 68 t(14;18) positive samples were sequenced to confirm that unique translocation breakpoints were amplified from each sample.

C.3. t(14;18)-FISH study

Commercially available FISH t(14;18) assays were run on 5-micron sections that were previously cut and archived from FARM study tumor blocks (Vysis, 32). All sections were stored at 4°C prior to use. Histologic subtype classifications were updated according to a modified version of the current World Health Organization (WHO) classification scheme based on morphology only with cases that would be classified as chronic lymphocytic leukemias/small lymphocytic lymphomas (CLL/SLL) are referred to as small lymphocytic lymphomas (SLL) since they were limited to solid tumors (33). The majority of cases classified as diffuse large cell lymphomas (DLCL) probably correspond to diffuse large B cell lymphomas (DLBCL); however, B-cell status was not confirmed. Cases classified as Burkitt lymphomas (BL) may include both Burkitt and Burkitt-like lymphomas.

t(14;18) translocations were identified using the LSI® *IGH*/*BCL2* Dual Color, Dual Fusion Translocation Probe (Vysis, 32). Assays were run according to manufacturer's instructions with minor modifications, as described in detail previously (Chang et al 2007, submitted for publication, 34). Briefly, we began by assaying cases with at least two 5-micron sections available (N = 230) for any *IGH* translocation. Deparaffinization and pretreatment of archival 5-micron sections was performed using the Vysis® VP2000™ Processor (31). A 6-minute denaturation was followed by a 16-18 hour hybridization step (HYBrite™ machine, Abbott Molecular Inc., 32). Finally, a rapid wash procedure was

performed to remove unbound probe, and DAPI II counterstain was applied over the target area. Newly assayed slides were stored at -20°C until scoring.

FISH assays were scored using the 40x and 100x oil objectives on a Zeiss Axioskope 2 (Carl Zeiss, 35) equipped with a triple bandpass filter set (DAPI/Green/Orange V.2,32). For each sample, a minimum of 25 non-overlapping lymphocytes with complete FISH signals were scored; samples with less than 25 readable cells were classified as unreadable. In addition, samples were classified as unreadable if the signal strength of one or both FISH probes was inadequate to visualize signals or distinguish fusion signals from signals produced by individual probes. t(14;18) assays were scored by two investigators. Cases were classified as translocation positive if more than 30% of cells showed abnormal signal patterns. Individual scores were concordant for 121 of 169 assays. Samples with discordant results were assigned a consensus classification based on a joint review, including 22 assays that were initially classified as positive by one reviewer and negative by the other, and 26 that were initially classified as unreadable by one scorer only (with 20 subsequently classified as positive or negative, and 6 classified as unreadable based on joint review.)

C.4. bcl-2 expression by IHC

bcl-2 immunostaining was performed on paraffin sections from 229 FARM study cases using a mouse monoclonal antibody corresponding to bcl-2 amino acids 41-54 (clone 124; Dako, 36). Cases were classified as bcl-2 positive if more than 20% of cells showed cytoplasmic staining. Independent scores assigned by two investigators were concordant for 201 of 219 cases (92%) that were adequate for scoring. Cases with discordant results were assigned a consensus classification after review (7 positive, 11 negative for bcl-2).

C.5. Data analysis

Ever use of any tobacco and specific tobacco products (cigarettes, cigars, pipe tobacco, chewing tobacco, snuff) was defined as daily use for at least 3 months. Cigarette smoking was categorized according to average intensity of use (0, 1-10, 11-20, >20 cigarettes per day), age of first use (18 or less, over age 18), and pack-years (0, >0-20, >20-40, >40). In addition, we estimated associations between NHL case-subtypes and other putative NHL risk factors including, family history of hemolymphatic cancer (in a first- or second-degree relative), hair dye use (ever use of hair dye at least once a month for at least one year, or occupational exposure to hair dyes on any job held for 1 or more years), occupation as farmer (ever worked on a farm for 6 continuous months or longer since the age 18), weekly alcohol use (ever drank beer, wine or hard liquor weekly for at least one year), education (<=12 years, >12 years), and marital status (married/widowed, divorced/separated, never married).

Case-only (case-case) analyses were performed using unconditional logistic regression models to determine whether estimates were comparable between molecular subtypes, including t(14;18)-positive vs. –negative case-subtype comparisons and bcl-2 positive vs. -negative case-subtype comparisons (SAS 9.1, 37). Case-subtype: control associations were estimated by polytomous regression models with the expectation-maximization (EM) algorithm used to reduce potential bias caused by missing case-subtype data (for cases for whom tumor blocks could not be retrieved or that were not successfully assayed) as previously described (38) (Stata release 9.0, 39).

All models included the matching factors state (Minnesota vs. Iowa), age at diagnosis (coded using upper and lower tail-restricted quadratic splines), and proxy status. Covariates examined were factors associated with NHL in past studies (e.g. alcohol use (40), family

history of hemolymphatic (HLP) cancer (41) and farming (42) as well as demographic factors (age (43) and education (43,44)). For the full logistic model of each main exposure evaluated, potential confounders were selected based on being a common cause of both the exposure and disease using conceptual models (45). No confounding was evident based a 0.15 or greater change in the beta coefficient of the main exposure variable when the potential confounder was removed from the model; therefore, final models included the matching factors only.

D. Results

Overall, cases were more likely to be classified as t(14;18)-positive based on FISH (81 of 154 cases, 53%) than PCR (68 of 182 cases, 37%). Additionally, detection of t(14;18) among follicular lymphomas by FISH (81%, 35 of 43 FL cases assayed) was higher than by PCR (44%) (Chapter 5) (34). The majority of cases with discordant results for FISH versus PCR (32 of 40 cases with data for both assays) were PCR-negative/ FISH-positive (18 FL, 4 DLCL, 94% bcl-2 positive), consistent with our expectation that FISH assays would have a higher sensitivity to detect t(14;18) than our previous PCR assay (15-21).

Tobacco use was common in the study population, with only 23% of 1245 controls and 17% of 154 successfully assayed cases reporting no use of tobacco products. Any tobacco use was associated with t(14;18)-negative NHL (OR=1.98, 95% CI=1.09-3.59), but not t(14;18)-positive NHL (OR=0.94, 95% CI=0.63-1.41) (Table 6.1). In addition, t(14;18)-negative NHL was associated with any cigarette use (OR=1.53, 95% CI=0.74-3.17), cigar use (OR=1.58, 95% CI=0.98-2.55), and use of both cigarettes with other forms of tobacco (OR=2.09, 95% CI=1.12-3.89). The association between cigarette use and t(14;18)-negative NHL was consistent for all categories of cigarettes per day, pack-years and age at first use,

without evidence of a positive dose-response relation. In contrast, associations between the same exposures and t(14;18)-positive NHL were null or inverse (OR's 0.6-1.0). Case-case effect estimates for t(14;18)-positive vs. t(14;18)-negative NHL were often close to 0.5 or below for cigarette exposures, consistent with associations with t(14;18)-positive NHL that were generally below the null, while associations with t(14;18)-negative NHL were above the null (Table 6.1).

t(14;18)-positive NHL was imprecisely associated with tobacco use other than cigarette smoking (OR=1.57, 95% CI=0.88-2.79), pipe use (OR=1.31, 95% CI=0.84-2.06), and chewing tobacco use (OR=1.45, 95% CI=0.85-2.5). In addition, there was evidence of a stronger association when chewing tobacco use began before age 18 based on a small number of exposed cases (OR=2.12, 95% CI=0.97-4.64, 6 exposed cases).

Based on bcl-2 expression detected by IHC, 168 cases were bcl-2-positive (77%) and 51 cases were bcl-2-negative. As expected, the majority of t(14;18)-positive cases were bcl-2-positive (66 of 76 cases), with only 10 t(14;18)-positive cases classified as bcl-2-negative. The majority of t(14;18)-negative cases also were bcl-2-positive (40 of 69 cases), with only 29 t(14;18)-negative cases classified as bcl-2-negative. Any tobacco use appeared to be more strongly associated with bcl-2-negative NHL (OR=1.79, 95% CI=0.84-3.79) than bcl-2-positive NHL (OR=1.18, 95% CI=0.88-1.6) (Table 6.2). However, cigarette use, pack-years, and intensity were not clearly associated with either bcl-2 case-subtype. Consistent with these findings, case-case estimates for bcl-2 positive NHL versus bcl-2 negative NHL were close to null (Table 6.2). Associations between tobacco use and other risk factors were not estimated for case-subtypes defined by the joint distribution of t(14;18) and bcl-2 expression due to small numbers of cases within subgroups.

Estimated associations for other non-occupational exposures and t(14;18) case-subtypes were generally consistent with previously reported estimates for t(14;18) case-subtypes classified based on PCR (Table 6.3) (12). Consistent with previous findings, a positive family history of hemolymphatic cancer was associated with t(14;18)-negative lymphoma (OR=2.9, 95% CI=1.77-4.76), but not t(14;18)-positive lymphoma (OR=0.91, 95% CI=0.36-2.34). However, a positive family history was associated with both bcl-2 negative (OR=2.31, 95% CI=1.11-4.80) and bcl-2 positive (OR=1.7 95% CI=1.1-2.63) case-subtypes (Table 6.3). FISH results suggest a weaker association between hair dye use and t(14;18)-positive NHL (OR=1.36, 95% CI=0.67-2.73) than t(14;18)-negative lymphoma (OR=2.73, 95% CI=1.56-4.76), while IHC results suggest a stronger association between hair dye use and bcl-2 positive NHL (OR=2.16, 95% CI=1.4-3.32), than bcl-2 negative NHL (OR=1.37, 95% CI=0.5-3.79); however, all estimates were based on small numbers of exposed cases in each case-subgroup.

E. Discussion

Using FISH assays, we detected a number of t(14;18)-positive cases that had been previously classified as t(14;18)-negative based on PCR. Although case-subtype:control associations for many exposures were similar between the current study and the previous PCR-based study, we noted a positive association between t(14;18)-negative NHL and tobacco use that was not apparent based on our previous analysis. A previous population-based case-control study of tobacco use and t(14;18) NHL in Nebraska also reported an association between smoking and t(14;18)-negative NHL (OR=1.9, 95% CI=1.1-3.3), but the association was specific to women, with no t(14;18) case-subtype associations evident among

men (13). The Nebraska study investigators also reported that a positive family history of a hematopoietic cancer was associated with both t(14;18)-subtypes (OR's 1.9-2.2) among both men and women, while only t(14;18)-negative NHL was associated with family history in our study. Finally, we noted an association between hair-dye use and t(14;18)-negative NHL (and to a lesser extent, t(14;18)-positive NHL), while hair-dye use was not associated with either case-subtype in the Nebraska study (13).

Differences between the current study and the Nebraska study of t(14;18) NHL may reflect differences between the two study populations, random variation due to small sample sizes, bias due to sampling error or uncontrolled confounding, or differences in case-subtyping (13). Although both studies used the same commercial FISH t(14;18) assay kits, we scored assays run on standard sections cut through the entire biopsy sample for each case, while Nebraska study samples were scored based on a review of four representative 0.6-mm cores from each biopsy that were included in a tissue micoarray (TMA). Use of TMAs reduces assay costs and increases scoring efficiency, but it is also possible that neoplastic t(14;18)-positive lymphocytes may not be represented in TMA cores for some cases. We noted a higher proportion of t(14;18)-positive cases among all cases (53% vs. 38%) and among follicular lymphomas (81% vs. 67%) than those in the Nebraska study, which would be consistent with differences in the prevalence of t(14;18)-NHL between the two study populations, or with reduced sensitivity due to the use of TMAs in the Nebraska study.

The association between cigarette smoking and t(14;18)-negative NHL was unexpected, given results of several previous studies that found a positive association between smoking and follicular lymphoma (1-7), which is largely t(14;18)-positive (14-21), as well as prior evidence of increased t(14;18) in the peripheral blood lymphocytes of smoker

compared with non-smokers (10,11). The lack of positive dose-response for cigarette smoking suggests a low-dose threshold or that smoking is acting as a proxy for another unmeasured factor, though chance association or bias are still possible. Although cigarette use was consistently associated with t(14;18)-negative NHL, the evaluation for other tobacco products including daily pipe use, chewing tobacco, and cigar use was inconclusive based on small numbers (see also Appendices L and M).

t(14;18)-negative NHL pathways are likely to be etiologically heterogeneous. Attempting to find several other common translocations (any *IGH*, *BCL6*, and t(8;14)) by running FISH assays on FARM study samples (34), we detected 14 cases positive for other *IGH* translocations and 3 cases positive for *BCL6* translocations among the t(14;18)-negative cases and two *BCL6*-positive and one t(8;14)-positive cases among the t(14;18)-positive cases. Additional molecular characterization of t(14;18)-negative cases will require studies of sufficient size to estimate more precise associations with case-subtypes and might help clarify whether the association between tobacco use and t(14;18)-negative NHL may have been causal versus an artifact of chance or bias.

