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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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Epidemiology.

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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 \textit{IGH}), t(14;18), t(8;14) and \textit{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. \textit{BCL6} translocations, t(8;14), and other \textit{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
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
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<tr>
<td>OR</td>
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<tr>
<td>PCNSL</td>
<td>primary central nervous system lymphoma</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pro-B cells</td>
<td>progenitor B-cells</td>
</tr>
<tr>
<td>sBL</td>
<td>sporadic Burkitt lymphoma</td>
</tr>
<tr>
<td>SEER</td>
<td>Surveillance, Epidemiology, and End Results</td>
</tr>
<tr>
<td>SLL</td>
<td>small lymphocytic lymphoma</td>
</tr>
<tr>
<td>REAL</td>
<td>Revised European-American Lymphoma</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>TB</td>
<td>tuberculosis</td>
</tr>
<tr>
<td>TMA</td>
<td>tissue microarrays</td>
</tr>
<tr>
<td>US</td>
<td>United States</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>WF</td>
<td>Working Formulation</td>
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<td>WHO</td>
<td>World Health Organization</td>
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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 \textit{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 \textit{IGH} translocations, t(14;18), t(8;14), \textit{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)
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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).

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-nonamer 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 antiapoptotic 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 1). 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 hematopoietic 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)
Hepatitis 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, organophosphate 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 case-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. \textit{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)

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>B-chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL)</td>
<td>B-cell chronic lymphocytic leukaemia</td>
<td>Small lymphocytic, consistent with CLL</td>
</tr>
<tr>
<td>Mantle cell lymphoma</td>
<td>Mantle cell lymphoma</td>
<td>Malignant lymphoma, diffuse, small cleaved cell type</td>
</tr>
<tr>
<td>Follicular lymphoma</td>
<td>Follicle centre lymphoma, follicular</td>
<td>Follicular small cleaved, mixed, large, or small non-cleaved cell</td>
</tr>
<tr>
<td>Extranodal marginal zone B-cell lymphoma of MALT type</td>
<td>Marginal zone B-cell lymphoma</td>
<td>Small lymphocytic, lymphoplasmacytoid, diffuse small cleaved cell</td>
</tr>
<tr>
<td>Nodal marginal zone B-cell lymphoma</td>
<td>Marginal zone B-cell lymphoma</td>
<td>Small lymphocytic, plasmacytoid, follicular or diffuse small cleaved cell, follicular or diffuse mixed small and large cell</td>
</tr>
<tr>
<td>Splenic marginal zone B-cell lymphoma</td>
<td>Splenic marginal zone B-cell lymphoma</td>
<td>Small lymphocytic lymphoma</td>
</tr>
<tr>
<td>Diffuse large B-cell lymphoma</td>
<td>Diffuse large B-cell lymphoma</td>
<td>Diffuse large cell, large cell immunoblastic, diffuse mixed small and large cell</td>
</tr>
<tr>
<td>Burkitt/Burkitt-like lymphoma</td>
<td>Burkitt lymphoma; high-grade B-cell lymphoma, Burkitt-like (provisional entity)</td>
<td>Small non-cleaved cell, Burkitt type</td>
</tr>
</tbody>
</table>
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)
Table 2.2. NHL translocations and associated World Health Organization (WHO) subtypes*

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

*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)

<table>
<thead>
<tr>
<th>Race/Ethnicity</th>
<th>Men (cases per 100,000)</th>
<th>Women (cases per 100,000)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Races</td>
<td>23.2</td>
<td>15.8</td>
</tr>
<tr>
<td>White</td>
<td>24.4</td>
<td>16.7</td>
</tr>
<tr>
<td>Black</td>
<td>17.6</td>
<td>11.6</td>
</tr>
<tr>
<td>Asian/Pacific Islander</td>
<td>16.8</td>
<td>11.5</td>
</tr>
<tr>
<td>American Indian/Alaska Native</td>
<td>10.2</td>
<td>7.5</td>
</tr>
<tr>
<td>Hispanic</td>
<td>18.8</td>
<td>13.4</td>
</tr>
<tr>
<td>Study</td>
<td>Design</td>
<td>Population</td>
</tr>
<tr>
<td>---------------</td>
<td>---------------------------------------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>Chiu 2007</td>
<td>Population-based case-control</td>
<td>Nebraska men and women, cases=172,</td>
</tr>
<tr>
<td>Besson 2006</td>
<td>Population based case-control (7 European</td>
<td>Epilymph: Czech Rep, Finland, France,</td>
</tr>
<tr>
<td>Morton 2005</td>
<td>Pooled analysis of 8 pop based and 1 hospital based case-control 1998-2004</td>
<td>InterLymph, cases=6,594, controls=8,892</td>
</tr>
<tr>
<td>Bracci 2005</td>
<td>Bay area population-based case-control 1988-1995</td>
<td>Cases=1,593; Controls=2,515, HIV-negative participants</td>
</tr>
<tr>
<td>Stagnaro 2004</td>
<td>Population-based case-control (11 Italian areas)</td>
<td>Italian, 1450 cases, 1779 controls</td>
</tr>
</tbody>
</table>
Table 2.4. Selected studies on smoking and NHL (continued)