Strengths of the study include use of a large population-based control group, the availability of detailed exposure data from both cases and controls, the use of FISH to determine t(14;18)-subtypes, and the availability of bcl-2 expression data in addition to information on t(14;18). However, due to small numbers of cases we were unable to evaluate case-subtypes defined by both bcl-2 and t(14;18).

Among the limitations, only 25% of the 622 original FARM study NHL cases were successfully assigned a case-subtype, and only 20% of participants reported never use of cigarettes, resulting in small case subgroups and imprecise estimates. The majority of

missing cases were from Minnesota (56% of 461 total missing), consequently, case-subtype:control estimates may have been biased if missing outcome data were associated with exposures. We used an EM-based method to impute missing case-subtype data, as previously described; however, bias due to missing data cannot be ruled out (38).

In summary, our data suggest that smoking and a family history of hemolymphatic cancer may be associated with t(14;18)-negative NHL. In order to clarify the role of smoking and other potential risk factors in the etiology of t(14;18) NHL, future studies should incorporate histologic subtypes assigned in clinical settings where the role of molecular analyses is increasing in the routine pathologic evaluation of lymphoma cases (46); subtypes defined by molecular characteristics in addition to t(14;18) should be pursued as well. Alternatively, improved methods to estimate the incidence and prevalence of t(14;18) translocations in the peripheral blood lymphocytes of people without cancer may help clarify whether or how smoking and other exposures influence the pathogenesis of t(14;18)-positive lymphomas (47-49). Improvements on these studies, including better control for confounding and detection of additional genetic events, could generate hypotheses in regards to identifying genotoxic exposures that may be associated with NHL molecular subtypes (50-53).

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Table 6.1. Adjusted* odds ratios for use of tobacco products among t(14;18)-positive or –negative NHL cases† compared with controls, and for t(14;18)-positive cases compared with –negative cases†

Exposures	Controls	t(14;18)-positive vs. controls‡			t(14;18)-negative vs. controls‡			t(14;18)-positive vs. t(14;18)-negative§	
	N	N	OR	95% CI	N	OR	95% CI	OR	95% CI
No tobacco use	286	17	1.0		9	1.0			
Used any tobacco	959	64	0.94	0.63 - 1.41	63	1.98	1.09 - 3.59	0.59	0.24-1.44
Tobacco by product									
Cigarettes	825	51	0.57	0.33 - 0 .98	56	1.53	0.74 - 3.17	0.41	0.13-1.26
Pipe	293	26	1.31	0.84 - 2.06	20	0.86	0.53 - 1.38	1.54	0.72-3.28
Cigar	167	7	0.69	0.34 - 1.39	14	1.58	0.98 - 2.55	0.45	0.17-1.32
Chew tobacco	115	13	1.45	0.85 - 2.50	9	1.03	0.54 - 1.98	1.48	0.54-4.10
Snuff	141	9	1.04	0.56 - 1.94	8	0.77	0.4 - 1.48	1.27	0.42-3.78
No tobacco use	286	17	1.0		9	1.0			
Cigarettes only	457	26	0.83	0.5 - 1.37	28	2.10	1.12 - 3.94	0.53	0.20-1.44
Cigarettes and other	368	25	0.87	0.54 - 1.4	28	2.09	1.12 - 3.89	0.52	0.19-1.40
Other tobacco only	132	13	1.57	0.88 - 2.79	7	1.38	0.58 - 3.26	1.32	0.35-5.08
Cigarette pack-years									
0-20 pack-yrs	216	17	1.0	0.6 - 1.67	11	1.68	0.89 - 3.16	0.81	0.29-2.28
20-40 pack-yrs	207	11	0.8	0.45 - 1.41	8	1.27	0.62 - 2.59	0.78	0.24-2.51
40+ pack-yrs	342	20	0.6	0.37 -0 .96	33	1.99	1.19 - 3.34	0.26	0.10-0.63
Cigarettes/day									
0	418	30	1.0		16	1.0			
>0-10	176	11	0.85	0.48 - 1.51	10	1.82	0.99 - 3.35	0.56	0.19-1.68
>10-20	363	21	0.7	0.43 - 1.11	23	1.86	1.1 - 3.15	0.42	0.17-1.05
>20	259	17	0.7	0.42 - 1.15	22	1.72	1.0 - 2.98	0.36	0.14-0.92
Product/age started									
Cigarettes									
>18	332	22	0.81	0.51 - 1.28	19	1.69	0.97 - 2.93	0.62	0.25-1.51
≤18	493	29	0.71	0.46 - 1.08	37	1.96	1.19 - 3.21	0.39	0.17-0.89
Chewing tobacco									
>18	86	7	1.21	0.63 - 2.33	6	1.16	0.57 - 2.36	1.08	0.31-3.77
≤18	29	6	2.12	0.97 - 4.64	3	1.16	0.39 - 3.5	1.75	0.40-7.72

*Adjusted for state, age (restricted quadratic splines), and proxy status

†Case-subtypes based on fluorescence in situ hybridization (FISH) assays

‡Estimates from expectation-maximization (EM) polytomous regression models including controls and both case-subtypes

§Estimates from unconditional logistic regression models restricted to assayed cases

Table 6.2. Adjusted* odds ratios for use of tobacco products among bcl-2-positive or –negative NHL cases† compared with controls, and for bcl-2-positive cases compared with –negative cases†

Exposures	Controls	bcl-2-positive vs. controls‡			bcl-2-negative vs. controls‡			bcl-2-positive vs. bcl-2-negative §	
	N	N	OR	95% CI	N	OR	95% CI	OR	95% CI
No tobacco use	286	32	1.0		7	1.0			
Used any tobacco	959	136	1.18	0.88 - 1.6	43	1.79	0.84 - 3.79	0.71	0.29-1.76
Tobacco by product									
Cigarettes	825	119	1.07	0.68 - 1.66	35	0.56	0.26 - 1.18	1.92	0.68-5.43
Pipe	293	43	1.08	0.8 - 1.47	11	1.0	0.51 - 1.94	1.24	0.55-2.75
Cigar	167	22	1.08	0.75 - 1.55	7	1.18	0.55 - 2.52	0.93	0.36-2.40
Chew tobacco	115	20	1.26	0.84 - 1.91	5	1.18	0.49 - 2.86	1.17	0.38-3.66
Snuff	141	14	0.73	0.46 - 1.18	7	1.51	0.67 - 3.38	0.49	0.16-1.47
No tobacco use	286	32	1.0		7	1.0			
Cigarettes only	457	67	1.18	0.85 - 1.65	21	1.66	0.74 - 3.75	0.78	0.29-2.08
Cigarettes and other	368	52	1.2	0.85 - 1.69	14	1.67	0.71 - 3.92	0.80	0.29-2.25
Other tobacco only	132	17	1.16	0.72 - 1.89	8	2.89	1.09 - 7.66	0.38	0.11-1.34
Cigarette pack-years									
0-20 pack-yrs	216	31	1.11	0.75 - 1.63	10	1.62	0.8 - 3.28	1.02	0.38-2.69
20-40 pack-yrs	207	30	1.13	0.79 - 1.61	4	0.44	0.15 - 1.26	3.00	0.82-10.93
40+ pack-yrs	342	50	1.03	0.74 - 1.43	18	1.2	0.64 - 2.25	0.89	0.39-2.05
Cigarettes/day									
0	418	49	1.0		15	1.0			
>0-10	176	24	1.21	0.82 - 1.79	6	1.14	0.49 - 2.67	1.31	0.43-3.95
>10-20	363	52	1.09	0.79 - 1.5	15	1.12	0.58 - 2.16	1.18	0.50-2.79
>20	259	39	1.07	0.75 - 1.52	12	1.01	0.5 - 2.06	1.11	0.45-2.72
Product/age started									
Cigarettes									
>18	332	51	1.14	0.82 - 1.57	14	1.05	0.54 - 2.07	1.24	0.53-2.91
≤18	493	68	1.14	0.84 - 1.53	21	1.13	0.61 - 2.1	1.09	0.49-2.42
Chewing tobacco									
>18	86	11	1.15	0.7 - 1.89	3	1.32	0.46 - 3.79	0.85	0.21-3.38
≤18	29	9	1.69	0.88 - 3.26	2	1.43	0.34 - 6.07	1.10	0.21-5.65

*Adjusted for state, age (restricted quadratic splines), and proxy status
†Case-subtypes based on immunohistochemistry (IHC) assays
‡Estimates from expectation-maximization (EM) polytomous regression models including controls and both case-subtypes
§Estimates from unconditional logistic regression models restricted to assayed cases

Table 6.3. Adjusted*† odds ratios for selected exposures among t(14;18)-positive or –negative NHL cases‡ compared with controls and for bcl-2-positive or –negative NHL cases§ compared with controls

Exposures	Controls	t(14;18)-positive vs. controls			t(14;18)-negative vs. controls			bcl-2-positive vs. controls			bcl-2-negative vs. controls		
	N	N	OR	95% CI	N	OR	95% CI	N	OR	95% CI	N	OR	95% CI
Farmer	698	52	1.34	0.88 - 2.04	40	0.97	0.64 - 1.46	99	1.10	0.85 - 1.43	29	1.26	0.73 - 2.18
Any hair dye	58	8	1.36	0.67 - 2.73	12	2.73	1.56 - 4.76	21	2.16	1.40 - 3.32	4	1.37	0.50 - 3.79
Education ≤ 12 years	878	60	1.26	0.81 - 1.95	49	0.87	0.58 - 1.31	118	1.09	0.82 - 1.45	34	0.91	0.52 - 1.60
Drank alcohol weekly	686	36	0.73	0.49 - 1.07	41	1.16	0.78 - 1.74	87	0.86	0.66 - 1.11	30	1.12	0.64 - 1.94
HLP in first-degree relatives	51	3	1.32	0.48 - 3.60	6	2.60	1.36 - 5.00	11	1.63	0.94 - 2.84	6	2.97	1.33 - 6.63
HLP in first or second-degree relatives	78	4	0.91	0.36 - 2.34	10	2.90	1.77 - 4.76	19	1.70	1.10 - 2.63	8	2.31	1.11 - 4.80

*Adjusted for state, age (restricted quadratic splines), and proxy status

†Estimates from expectation-maximization (EM) polytomous regression models including controls and both case-subtypes

‡Case-subtypes based on fluorescence in situ hybridization (FISH) assays

§Case-subtypes based on immunohistochemistry (IHC) assays

G. References

1. Morton LM, Hartge P, Holford TR, Holly EA, Chiu BC, Vineis P, Stagnaro E, Willett EV, Franceschi S, La Vecchia C, Hughes AM, Cozen W, Davis S, Severson RK, Bernstein L, Mayne ST, Dee FR, Cerhan JR, Zheng T. Cigarette smoking and risk of non-Hodgkin lymphoma: a pooled analysis from The International Lymphoma Epidemiology Consortium (InterLymph). Cancer Epidemiol Biomarkers Prev. 2005; 14(4):925-33.

2. Parker AS, Cerhan JR, Dick F, Kemp J, Habermann TM, Wallace RB, Sellers TA, Folsom AR. Smoking snd risk of non-Hodgkin lymphoma subtypes in a cohort of older women. Leuk Lymphoma. 2000; 37(3-4): 341-9.

3. Herrinton LJ, Friedman GD. Cigarette smoking and risk of non-Hodgkin lymphoma subtypes. Cancer Epidemiol Biomarkers Prev. 1998;7(1):25-8.

4. Stagnaro E, Tumino R, Parodi S, Crosignani P, Fontana A, Masala G, Miligi L, Nanni O, Ramazzotti V, Rodella S, Senoiri Constantini A, Vigano C, Vindigni C, Vineis P. Non-Hodgkin lymphoma and type of tobacco smoke. Cancer Epidemiol Biomarkers Prev. 2004; 13(3): 431-7.

5. Stagnaro E, Ramazzotti V, Crosignani P, Fontana A, Masala G, Miligi L, Nanni O, Neri M, Rodella S, Costantini AS, Tumino R, Vigano C, Vindigni C, Vineis P. Smoking and hematolymphopoietic malignancies. Cancer Causes Control. 2001; 12(4): 325-34.

6. Morton LM, Holford TR, Leaderer B, Boyle P, Zahm SH, Zhang Y, Flynn S, Tallini G, Zhang B, Owens PH, Zheng T. Cigarette smoking and risk of non-Hodgkin lymphoma subtypes among women. Br J Cancer. 2003; 89(11): 2087-92.

7. Schollkopf C, Smedby KE, Hjalgrim H, Rostgaard K, Gadeberg O, Roos G, Porwit-Macdonald A, Glimelius B, Adami HO, Melbye M. Cigarette smoking and risk of non-Hodgkin lymphoma--a population-based case-control study. Cancer Epidemiol Biomarkers Prev. 2005 Jul;14(7):1791-6.

8. Godon A, Moreau A, Talmant P, Baranger-Papot L, Genevieve F, Milpied N, Zandecki M, Avet-Loiseau H.. Is t(14;18)(q32;q21) a constant finding in follicular lymphoma? An interphase FISH study on 63 patients. Leukemia 2003; 17: 255-259.

9. Limpens J, Stad R, Vos C, de Vlaam C, de Jong D, van Ommen GJ, Schuuring E, Kluin PM. Lymphoma-associated translocation t(14;18) in blood B cells of normal individuals. Blood. 1995 May 1;85(9):2528-36.

10. Rauzy O, Galoin S, Chale JJ, Adoue D, Albarede JL, Delsol G, al Saati T. Detection of t(14;18) carrying cells in bone marrow and peripheral blood from patients affected by non-lymphoid diseases. Mol Pathol. 1998 Dec;51(6):333-8.

11. Bell DA, Liu Y, Cortopassi GA. Occurrence of bcl-2 oncogene translocation with increased frequency in the peripheral blood of heavy smokers. JNCI 1995; 87(3): 223-4.

12. Schroeder JC, Olshan AF, Baric R, Dent GA, Weinberg CR, Yount B, Cerhan JR, Lynch CF, Schuman LM, Tolbert PE, Rothman N, Cantor KP, Blair A. A case-control study of tobacco use and other non-occupational risk factors for t(14;18) subtypes of non-Hodgkin lymphoma (United States). Cancer Causes Control. 2002 Mar;13(2):159-68.

13. Chiu BC, Dave BJ, Blair A, Gapstur SM, Chmiel JS, Fought AJ, Zahm SH, Weisenburger DD. Cigarette smoking, familial hematopoietic cancer, hair dye use, and risk of t(14;18)-defined subtypes of non-Hodgkin lymphoma. Am J Epidemiol. 2007 Mar 15;165(6):652-9. Epub 2007 Jan 4.

14. Vega F, Medeiros LJ. Chromosomal translocations involved in non-Hodgkin lymphomas. Arch Pathol Lab Med. 2003 Sep;127(9):1148-60.

15. Poetsch M, Weber-Matthiesen K, Plendl HJ, Grote W, Schlegelberger B. Detection of the t(14;18) chromosomal translocation by interphase cytogenetics with yeast-artificial-chromosome probes in follicular lymphoma and nonneoplastic lymphoproliferation. J Clin Oncol. 1996 Mar;14(3):963-9.

16. Barrans SL, Evans PA, O'Connor SJ, Owen RG, Morgan GJ, Jack AS.The detection of t(14;18) in archival lymph nodes: development of a fluorescence in situ hybridization (FISH)-based method and evaluation by comparison with polymerase chain reaction. J Mol Diagn. 2003 Aug;5(3):168-75.

17. Godon A, Moreau A, Talmant P, Baranger-Papot L, Genevieve F, Milpied N, Zandecki M, Avet-Loiseau H.. Is t(14;18)(q32;q21) a constant finding in follicular lymphoma? An interphase FISH study on 63 patients. Leukemia 2003; 17: 255-259.

18. Einerson RR, Kurtin PJ, Dayharsh GA, Kimlinger TK, Remstein ED. FISH is superior to PCR in detecting t(14;18)(q32;q21)-IgH/bcl-2 in follicular lymphoma using paraffin-embedded tissue samples. Am J Clin Pathol. 2005 Sep;124(3):421-9.

19. Belaud-Rotureau MA, Parrens M, Carrere N, Turmo M, Ferrer J, de Mascarel A, Dubus P, Merlio JP. Interphase fluorescence in situ hybridization is more sensitive than BIOMED-2 polymerase chain reaction protocol in detecting IGH-BCL2 rearrangement in both fixed and frozen lymph node with follicular lymphoma. Hum Pathol. 2007 Feb;38(2):365-72. Epub 2006 Nov 28.

20. Streubel B, Scheucher B, Valencak J, Huber D, Petzelbauer P, Trautinger F, Weihsengruber F, Mannhalter C, Cerroni L, Chott A. Molecular cytogenetic evidence of t(14;18)(IGH;BCL2) in a substantial proportion of primary cutaneous follicle center lymphomas. Am J Surg Pathol. 2006 Apr;30(4):529-36.

21. Medeiros LJ, Carr J. Overview of the role of molecular methods in the diagnosis of malignant lymphomas. Arch Pathol Lab Med. 1999 Dec;123(12):1189-207.

22. Cook JR. Paraffin section interphase fluorescence in situ hybridization in the diagnosis and classification of non-hodgkin lymphomas. Diagn Mol Pathol. 2004 Dec;13(4):197-206.

23. Spagnolo DV, Ellis DW, Juneja S, Leong AS, Miliauskas J, Norris DL, Turner J. The role of molecular studies in lymphoma diagnosis: a review. Pathology. 2004 Feb;36(1):19-44.

24. Papakonstantinou G, Verbeke C, Hastka J, Bohrer M, Hehlmann R. bcl-2 expression in non-Hodgkin lymphomas is not associated with bcl-2 gene rearrangements. Br J Haematol. 2001 May;113(2):383-90.

25. Brown LM, Blair A, Gibson R, Everett GD, Cantor KP, Schuman LM, Burmeister LF, Van Lier SF, Dick F. Pesticide exposures and other agricultural risk factors for leukemia among men in Iowa and Minnesota. Cancer Res. 1990 Oct 15;50(20):6585-91.

26. Blair A, Linos A, Stewart PA, Burmeister LF, Gibson R, Everett G, Schuman L, Cantor KP. Evaluation of risks for non-Hodgkin lymphoma by occupation and industry exposures from a case-control study. Am J Industrial Med 1993; 23: 301-312.

27. Brown LM, Everett GD, Gibson R, Burmeister LF, Schuman LM, Blair A. Smoking and risk of non-Hodgkin lymphoma and multiple myeloma. Cancer Causes Control. 1992 Jan;3(1):49-55.

28. Cantor KP, Blair A, Everett G, VanLier S, Burmeister L, Dick FR, Gibson RW, Schuman L. Hair Dye Use and Risk of Leukemia and Lymphoma. Am J Public Health. 1988; 78(5): 570-1.

29. Cantor KP, Blair A, Everett G, Gibson R, Burmeister LF, Brown LM, Schuman L, Dick FR. Pesticides and other agricultural risk factors for non-Hodgkin lymphoma among men in Iowa and Minnesota. Cancer Res. 1992;52(9):2447-55.

30. Pottern LM, Linet M, Blair A, Dick F, Burmeister LF, Gibson R, Schuman LM, Fraumeni JF Jr. Familial cancers associated with subtypes of leukemia and non-Hodgkin lymphoma. Leuk Res. 1991;15(5):305-14.

31. Harris NL, Jaffe ES, Stein H, Banks PM, Chan JK, Cleary ML, Delsol G, De Wolf-Peeters C, Falini B, Gatter KC, et al. A revised European-American classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group. Blood. 1994 Sep 1;84(5):1361-92.

32. Vysis, Abbott Molecular Inc. (2007) Des Plaines, IL.

33. Jaffe ES, Harris NL, Stein H, Vardiman JW (editors). Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues: Lyon. IARCPress, 2001.

34. Chang et al. Non-Hodgkin lymphoma subtypes defined by common translocations: utility of fluorescence in situ hybridization (FISH) in a case-control study (unpublished).

35. Carl Zeiss (2007), Thornwood, NY.

36. Dako Denmark A/S (2007), Copehagen, Denmark.

37. SAS Institute Inc (2002-2003) SAS version 9.1, Cary, NC.

38. Schroeder JC, Weinberg CR. Use of missing-data methods to correct bias and improve precision in case-control studies in which cases are subtyped but subtype information is incomplete. Am J Epidemiol. 2001 Nov 15;154(10):954-62.

39. StataCorp LP (2007) Stata Statistical Software, 9.0 ed. College Station, TX.

40. Morton LM, Zheng T, Holford TR, Holly EA, Chiu BC, Costantini AS, Stagnaro E, Willett EV, Dal Maso L, Serraino D, Chang ET, Cozen W, Davis S, Severson RK, Bernstein L, Mayne ST, Dee FR, Cerhan JR, Hartge P; InterLymph Consortium. Alcohol consumption and risk of non-Hodgkin lymphoma: a pooled analysis. Lancet Oncol. 2005;6(7):469-76.

41. Wang SS, Slager SL, Brennan P, Holly EA, De Sanjose S, Bernstein L, Boffetta P, Cerhan JR, Maynadie M, Spinelli JJ, Chiu BC et al. Family history of hematopoietic malignancies and risk of non-Hodgkin lymphoma (NHL): a pooled analysis of 10 211 cases and 11 905 controls from the International Lymphoma Epidemiology Consortium (InterLymph). Blood. 2007 Apr 15;109(8):3479-88. Epub 2006 Dec 21.

42. Keller-Byrne JE, Khuder Sa, Schaub Ea, Mcafee O. A Meta-analysis of non-Hodgkin lymphoma among farmers in the central United States. Am J of Indus Medicine 1997; 31: 442-4.

43. Alexander DD, Mink PJ, Adami HO, Chang ET, Cole P, Mandel JS, Trichopoulos D. The non-Hodgkin lymphomas: A review of the epidemiologic literature. Int J Cancer. 2007;120 Suppl 12:1-39.

44. Cantor KP, Fraumeni JF Jr. Distribution of non-Hodgkin lymphoma in the United States between 1950 and 1975. Cancer Res. 1980 Aug;40(8 Pt 1):2645-52.

45. Hernan MA, Hernandez-Diaz S, Werler MM, Mitchell AA. Causal knowledge as a prerequisite for confounding evaluation: an application to birth defects epidemiology. Am J Epidemiol. 2002 Jan 15;155(2):176-84.

46. Campbell LJ. Cytogenetics of Lymphomas. Pathology 2005; 37(6): 493-407.

47. Roulland S, Lebailly P, Lecluse Y, Briand M, et al. Characterization of the t(14;18) *BCL2-IGH* translocation in farmers occupationally exposed to pesticides. Cancer Res 2004; 64:2264-9.

48. Bentham G, Wolfreys AM, Liu Y, Cortopassi G, Green MH, Arlett CF, Cole J. Frequencies of hprt(-) mutations and *bcl-2* translocations in circulating human lymphocytes are correlated with United Kingdom sunlight records. Mutagenesis. 1999;14(6):527-32.

49. Baccarelli A, Hirt C, Pesatori AC, Consonni D, Patterson DG Jr, Bertazzi PA, Dolken G, Landi MT. t(14;18) translocations in lymphocytes of healthy dioxin-exposed individuals from Seveso, Italy. Carcinogenesis. 2006 Oct;27(10):2001-7. Epub 2006 Mar 16.

50. Libra M, De Re V, De Vita S, Gasparotto D, Gloghini A, Rupolo M, Degan M, Marzotto A, Stivala F, Carbone A, Boiocchi M. Low frequency of *bcl-2* rearrangement in HCV-associated non-Hodgkin lymphoma tissue. Leukemia. 2003;17(7):1433-6.

51. Fuscoe JC, Setzer RW, Collard DD, Moore MM. Quantification of t(14;18) in the lymphocytes of healthy adult humans as a possible biomarker for environmental exposures to carcinogens. Carcinogenesis. 1996 May;17(5):1013-20.

52. Janz S, Potter M, Rabkin CS. Lymphoma- and leukemia-associated chromosomal translocations in healthy individuals. Genes Chromosomes Cancer. 2003 Mar;36(3):211-23.

53. Schmitt C, Balogh B, Grundt A, Buchholtz C, Leo A, Benner A, Hensel M, Ho AD, Leo E. The *bcl-2/IgH* rearrangement in a population of 204 healthy individuals: occurrence, age and gender distribution, breakpoints, and detection method validity. Leuk Res. 2006 Jun;30(6):745-50. Epub 2005 Nov 16.