<table>
<thead>
<tr>
<th>Study</th>
<th>Design</th>
<th>Population</th>
<th>Exposure categories</th>
<th>Estimate</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Willet 2004</td>
<td>population-based case-</td>
<td>England, age 18-64, 700 cases</td>
<td>ever smoking cigarettes, cigars, or pipe once/day for 6 months, age or year started,</td>
<td>no association between smoking and NHL or subtypes; no assoc between age and NHL or subtypes; no dose-response observed</td>
<td></td>
</tr>
<tr>
<td></td>
<td>control</td>
<td></td>
<td>amt/day, age or year stopped</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morton 2003</td>
<td>population-based</td>
<td>Conn. Women; 601 cases and 718 controls</td>
<td>smoker=at least 100 cigarettes; age started, ave # cigarettes/day, years smoked at least 1 cig/day, age stopped</td>
<td>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</td>
<td>increased risk only seen for follicular</td>
</tr>
<tr>
<td>Besson 2003</td>
<td>hospital case-control</td>
<td>France; 180 cases, 2 groups of 180 hospital-based controls</td>
<td>smoking status, smoking duration, cigs/day, age smoking initiated and termin</td>
<td>for NHL, current smoker (women) OR=2.40, 1.19-4.84; smoking for &gt;31 y, OR=5.04, 1.40-18.12; for FL, ORs elevated but CI wide</td>
<td>differences between genders may be biologic diff; no effect on other subtypes except for FL</td>
</tr>
<tr>
<td>Schroeder 2002</td>
<td>population-based case-</td>
<td>Iowa and Minnesota men, cases=182, controls=1245</td>
<td>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</td>
<td>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</td>
<td>Estimates were adjusted for age and state. EM algorithm was applied to reduce imprecision and bias.</td>
</tr>
<tr>
<td>Stagnaro 2001</td>
<td>Italian pop-based case-</td>
<td>Italian, 3357 cases, 2391 controls</td>
<td>former smokers stopped at 1 year b/c study, cigarettes/day and years--3 levels</td>
<td>current smk, OR=1.2, 1.0-1.5 (NHL); current for FL, OR=2, 1.3-3.0; &gt;36 years OR=2.3, 1.4-3.8</td>
<td>smoking an RF for NHL with a consistent assoc only for FL in women</td>
</tr>
<tr>
<td>Parker 2000</td>
<td>cohort study</td>
<td>Iowa women, 37,336 women, 200 cases (random sample of Iowa women)</td>
<td>current, former, never; pack-years</td>
<td>former smk and FL OR=1.6, 0.7-3.4; current sk and FL OR=2.3, 1.0-5.0</td>
<td>no assoc between smoking history and NHL overall; or for low/intermed--positive for high grade but small cases; suggestive association for FL</td>
</tr>
</tbody>
</table>
Table 2.4. Selected studies on smoking and NHL (continued)