CHAPTER 7

DISCUSSION

A. Study Aims and Findings

A.1. Utility of FISH

We extended a previous analysis (1) of the NCI's Factors Affecting Rural Men (FARM) study samples by using fluorescence in situ hybridization (FISH) assays to identify tumors with translocations involved with the immunoglobulin heavy chain gene (*IGH)* promoter on chromosome 14, including any *IGH* translocation, t(8;14) involving *IGH* and the *C-MYC* gene on chromosome 8, and translocations involving *BCL-6* on chromosome 3. In addition, we used FISH to identify t(14;18) involving *IGH* and *BCL2* on chromosome 18 in samples that were previously classified based on PCR and also evaluated bcl-2 protein expression in tumor samples based on immunohistochemistry.

Our first aim was to evaluate the utility of FISH for detecting common translocations in paraffin-embedded NHL tumor sections. To increase efficiency, we had considered using the *IGH* assay as an intitial screen such that translocation-specific assays would be run only on cases that were first determined to have a translocation involving *IGH*. However, we concluded that the *IGH* assay was not reliable enough to use in this fashion because it failed to detect five t(14;18)-positive cases detected in the FISH assays. In addition, the majority of *IGH*-positive cases were t(14;18)-positive (75 of 94 *IGH*-positive cases); consequently, associations between exposures and case-subtypes defined by any *IGH* translocation would be largely determined by t(14;18) status.

The number of positive cases detected by the *BCL6*- (n=5) and t(8;14)-FISH probes (n=1) in our case population was insufficient for meaningful epidemiologic analyses. Previous studies have shown that these translocations are less common compared to t(14;18) (2,3). None of the 10 Burkitt lymphoma cases run with t(8;14)-FISH in our study population were t(8;14)-positive, contrary to expectations that 75-100% would be (4-8); however, cases may have had *CMYC* translocations with partner genes other than *IGH* (e.g. t(8;22) or t(2;8)), which occur in 20% of BL/BLL cases (9).

Follicular lymphoma cases were more likely to be classified as t(14;18)-positive based on FISH (81%) than PCR (44%). This finding supports the higher sensitivity of FISH versus PCR to detect t(14;18) (10-14), given that 83-100% of follicular lymphoma cases are expected to be t(14;18)-positive (2,15-18). The success rate for classifying t(14;18) status was somewhat higher for PCR (73%) than FISH (67%); however, we believe that up to 28% of cases classified as t(14;18)-negative based on PCR may have been false-negative. One other study found a higher success rate with FISH (86%) than PCR (50%) (14), but this may reflect differences in assay protocols or characteristics of study samples. For example, archival samples assayed in our study were 23 to 26 years old.

A.2. Tobacco and t(14;18)-NHL

The second aim of our study was to determine whether risk factors varied among NHL molecular subtypes. We evaluated tobacco use as a risk factor across NHL case-subtypes defined by t(14;18) and bcl-2 expression. In previous studies, smoking was more strongly associated with follicular lymphomas, which are almost always t(14;18)-positive (19), than with NHL overall or other lymphoma subtypes (20-26). Additionally, studies have

found associations between smoking and the frequency of t(14;18)-positive cells in the bone marrow and peripheral blood of healthy individuals (27,28). These observations suggested that smoking may act directly or indirectly to increase the risk of t(14;18)-positive tumors. Therefore, the positive associations found with t(14;18)-negative NHL for any tobacco use (OR=1.98, 95% CI=1.09-3.59) and cigarette use only (OR=2.10, 95% CI=1.12-3.94), and the null associations with t(14;18)-positive NHL (any tobacco, OR=0.94, 95% CI=0.63-1.41; cigarette use only, OR=0.83, 95% CI=0.5-1.37) were unexpected. The associations with t(14;18)-negative NHL persisted when cigarette smoking was classified according to pack-years, intensity (cigarettes per day), and age at first use, without evidence of a positive dose-response or difference by age at intiation.

In the previous analysis of t(14;18)-NHL based on PCR, smoking was not clearly associated with either subtype (1); however, we believe that case-classification was improved in the current study as a consequence of the use of FISH to classify cases. The only other population-based case-control study of risk factors for t(14;18) subtypes that we are aware of reported a positive association between smoking and t(14;18)-negative NHL, but the association was only evident among women (OR=1.9, 95% CI=1.1-3.3, based on 64 cases), and there was no evidence of an association among men (29). Differences between the current study and the Nebraska study of t(14;18) NHL may reflect differences between the two study populations, random variation due to small samples sizes, bias due to sampling error or uncontrolled confounding, or differences in case-subtyping.

Overall, bcl-2 expression assays did not clarify the nature of the subtype-specific association between t(14;18)-negative NHL and cigarette smoking. We did not see evidence of associations between smoking and bcl-2 negative NHL, which suggests that the

associations observed with t(14;18)-negative NHL were not a function of bcl-2 negative status. However, the majority of t(14;18)-negative and t(14;18)-positive cases were positive for bcl-2 expression (87% and 58% respectively); consequently, associations with bcl-2-negative case-subtypes and joint t(14;18)-bcl-2-negative case-subtypes were difficult to interpret due to the small numbers within case-subtype strata.

B. Strengths and Limitations

Strengths of the study included use of a large population-based control group, the availability of detailed exposure data from both cases and controls, the use of FISH to determine t(14;18)-subtypes, and the availability of bcl-2 expression data in addition to information on t(14;18). However, we were unable to interpret effect estimates for case-subtypes defined by both bcl-2 and t(14;18) due to small numbers of cases within subtype groups.

The small number of cases hampered the precision and stability of estimates. Although 622 cases were enrolled in the study, tumor blocks were available for less than 40% of the cases. The number of successfully assayed cases was reduced to 25% of the original FARM study cases, and the majority of missing cases (56%) were from one of the two states included in the study. Use of the EM algorithm to impute missing case subtype data may have reduced bias improved precision (34), but small number of cases still hampered precision, and bias due to misclassification, uncontrolled confounding, and other sources of systematic error cannot be ruled out.

Unlike PCR, which requires that DNA be extracted from tumor samples, FISH can be performed and visualized directly on histologic sections. With the tissue architecture

preserved, the scorer has the ability to focus on appropriate regions of the tumor, therefore enhancing specificity for detecting translocations in neoplastic versus non-neoplastic lymphocytes. In addition, the fluorescent probes in FISH can hybridize to long regions of DNA; in contrast, PCR cannot detect widely dispersed breakpoints or breakpoints outside of the PCR primers used (30). Disadvantages of FISH include the difficulty of scoring cells that are overlapping or truncated, and the loss of signal strength on exposure to fluorescent light, and over time (though we observe signals lasting for several months). As a consequence, many samples were classified as "unreadable," which reduced the number of cases with known subtypes for the analysis. In addition, FISH probes were expensive, costing about $1000 for 20 assays, and scoring individual sections was labor intensive.

C. Future Directions

Our results support the use of FISH for detecting t(14;18) translocations in archival paraffin-embedded tumor blocks from case-control studies. We found that cigarette smoking was consistently associated with t(14;18)-negative NHL, however the reason for this association is unclear given that t(14;18)-negative pathways are likely to be etiologically heterogeneous, and given prior information suggesting that tobacco use may increase the prevalence of t(14;18) in bone marrow and peripheral lymphocytes (27,28) and thus increase the risk of t(14;18)-positive follicular lymphoma. Additional molecular characterization of t(14;18)-negative cases, which accounted for nearly 50% of the cases evaluated, might help clarify whether the association between tobacco use and t(14;18)-negative NHL may have been causal versus an artifact of chance or bias.

Future studies should consider incorporating strategies to minimize cost and labor including the use of tissue microarrays (TMAs) that include representative cores from multiple case biopsies (29,31) and automated FISH scoring (32,33). Alternatively, new studies may be able to take advantage of molecular analyses that are increasingly common as part of the routine pathologic evaluation of lymphoma cases. Specifically, many pathologists now attempt to perform a cytogenetic analysis of all tumor biopsies, with translocation-specific FISH assays used when cytogenetic analyses are unsuccessful. Incorporating these data would allow epidemiologic analyses of molecular subtypes without the expense and difficulty of obtaining archival tumor blocks and running and scoring FISH assays (35).

D. References

1. Schroeder JC, Olshan AF, Baric R, Dent GA, Weinberg CR, Yount B, Cerhan JR, Lynch CF, Schuman LM, Tolbert PE, Rothman N, Cantor KP, Blair A. A case-control study of tobacco use and other non-occupational risk factors for t(14;18) subtypes of non-Hodgkin lymphoma (United States). Cancer Causes Control. 2002 Mar;13(2):159-68.

2. Bernicot I, Douet-Guilbert N, Le Bris MJ, Morice P, Abgrall JF et al. Characterization of *IGH* rearrangements in non-Hodgkin B-cell lymphomas by fluorescence in situ hybridization. Anticancer Res 2005; 25(5): 3179-82.

3. Cook JR, Shekhter-Levin S, Swerdlow SH. Utility of routine classical cytogenetic studies in the evaluation of suspected lymphomas: results of 279 consecutive lymph node/extranodal tissue biopsies. Am J Clin Pathol. 2004 Jun;121(6):826-35.

4. Nishida K, Taniwaki M, Misawa S, Abe T. Nonrandom rearrangement of chromosome 14 at band q32.33 in human lymphoid malignancies with mature B-cell phenotype. Cancer Res. 1989 Mar 1;49(5):1275-81.

5. Mossafa H, Damotte D, Jenabian A, Delarue R, Vincenneau A, Amouroux I, Jeandel R, Khoury E, Martelli JM, Samson T, Tapia S, Flandrin G, Troussard X. Non-Hodgkin lymphomas with Burkitt-like cells are associated with c-Myc amplification and poor prognosis. Leuk Lymphoma. 2006 Sep;47(9):1885-93.

6. Pienkowska-Grela B, Witkowska A, Grygalewicz B, Rymkiewicz G, Rygier J, Woroniecka R, Walewski J. Frequent aberrations of chromosome 8 in aggressive B-cell non-Hodgkin lymphoma. Cancer Genet Cytogenet. 2005 Jan 15;156(2):114-21.

7. Braziel RM, Arber DA, Slovak ML, Gulley ML, Spier C, Kjeldsberg C, Unger J, Miller TP, Tubbs R, Leith C, Fisher RI, Grogan TM. The Burkitt-like lymphomas: a Southwest Oncology Group study delineating phenotypic, genotypic, and clinical features. Blood. 2001 Jun 15;97(12):3713-20.

8. Yano T, van Krieken JH, Magrath IT, Longo DL, Jaffe ES, Raffeld M. Histogenetic correlations between subcategories of small noncleaved cell lymphomas. Blood. 1992 Mar 1;79(5):1282-90.

9. Janz S. *MYC* translocations in B cell and plasma cell neoplasms. DNA Repair (Amst). 2006 Sep 8;5(9-10):1213-24. Epub 2006 Jul 11.

10. Barrans SL, Evans PA, O'Connor SJ, Owen RG, Morgan GJ, Jack AS. The detection of t(14;18) in archival lymph nodes: development of a fluorescence in situ hybridization (FISH)-based method and evaluation by comparison with polymerase chain reaction. J Mol Diagn. 2003 Aug;5(3):168-75.

11. Godon A, Moreau A, Talmant P, Baranger-Papot L, Genevieve F, Milpied N, Zandecki M, Avet-Loiseau H.. Is t(14;18)(q32;q21) a constant finding in follicular lymphoma? An interphase FISH study on 63 patients. Leukemia 2003; 17: 255-259.

12. Belaud-Rotureau MA, Parrens M, Carrere N, Turmo M, Ferrer J, de Mascarel A, Dubus P, Merlio JP. Interphase fluorescence in situ hybridization is more sensitive than BIOMED-2 polymerase chain reaction protocol in detecting *IGH-BCL2* rearrangement in both fixed and frozen lymph node with follicular lymphoma. Hum Pathol. 2007 Feb;38(2):365-72. Epub 2006 Nov 28.

13. Poetsch M, Weber-Matthiesen K, Plendl HJ, Grote W, Schlegelberger B. Detection of the t(14;18) chromosomal translocation by interphase cytogenetics with yeast-artificial-chromosome probes in follicular lymphoma and nonneoplastic lymphoproliferation. J Clin Oncol. 1996 Mar;14(3):963-9.

14. Vaandrager JW, Schuuring E, Raap T, Philippo K, Kleiverda K, Kluin P. Interphase FISH detection of BCL2 rearrangement in follicular lymphoma using breakpoint-flanking probes. Genes Chromosomes Cancer. 2000 Jan;27(1):85-94.

15. Richmond J, Bryant R, Trotman W, Beatty B, Lunde J. FISH detection of t(14;18) in follicular lymphoma on Papanicolaou-stained archival cytology slides. Cancer. 2006 Jun 25;108(3):198-204.

16. Gong Y, Caraway N, Gu J, Zaidi T, Fernandez R, Sun X, Huh YO, Katz RL. Evaluation of interphase fluorescence in situ hybridization for the t(14;18)(q32;q21) translocation in the diagnosis of follicular lymphoma on fine-needle aspirates: a comparison with flow cytometry immunophenotyping. Cancer. 2003 Dec 25;99(6):385-93.

17. Fan YS, Rizkalla K. Comprehensive cytogenetic analysis including multicolor spectral karyotyping and interphase fluorescence in situ hybridization in lymphoma diagnosis: a summary of 154 cases. Cancer Genetics and Cytogenetics 2001; 143: 73-79.

18. Einerson RR, Kurtin PJ, Dayharsh GA, Kimlinger TK, Remstein ED. FISH is superior to PCR in detecting t(14;18)(q32;q21)-IgH/bcl-2 in follicular lymphoma using paraffin-embedded tissue samples. Am J Clin Pathol. 2005 Sep;124(3):421-9.

19. Vega F, Medeiros LJ. Chromosomal translocations involved in non-Hodgkin lymphomas. Arch Pathol Lab Med. 2003 Sep; 127(9): 1148-60.

20. Morton LM, Hartge P, Holford TR, Holly EA, Chiu BC, Vineis P, Stagnaro E, Willett EV, Franceschi S, La Vecchia C, Hughes AM, Cozen W, Davis S, Severson RK, Bernstein L, Mayne ST, Dee FR, Cerhan JR, Zheng T. Cigarette smoking and risk of non-hodgkin lymphoma: a pooled analysis from the International Lymphoma Epidemiology Consortium (InterLymph). Cancer Epidemiol Biomarkers Prev. 2005; 14(4):925-33.

21. Parker AS, Cerhan JR, Dick F, Kemp J, Habermann TM, Wallace RB, Sellers TA, Folsom AR. Smoking snd risk of non-Hodgkin lymphoma subtypes in a cohort of older women. leuk lymphoma. 2000; 37(3-4): 341-9.

22. Herrinton LJ, Friedman GD. Cigarette smoking and risk of non-Hodgkin lymphoma subtypes. Cancer Epidemiol Biomarkers Prev. 1998;7(1):25-8.

23. Stagnaro E, Tumino R, Parodi S, Crosignani P, Fontana A, Masala G, Miligi L, Nanni O, Ramazzotti V, Rodella S, Senoiri Constantini A, Vigano C, Vindigni C, Vineis P. Non-Hodgkin lymphoma and type of tobacco smoke. Cancer Epidemiol Biomarkers Prev. 2004; 13(3): 431-7.

24. Stagnaro E, Ramazzotti V, Crosignani P, Fontana A, Masala G, Miligi L, Nanni O, Neri M, Rodella S, Costantini AS, Tumino R, Vigano C, Vindigni C, Vineis P. Smoking and hematolymphopoietic malignancies. Cancer Causes Control. 2001; 12(4): 325-34.

25. Morton LM, Holford TR, Leaderer B, Boyle P, Zahm SH, Zhang Y, Flynn S, Tallini G, Zhang B, Owens PH, Zheng T. Cigarette smoking and risk of non-Hodgkin lymphoma subtypes among women. Br J Cancer. 2003; 89(11): 2087-92.

26. Schollkopf C, Smedby KE, Hjalgrim H, Rostgaard K, Gadeberg O, Roos G, Porwit-Macdonald A, Glimelius B, Adami HO, Melbye M. Cigarette smoking and risk of non-Hodgkin lymphoma--a population-based case-control study. Cancer Epidemiol Biomarkers Prev. 2005 Jul;14(7):1791-6.

27. Bell DA, Liu Y, Cortopassi GA. Occurrence of *bcl-2* oncogene translocation with increased frequency in the peripheral blood of heavy smokers. JNCI 1995; 87(3): 223-4.

28. Rauzy O, Galoin S, Chale JJ, Adoue D, Albarede JL, Delsol G, al Saati T. Detection of t(14;18) carrying cells in bone marrow and peripheral blood from patients affected by non-lymphoid diseases. Mol Pathol. 1998 Dec;51(6):333-8.

29. Chiu BC, Dave BJ, Blair A, Gapstur SM, Chmiel JS, Fought AJ, Zahm SH, Weisenburger DD. Cigarette smoking, familial hematopoietic cancer, hair dye use, and risk of t(14;18)-defined subtypes of non-Hodgkin lymphoma. Am J Epidemiol. 2007 Mar 15;165(6):652-9. Epub 2007 Jan 4.

30. Medeiros LJ, Carr J. Overview of the role of molecular methods in the diagnosis of malignant lymphomas. Arch Pathol Lab Med. 1999 Dec;123(12):1189-207.

31. Iqbal J, Sanger WG, Horsman DE, Rosenwald A, et al. BCL2 translocation defines a unique tumor subset within the germinal center B-cell-like diffuse large B-cell lymphoma. Am J Pathol. 2004 Jul;165(1):159-66.

32. Reichard KK, Hall BK, Corn A, Foucar MK, Hozier J. Automated analysis of fluorescence in situ hybridization on fixed, paraffin-embedded whole tissue sections in B-cell lymphoma. Mod Pathol. 2006 Aug;19(8):1027-33. Epub 2006 May 5.

33. Bayani J, Squire JA. Application and interpretation of FISH in biomarker studies. Cancer Lett. 2007 Apr 28;249(1):97-109. Epub 2007 Feb 6.

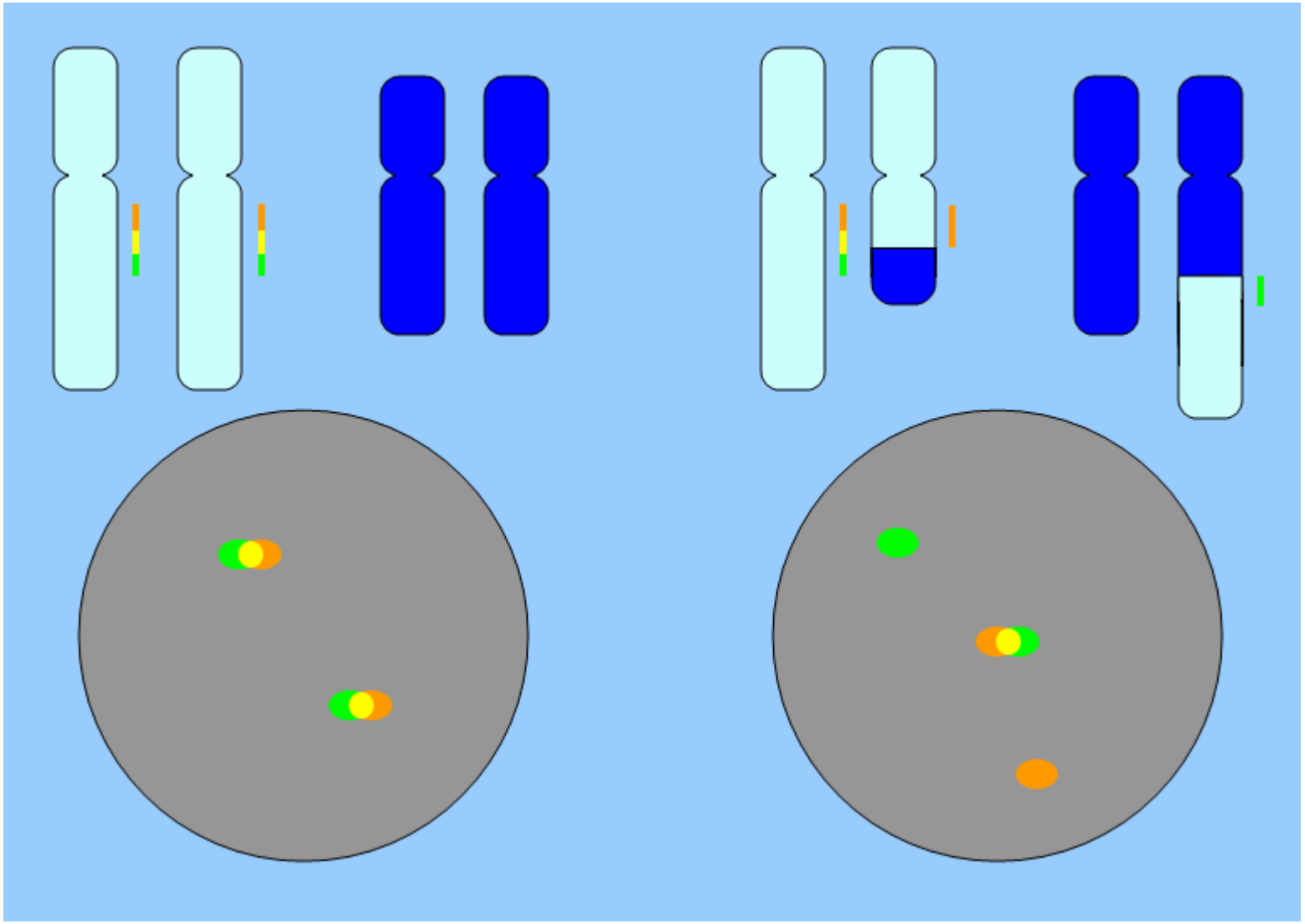
34. Schroeder JC, Weinberg CR. Use of missing-data methods to correct bias and improve precision in case-control studies in which cases are subtyped but subtype information is incomplete. Am J Epidemiol. 2001 Nov 15;154(10):954-62.

35. Campbell LJ. Cytogenetics of Lymphomas. Pathology 2005; 37(6): 493-407.

CHAPTER 8

APPENDIX

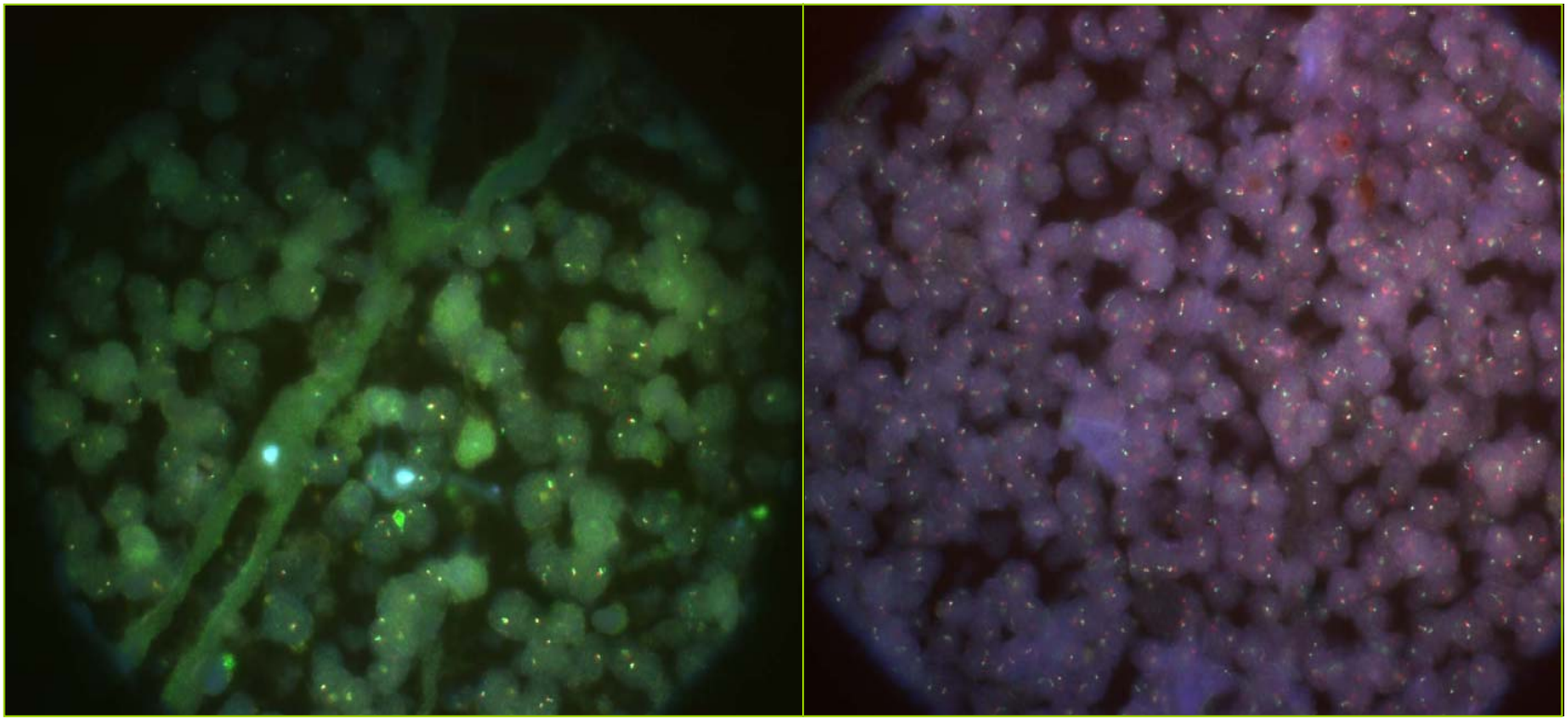
Appendix A. Expected FISH signal pattern of break-apart probe in negative and positive cells