<table>
<thead>
<tr>
<th>Study</th>
<th>Design</th>
<th>Population</th>
<th>Exposure categories</th>
<th>Estimate</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freedman 1998</td>
<td>pop-based case-control</td>
<td>Vietnam vets, 1190 cases, 2299 controls</td>
<td>packs per day, years smoking, age at start, years since quitting, pack-years; current smoking---men who stopped within 1 year of study</td>
<td>sig linear trends for packs/day and pack-years for NHL and &gt;50 p-y OR=1.41, 1.1-1.8; low grade and &gt;50 pack-years OR=1.65 (1.2-2.5); high-grade OR=1.73, 1.1-2.8</td>
<td>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</td>
</tr>
<tr>
<td>Herrinton 1998</td>
<td>prospective, 11 year follow-up</td>
<td>Kaiser Perman members, 674 cases of incident NHL</td>
<td>current, past, years of regular cig smk, ave pack/day</td>
<td>smokers at increased risk of FL, former smk RR=1.9, 1.2-2.9, current smkrs RR=1.4, 0.9-2.2</td>
<td>smoking history only one point in time, no info on HIV</td>
</tr>
<tr>
<td>Zahm 1997 (135)</td>
<td>pooled analysis of 3 pop-based case-control</td>
<td>Nebraska, Kansas, IA, MN--1200 cases</td>
<td>age began smoking, cigarettes/day, age stopped, duration/intensity of smk, pack-years</td>
<td>no association with men, but smoking and NHL associated in women</td>
<td>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</td>
</tr>
<tr>
<td>Nelson 1997 (136)</td>
<td>population-based case-control</td>
<td>LA county, 378 cases</td>
<td>never, ever, former, current, cigarettes/day</td>
<td>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)</td>
<td>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</td>
</tr>
<tr>
<td>Brown 1992 (137)</td>
<td>population-based case-control</td>
<td>white men over 30 from Iowa health registry and MN</td>
<td>tobacco use, first and last year of use, age at first use, amt/day</td>
<td>risk of lymphoma, pipes or cigars, smokeless 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)</td>
<td>history of alcohol and tobacco use not associated with NHL in men; cocaine use sig in stepwise &amp; drug use in last 5 &amp; 10 y; increase in NHL coincides w/ epidemic of recreational drug use in US</td>
</tr>
<tr>
<td>Linet 1992 (138)</td>
<td>cohort study</td>
<td>white males of Lutheran Brotherhood Insurance Soc.; 286,731 PY; 1967-86; 49 NHL deaths</td>
<td>tobacco use, cig/day</td>
<td>used any tobacco risk of NHL elevated, RR=1.9 (0.8-4.5); &gt;1 pack/day RR=3.8 (1.4-10.1)</td>
<td>1st cohort study to show dose-response relationship--smoking only measured once in 1966, so risk may be underestimated</td>
</tr>
</tbody>
</table>
F. References


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 \textit{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


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 Jh 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 \textit{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 \textit{IGH}, t(14;18), \textit{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 described in Chapter 5.

B.3. Data Analysis

B.3.1. Univariate 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

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Controls</th>
<th>FISH t(14;18)-positive (n=81)</th>
<th>FISH t(14;18)-negative (n=73)</th>
<th>FISH t(14;18)-positive (n=68)</th>
<th>FISH t(14;18)-negative (n=114)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
<td>%</td>
<td>N</td>
</tr>
<tr>
<td>Farmer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>698</td>
<td>56.1</td>
<td>52</td>
<td>64.2</td>
<td>40</td>
</tr>
<tr>
<td>No</td>
<td>547</td>
<td>43.9</td>
<td>29</td>
<td>35.8</td>
<td>33</td>
</tr>
<tr>
<td>State</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Iowa</td>
<td>603</td>
<td>48.4</td>
<td>51</td>
<td>63.0</td>
<td>39</td>
</tr>
<tr>
<td>Minnesota</td>
<td>642</td>
<td>51.6</td>
<td>30</td>
<td>37.0</td>
<td>34</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
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<td></td>
</tr>
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<td>30-55</td>
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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

[Diagram showing the relationship between various factors and NHL case-subtypes]
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

\[ U_L = \text{Unmeasured shared lifestyle/environment/genetic factors} \]
\[ U_E = \text{Unmeasured environmental factors} \]
Figure 4.4. Conceptual model for the relation of hair dye use and covariates with NHL case-subtypes

$U_L = \text{Unmeasured lifestyle/religion}$

$U_E = \text{Unmeasured environmental factors}$
C. References


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 \textit{IGH}, t(14;18), t(8;14), and \textit{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. \textit{BCL6} translocations, t(8;14) and other \textit{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 \textit{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 \textit{IGH} promoter on chromosome 14 with an oncogene whose subsequent overexpression contributes to lymphomagenesis (1, 3-4). t(14;18) translocations involving \textit{IGH} and \textit{BCL2} at 18q21 cause overexpression of the anti-apoptotic bcl-2 protein, while t(8;14) translocations disrupt the normal regulation of the \textit{CMYC} transcription factor located at 8q24. Translocations involving \textit{BCL6} at 3q27 and \textit{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 \textit{IGH} translocations, t(8;14) translocations and \textit{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’-GAGAGTTGCTTTACGTGCTG-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.