Appendix B. Example of sections assayed with the *IGH* break-apart FISH probe

a) *IGH*-negative

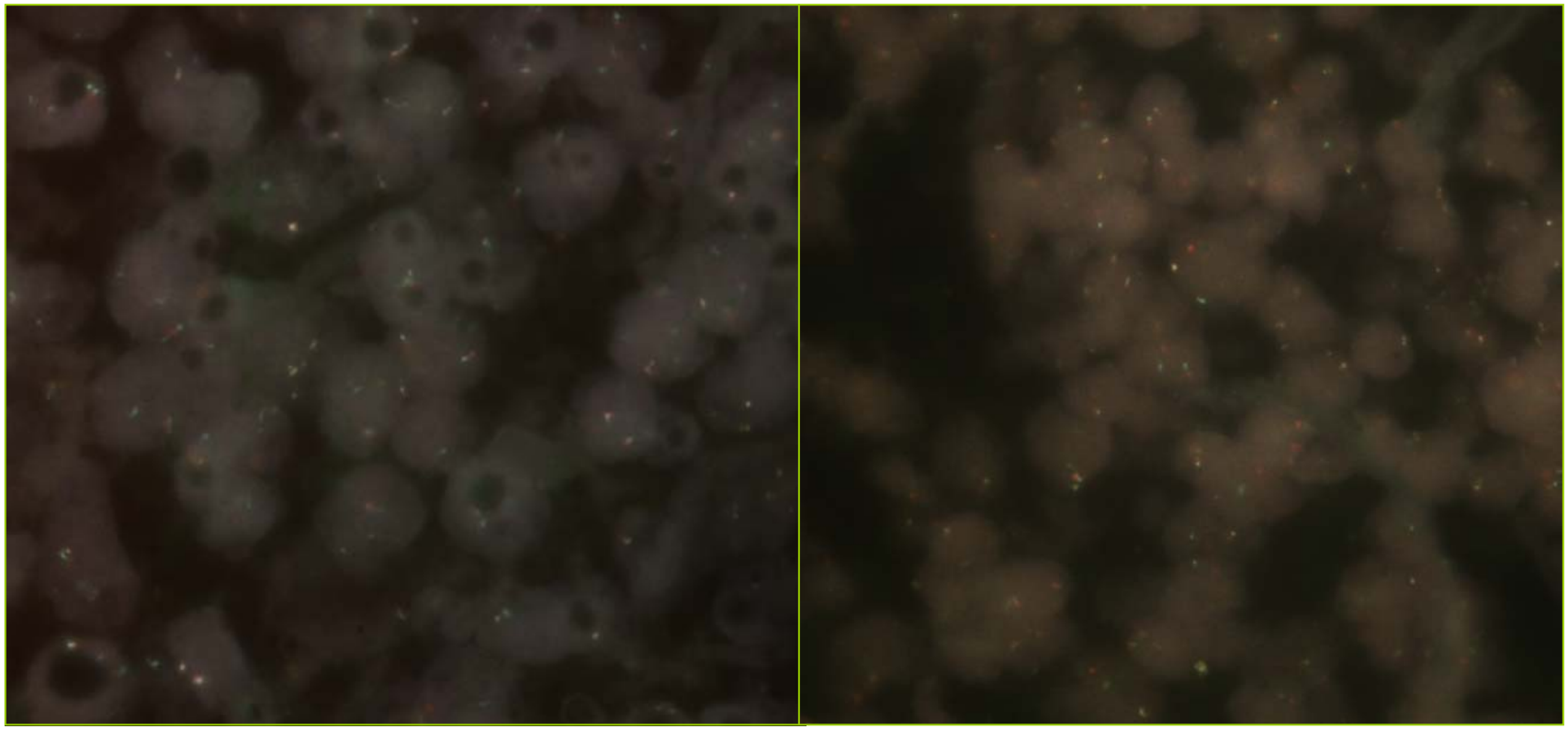
b) *IGH*-positive



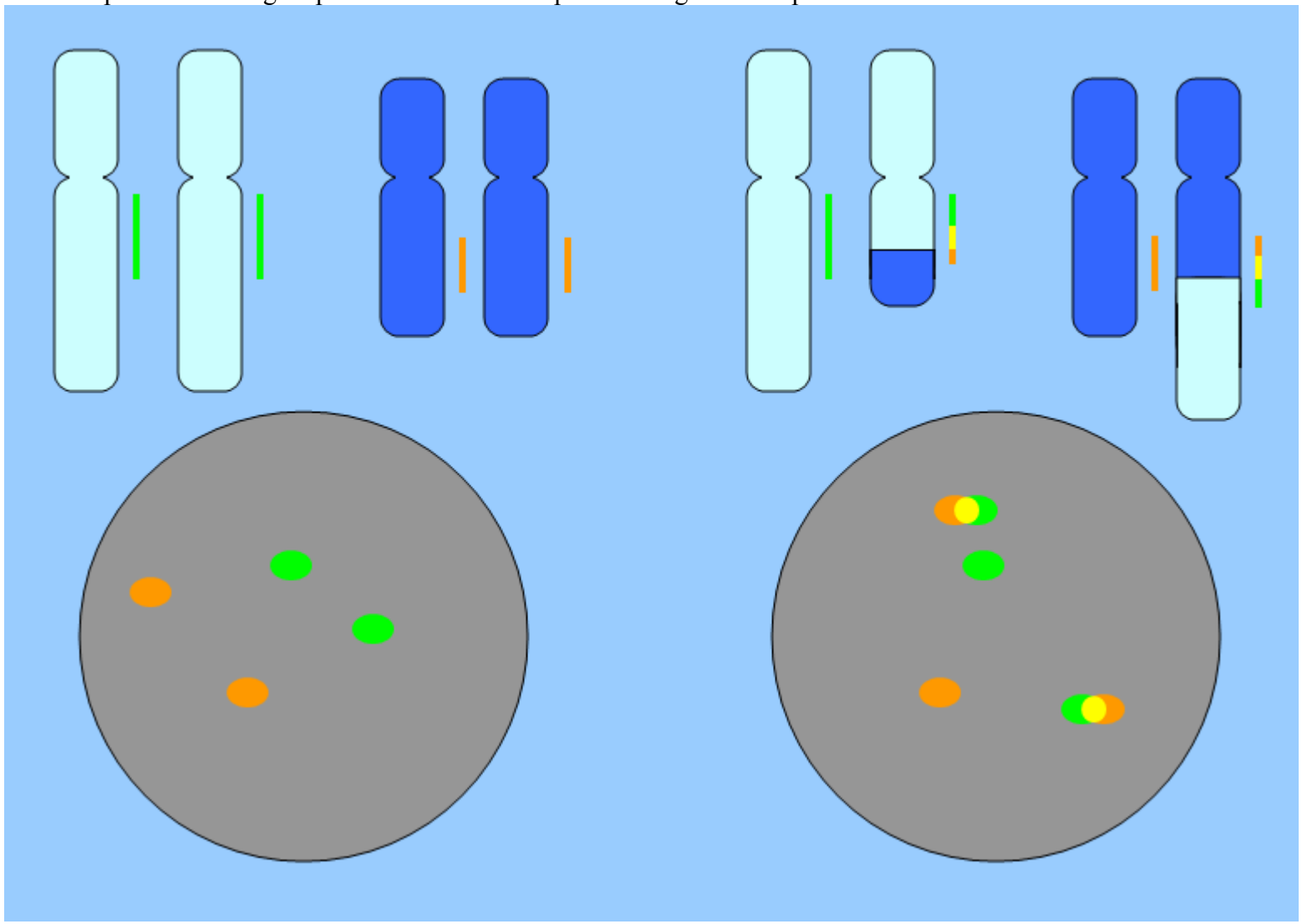
Appendix C. Example of sections assayed with the *BCL6* break-apart FISH probe

a) *BCL6*-negative

b) *BCL6*-positive



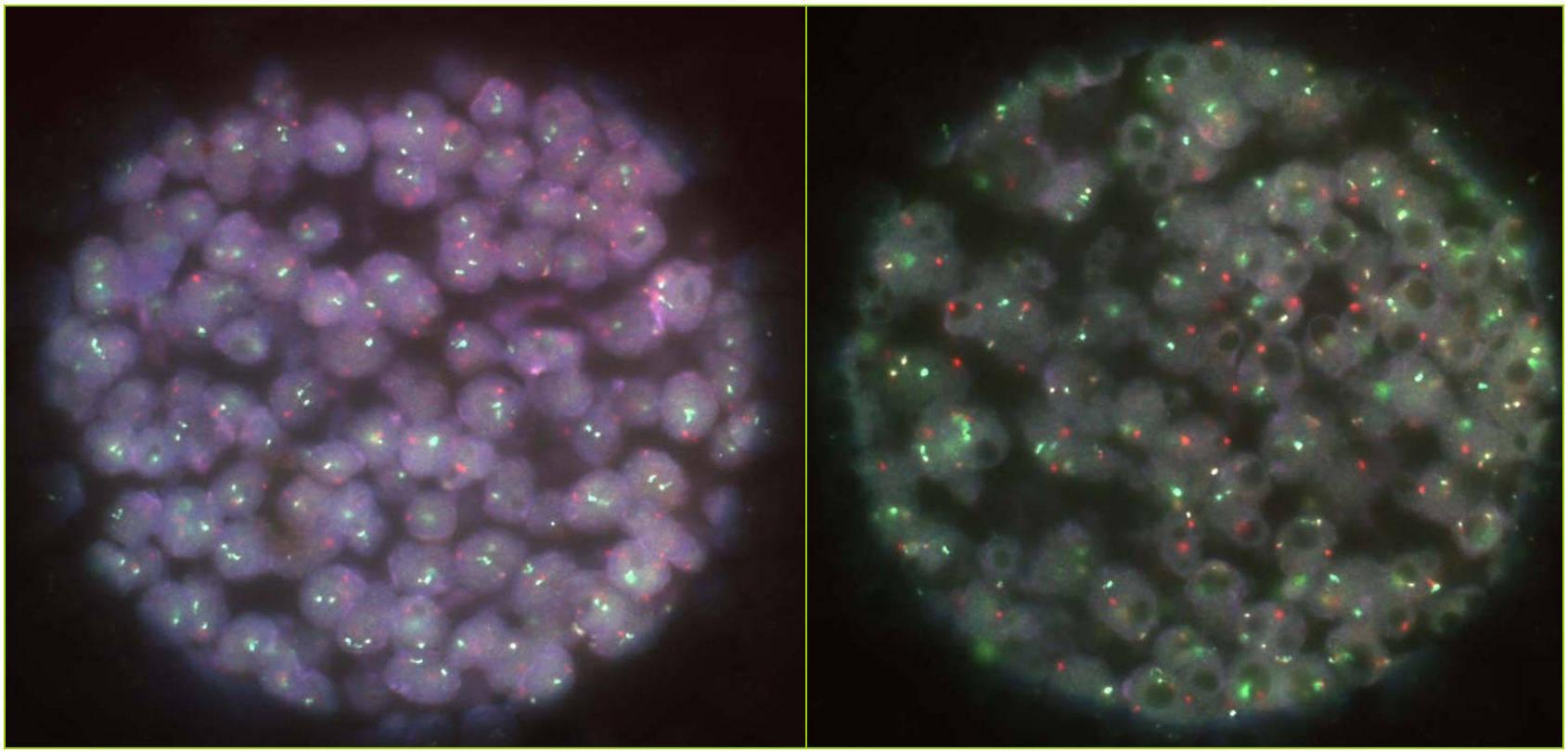
Appendix D. Expected FISH signal pattern of dual-fusion probe in negative and positive cells



Appendix E. Example of sections assayed with the t(14;18) dual-fusion FISH probe

a) t(14;18)-negative

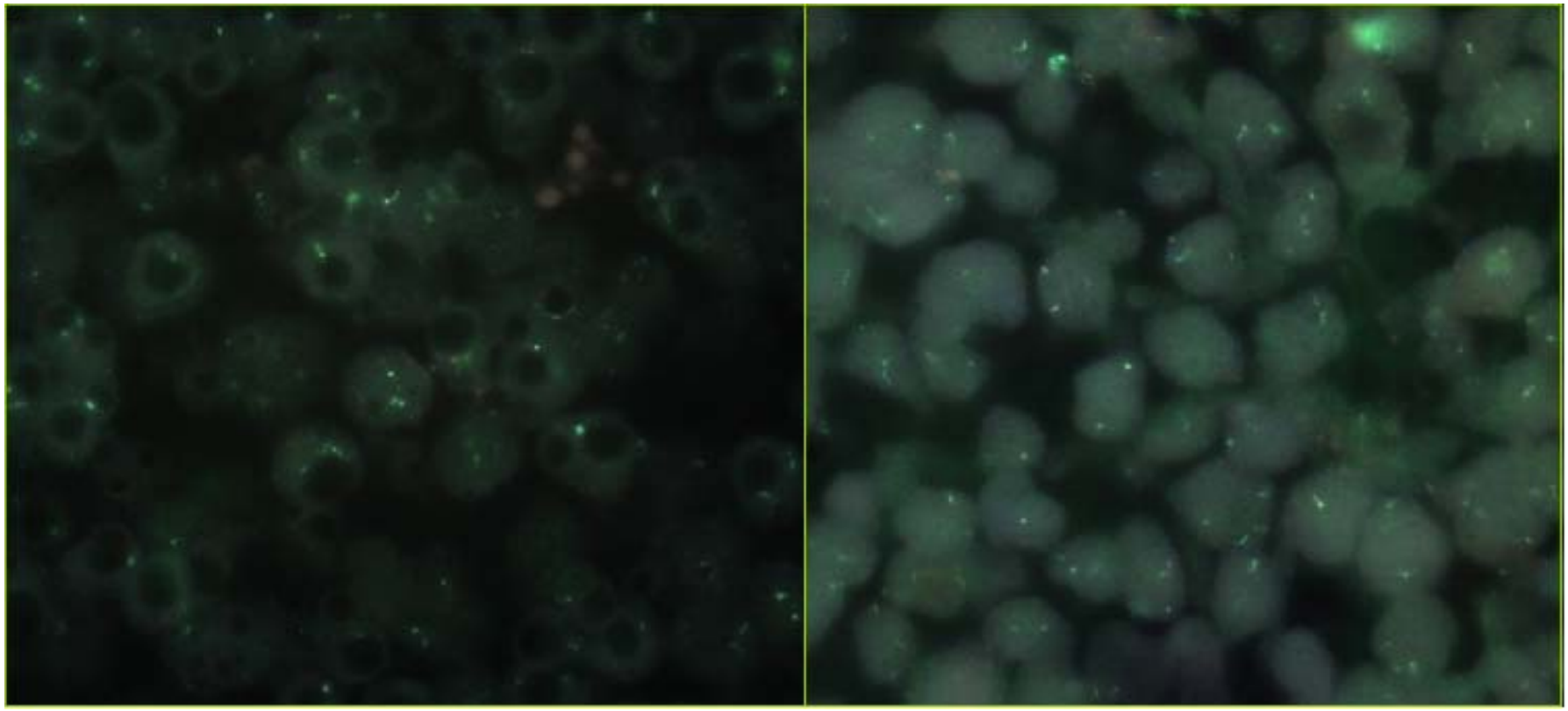
b) t(14;18)-positive



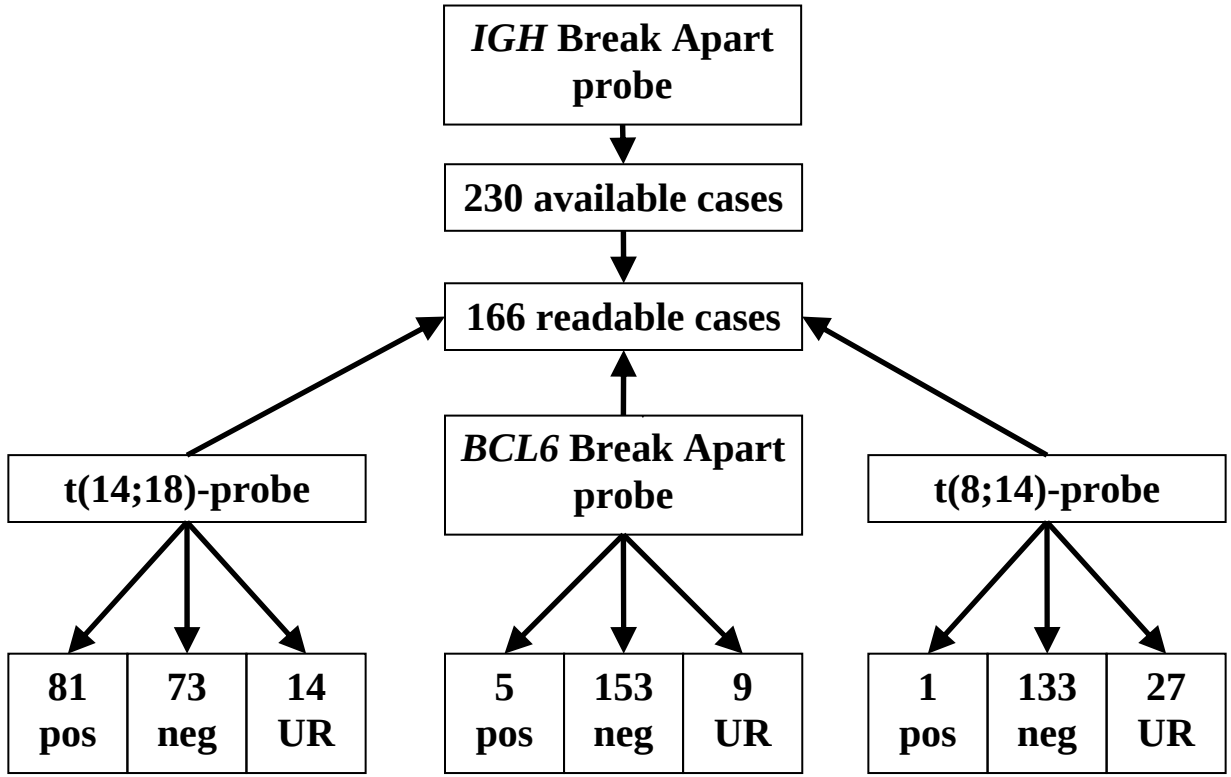
Appendix F. Example of sections assayed with the t(8;14) dual-fusion FISH probe

a) t(8;14)-negative

b) t(8;14)-positive



Appendix G. Strategy and frequency of translocations detected using FISH assays. Numbers of cases that were positive (pos), negative (neg) or unreadable (inadequate for scoring due to sample quality or signal strength, UR) for each FISH translocation assay. Sixty-four cases that were inadequate for the *IGH* assay were not subjected to any additional assays.



Appendix H. Adjusted* odds ratios for use of tobacco products among t(14;18)-positive/ bcl-2-positive, t(14;18)-negative/ bcl-2-positive, t(14;18)-positive/ bcl-2-negative, t(14;18)-negative/ bcl-2-negative NHL cases† compared with controls

Exposure	t(14;18)-positive/ bcl-2-positive (n=66)			t(14;18)-negative/ bcl-2-positive (n=40)			t(14;18)-positive/ bcl-2-negative (n=10)			t(14;18)-negative/ bcl-2-negative (n=29)		
	N	OR	95% CI	N	OR	95% CI	N	OR	95% CI	N	OR	95% CI
Used any tobacco	51	1.01	0.56- 1.84	35	2.07	0.80 -5.34	9	2.62	0.33 -20.94	26	3.92	0.92 -16.64
Tobacco by product												
Cigarettes	43	0.71	0.31-1.62	32	1.45	0.42 -5.03	6	0.16	0.04 -0.68	23	0.99	0.28 -3.55
Pipe	21	1.63	0.91 -2.93	10	0.91	0.43-1.94	2	0.69	0.14-3.42	9	1.23	0.53 -2.82
Cigar	5	0.52	0.20 -1.33	8	1.48	0.65 -3.34	2	1.61	0.32 - 7.98	5	1.17	0.43-3.19
Chew tobacco	12	2.33	1.16 -4.71	5	1.35	0.50 -3.65	1	0.94	0.11 -7.91	4	1.48	0.49 -4.50
Snuff	7	1.09	0.46 -2.55	2	0.38	0.09-1.64	1	1.24	0.14 -10.69	5	1.66	0.58 -4.74
No tobacco use	15			5			1			2		
Cigarettes only	20	0.79	0.40 -1.58	17	2.03	0.73 -5.61	5	2.73	0.31-23.77	10	3.00	0.65 -13.86
Cigarettes and other	23	1.16	0.59-2.27	15	2.29	0.82-6.38	1	0.77	0.05 -12.49	13	5.03	1.12 -22.56
Other tobacco only	8	1.49	0.60 -3.70	3	1.53	0.35 -6.67	3	11.36	1.12-115.72	3	4.09	0.66 -25.47
0-20 pack-yrs	12	0.86	0.41 -1.78	5	1.08	0.34 -3.40	4	1.60	0.39 -6.63	5	1.73	0.48-6.19
20-40 pack-yrs	11	0.85	0.40 -1.79	5	1.15	0.37-3.6	0			3	1.12	0.26 -4.82
40+ pack-yrs	17	0.81	0.42 -1.55	20	2.97	1.26 -6.99	2	0.46	0.08-2.57	13	3.21	1.10 - 9.34
Cigarettes/day												
>0-10	9	0.87	0.39-1.93	5	1.43	0.46 -4.45	1	0.55	0.06 -4.94	4	1.88	0.50 -7.11
>10-20	18	0.76	0.40 -1.45	8	1.59	0.63 4.00	3	0.64	0.14-2.93	11	2.40	0.81 -7.11
>20	14	0.86	0.43 -1.72	15	2.86	1.17 -7.04	2	0.63	0.11 -3.50	7	2.01	0.62 -6.55
Product/age started Cigarettes												
>18	17	0.85	0.44 -1.63	10	1.47	0.57-3.81	4	1.01	0.25-4.12	9	2.20	0.72 -6.69
≤18	26	0.85	0.47 -1.53	22	2.15	0.93-4.96	2	0.34	0.06 -1.86	14	2.15	0.76-6.11
Chewing tobacco												
>18	7	1.72	0.75 -3.95	3	1.14	0.34-3.83	0			3	1.85	0.53 -6.38
≤18	5	4.10	1.47 -11.43	2	2.53	0.56 -11.42	1	6.43	0.72 -57.49	1	2.08	0.26-16.43

Abbreviations: n, number of total cases; N, number of exposed cases; OR, odds ratio; CI, confidence interval

*Adjusted for state, age (restricted quadratic splines), and proxy status

†Case-subtypes based on fluorescence in situ hybridization (FISH) assays and immunohistochemistry (IHC) assays

Appendix I. Adjusted* odds ratios for selected exposures among t(14;18)-positive/ bcl-2-positive, t(14;18)-negative/ bcl-2-positive, t(14;18)-positive/ bcl-2-negative, t(14;18)-negative/ bcl-2-negative NHL cases† compared with controls

Exposure	t(14;18)-positive/ bcl-2-positive (n=66)			t(14;18)-negative/ bcl-2-positive (n=40)			t(1;418)-positive/ bcl-2-negative (n=10)			t(14;18)-negative/ bcl-2-negative (n=29)		
	N	OR	95% CI	N	OR	95% CI	N	OR	95% CI	N	OR	95% CI
Farmer	41	1.34	0.79-2.27	21	0.90	0.47-1.73	6	1.34	0.36-4.96	16	1.09	0.50-2.34
Any hair dye	8	2.55	1.15- 5.65	8	4.81	2.09 -11.07	0	n/a	n/a	4	3.12	1.04-9.41
Education ≤ 12 years	48	1.18	0.66-2.09	29	1.15	0.56-2.39	7	1.06	0.26-4.35	17	0.64	0.29-1.39
Drank alcohol weekly	28	0.56	0.34-0.94	21	0.85	0.44-1.62	6	1.16	0.32-4.22	17	1.12	0.51-2.46
HLP in first-degree relative	3	1.15	0.35 -3.81	2	1.19	0.28-5.10	0	n/a	n/a	4	3.67	1.22 -11.01
HLP in first or second-degree relative	4	0.92	0.32- 2.60	4	1.53	0.53 -4.42	0	n/a	n/a	6	3.61	1.42 -9.20

Abbreviations: n, number of total cases; N, number of exposed cases; OR, odds ratio; CI, confidence interval; HLP, hemolymphatic cancer

*Adjusted for state, age (restricted quadratic splines), and proxy status

†Case-subtypes based on fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC) assays

Appendix J. Adjusted* odds ratios for tobacco exposures among modified World Health Organization (WHO) subtypes compared with controls

Exposures	FL		DL		SLL		BL		Uncl	
	N	OR, 95% CI	N	OR, 95% CI	N	OR, 95% CI	N	OR, 95% CI	N	OR, 95% CI
No tobacco use	20	1.0	15	1.0	5	1.0	2	1.0	11	1.0
Used any tobacco	63	0.95 (0.56-1.61)	99	1.91 (1.09-3.36)	28	1.73 (0.66-4.55)	15	2.17 (0.48-9.79)	34	0.89 (0.44-1.78)
Tobacco by product										
Cigarettes	56	0.92 (0.40-2.13)	86	0.94 (0.50-1.78)	21	0.48 (0.19-1.24)	13	0.79 (0.16-3.80)	32	2.21 (0.50-9.72)
Pipe	18	0.95 (0.54-1.70)	30	1.0 (0.64-1.58)	7	0.63 (0.26-1.51)	6	2.04 (0.70-5.97)	11	1.12 (0.54-2.36)
Cigar	13	1.33 (0.70-2.53)	15	0.91 (0.51-1.63)	7	1.34 (0.55-3.28)	1	0.48 (0.06-3.75)	4	0.67 (0.23-1.95)
Chew tob	5	0.67 (0.26-1.74)	17	1.75 (0.98-3.12)	3	0.79 (0.23-2.76)	1	0.94 (0.12-7.62)	4	1.03 (0.35-3.07)
Snuff	5	0.65 (0.25-1.68)	14	0.99 (0.53-1.83)	2	0.39 (0.09-1.72)	4	2.37 (0.67-8.42)	3	0.68-0.20-2.36)
No tobacco use	20	1.0	15	1.0	5	1.0	2	1.0	11	1.0
Cigarettes only	32	0.95 (0.53-1.70)	49	1.91 (1.05-3.49)	13	1.83 (0.64-5.25)	6	1.57 (0.30-8.07)	20	1.04 (0.49-2.24)
Cigarettes and other	24	0.94 (0.51-1.74)	37	1.89 (1.01-3.51)	8	1.16 (0.37-3.6)	7	2.96 (0.59-14.8)	12	0.82 (0.35-1.89)
Other tobacco only	7	1.05 (0.43-2.60)	13	2.04 (0.93-4.46)	7	3.17 (0.96-10.5)	2	2.68 (0.35-20.5)	2	0.47 (0.1-2.16)
0-20 pack-yrs	17	0.98 (0.52-1.86)	24	1.58 (0.89-2.83)	8	1.31 (0.52-3.31)	2	1.05 (0.18-6.0)	5	0.64 (0.22-1.84)
20-40 pack-yrs	18	1.11 (0.59-2.08)	15	1.02 (0.53-1.97)	3	0.50 (0.14-1.83)	4	1.99 (0.47-8.39)	8	1.0 (0.42-2.55)
40+ pack-yrs	19	0.78 (0.42-1.44)	38	1.53 (0.91-2.58)	10	1.06 (0.44-2.55)	4	1.14 (0.27-4.79)	17	1.34 (0.63-2.84)
Cigarettes/day										
0	27	1.0	28	1.0	12	1.0	4	1.0	13	1.0
>0-10	10	0.87 (0.41-1.84)	14	1.16 (0.59-2.25)	7	1.36 (0.52-3.53)	5	2.74 (0.71-10.66)	4	0.68 (0.22-2.12)
>10-20	30	1.11 (0.64-1.91)	41	1.59 (0.95-2.65)	5	0.48 (0.16-1.39)	4	1.03 (0.25-4.30)	12	0.87 (0.39-1.96)
>20	14	0.66 (0.34-1.29)	29	1.57 (0.90-2.74)	9	1.31 (0.53-3.26)	3	1.01 (0.22-4.72)	14	1.42 (0.64-3.12)
Product/age started Cigarettes										
>18	20	0.91 (0.50-1.67)	36	1.48 (0.88-2.50)	12	1.33 (0.58-3.04)	5	1.45 (0.38-5.58)	11	0.97 (0.42-2.21)
≤18	36	0.96 (0.56-1.64)	50	1.39 (0.85-2.27)	9	0.65 (2.7-1.59)	8	1.48 (0.43-5.09)	21	1.14 (0.55-2.35)
Chewing tobacco										
>18	4	0.68 (0.24-1.94)	10	1.47 (0.73-2.96)	1	0.40 (0.05-3.03)	0	--	2	0.64 (0.15-2.73)
≤18	4	0.66 (0.09-4.98)	7	3.47 (1.44-8.33)	4	2.1 (0.46-9.6)	3	5.80 (0.68-50)	4	2.28 (0.51-10.3)

Percents are the proportion of cases in each exposure category out of the total number of WHO case-subtypes

Abbreviations: FL, follicular lymphoma; DL, diffuse large cell lymphoma; SLL,small lympocytic lymphoma; BL, Burkitt lymphoma; Uncl, unclassified; N, number of exposed cases; OR, odds ratio; CI, confidence interval

*Adjusted for state, age (restricted quadratic splines), and proxy status

Appendix K. Adjusted* odds ratios for selected exposures among modified World Health Organization (WHO) subtypes compared with controls

Exposures	FL		DL		SLL		BL		Uncl	
	N	OR, 95% CI	N	OR, 95% CI	N	OR, 95% CI	N	OR, 95% CI	N	OR, 95% CI
Farmer	40	0.85 (0.54-1.36)	68	1.21 (0.81-1.80)	21	1.20 (0.57-2.51)	7	0.69 (0.25-1.89)	24	0.96 (0.52-1.78)
Any hair dye	14	3.76 (1.97-7.17)	8	1.53 (0.71-3.31)	2	1.14 (0.27-4.93)	2	4.04 (0.84-19.4)	3	1.26 (0.37-4.21)
Education ≤ 12 years	52	0.85 (0.53-1.38)	81	0.99 (0.64-1.52	24	1.07 (0.49-2.37	13	1.65 (0.49-5.54)	32	1.06 (0.54-2.08)
Drank alcohol weekly	38	0.58 (0.37-0.92)	66	1.05 (0.71-1.57)	19	1.16 (0.57-2.39)	8	0.50 (0.19-1.35)	28	1.24 (0.66-2.32)
HLP in first-degree relative	4	1.18 (0.41-3.37)	7	1.47 (0.65-3.32)	4	3.18 (1.06-9.54)	3	5.25 (1.39-19.8)	4	2.14 (0.73-6.26)
HLP in first or second-degree relative	9	1.58 (0.75-3.3)	10	1.38 (0.69-2.76)	6	3.43 (1.34-8.76)	4	4.8 (1.48-15.7)	8	2.9 (1.31-6.58)

Percents are the proportion of cases in each exposure category out of the total number of WHO case-subtypes

Abbreviations: FL, follicular lymphoma; DL, diffuse large cell lymphoma; SLL,small lympocytic lymphoma; BL, Burkitt lymphoma; Uncl, unclassified; N, number of exposed cases; OR, odds ratio; CI, confidence interval; HLP, hemolymphatic cancer

*Adjusted for state, age (restricted quadratic splines), and proxy status

Appendix L. Adjusted* odds ratios for intensity of tobacco product use among t(14;18)-positive or –negative NHL cases† compared with controls

Exposures	Controls	t(14;18)-positive vs. controls‡			t(14;18)-negative vs. controls‡		
	N	N	OR	95% CI	N	OR	95% CI
Average amount used/day							
Bowls of pipe/day							
0	951	55	1.00		52	1.00	
>0-3	75	9	1.62	0.86 - 3.04	8	1.63	0.89 - 3.00
>3	182	16	1.18	0.74 - 1.88	10	0.80	0.44 - 1.44
Cigars/day							
0	1077	74	1.00		58	1.00	
1 to 2	104	5	0.74	0.33 - 1.66	9	1.60	0.9 - 2.83
3 to 4	56	2	0.64	0.19 - 2.18	5	1.86	0.93 - 3.72
Oz. chewing tobacco/day							
0	791	57	1.00		49	1.00	
1	374	22	0.89	0.42 - 1.88	15	0.93	0.42 - 2.04
2+	80	2	1.48	0.39 - 5.68	8	2.46	0.92 - 6.59
Pinches of snuff/day							
0	1103	72	1.00		64	1.00	
>0-6	67	4	0.84	0.35 - 2.04	3	0.67	0.26 - 1.77
>6	55	4	1.22	0.52 - 2.87	4	1.10	0.47 - 2.59

Abbreviations: N, number of exposed cases or controls; OR, odds ratio; CI, confidence interval
*Adjusted for state, age (restricted quadratic splines), and proxy status
†Case-subtypes based on fluorescence in situ hybridization (FISH) assays
‡Estimates from expectation-maximization (EM) polytomous regression models including controls and both case-subtypes

Appendix M. Adjusted* odds ratios for duration and age at first tobacco product use among t(14;18)-positive or –negative NHL cases† compared with controls

Exposures	Controls	t(14;18)-positive vs. controls‡			t(14;18)-negative vs. controls‡		
	N	N	OR	95% CI	N	OR	95% CI
Cigarette-years							
0	132	13	1.00		7	1.00	
>0 to 25	238	19	0.85	0.42 - 1.75	11	1.01	0.35 - 2.91
>25 to 45	331	16	0.44	0.23 - 0.85	25	1.48	0.69 - 3.19
>45	211	13	0.53	.27 - 1.04	16	1.46	0.65 - 3.26
Pipe-years							
0	665	38	1.00		43	1.00	
>0-10	117	11	1.18	0.66 - 2.1	10	0.86	0.47 - 1.58
>10	139	14	1.61	0.92 - 2.8	9	0.91	0.48 - 1.73
Cigar-years							
0	791	57	1.00		49	1.00	
>0-25	97	4	0.52	0.21 - 1.28	10	1.28	0.73 - 2.24
>25	47	3	1.04	0.29 - 3.7	4	2.18	0.88 - 5.39
Chewing tobacco-years							
0	841	51	1.00		54	1.00	
>0-10	39	4	1.79	0.78 - 4.12	2	0.95	0.26 - 3.49
>10	64	4	0.77	0.3 - 1.95	6	1.13	0.55 - 2.34
Snuff-years							
0	817	55	1.00		54	1.00	
>0-20	46	2	0.82	0.28 - 2.41	2	0.79	0.28 - 2.23
>20	80	6	1.33	0.64 - 2.78	5	0.75	0.32 - 1.78
Product/age started							
Pipe							
>18	228	21	1.31	0.86 - 2	12	0.86	0.5 - 1.47
≤ 18	65	5	0.90	0.38 - 2.13	8	1.81	0.98 - 3.34
Cigar							
>18	131	6	0.68	0.33 - 1.4	10	1.23	0.7 - 2.16
≤ 18	22	1	0.59	0.08 - 4.3	4	3.94	1.78 - 8.71
Snuff							
>18	95	6	1.36	0.74 - 2.52	2	0.51	0.15 - 1.76
≤ 18	46	3	0.74	0.24 - 2.29	6	1.46	0.68 - 3.12

Abbreviations: N, number of exposed cases or controls; OR, odds ratio; CI, confidence interval

*Adjusted for state, age (restricted quadratic splines), and proxy status

†Case-subtypes based on fluorescence in situ hybridization (FISH) assays

‡Estimates from expectation-maximization (EM) polytomous regression models including controls and both case-subtypes