*IGH* 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.

F. Acknowledgements

This study was supported in part by the UNC Lineberger Comprehensive Cancer Center (Population Sciences Research Award), the National Institute of Environmental Health Sciences (P30ES10126), the National Institutes of Health National Cancer Institute (R03 CA71617-01), and the Occupational Studies Section, National Cancer Institute. Special thanks to David W. Cowan from the Lineberger Comprehensive Cancer Center
Immunohistochemistry/Hybridization Core Laboratory for guidance with FISH assays; Stephen Oglesbee, Georgette Dent, and Boyd Yount from UNC-Chapel Hill; Wen-Yi Huang from NCI; and FARM study collaborators: Ken Cantor, Nat Rothman, Charles Lynch, Jim Cerhan, and Paige Tolbert.
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).

![Diagram of 14q32 region](image)

*IGH*, Break Apart

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).

![Diagram of 3q27 region](image)

*BCL6*, Break Apart
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 Jh 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 \textit{IGH} Dual Color, Break Apart probes for any \textit{IGH} translocation showing the expected signal pattern for a translocation-negative cell (2 fusion signals). b) Vysis LSI \textit{IGH} Dual Color, Break Apart probes for any \textit{IGH} translocation showing the expected signal pattern for a \textit{IGH}-positive cell (1 orange, 1 green, and 1 fusion). c) Vysis LSI \textit{IGH/BCL2} Dual Color, Dual Fusion probes for \textit{t(14;18)} showing the expected signal pattern for a translocation-negative cell (2 orange, 2 green). d) Vysis LSI \textit{IGH/BCL2} Dual Color, Dual Fusion probes for \textit{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

<table>
<thead>
<tr>
<th>Histologic Subtype</th>
<th>Cases Assayed*</th>
<th>Any translocation†</th>
<th>t(14;18)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td>Follicular</td>
<td>45</td>
<td>38</td>
<td>84</td>
</tr>
<tr>
<td>Diffuse Large Cell</td>
<td>73</td>
<td>37</td>
<td>51</td>
</tr>
<tr>
<td>Small lymphocytic</td>
<td>16</td>
<td>8</td>
<td>50</td>
</tr>
<tr>
<td>Burkitt/Burkitt-like</td>
<td>13</td>
<td>8</td>
<td>62</td>
</tr>
<tr>
<td>Mantel Cell</td>
<td>4</td>
<td>3</td>
<td>75</td>
</tr>
<tr>
<td>Unclassified</td>
<td>10</td>
<td>7</td>
<td>70</td>
</tr>
</tbody>
</table>

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 \textit{IGH}, \textit{t(14;18)}, \textit{BCL6}, or \textit{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

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

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

<table>
<thead>
<tr>
<th>t(14;18)-FISH</th>
<th>t(14;18)-PCR</th>
<th></th>
<th></th>
<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
<td>Unreadable‡</td>
<td>Not Assayed</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>39</td>
<td>32</td>
<td>10</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>8</td>
<td>53</td>
<td>10</td>
<td>2</td>
<td>73</td>
</tr>
<tr>
<td>Unreadable†</td>
<td>14</td>
<td>25</td>
<td>37</td>
<td>0</td>
<td>76</td>
</tr>
<tr>
<td>Not Assayed</td>
<td>7</td>
<td>4</td>
<td>9</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>68</strong></td>
<td><strong>114</strong></td>
<td><strong>66</strong></td>
<td><strong>2</strong></td>
<td><strong>250</strong></td>
</tr>
</tbody>
</table>

t(14;18) status was determined using Vysis LSI IGH/BCL2 Dual Color, Dual Fusion FISH assays and PCR assays with the J5 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

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

*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

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

*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

<table>
<thead>
<tr>
<th>Study</th>
<th>Location</th>
<th>t(14;18) by PCR</th>
<th>t(14;18) by FISH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cases assayed (N)</td>
<td>% positive</td>
</tr>
<tr>
<td>Einerson 2005 *† (57)</td>
<td>US</td>
<td>14</td>
<td>36</td>
</tr>
<tr>
<td>Barrans 2003 ‡‡ (58)</td>
<td>UK</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>Godon 2003§</td>
<td></td>
<td>(53)</td>
<td>France</td>
</tr>
<tr>
<td>Godon 2003† (53)</td>
<td>France</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Belaud-Rotureau 2006 ‡‡ (59)</td>
<td>France</td>
<td>39</td>
<td>64</td>
</tr>
<tr>
<td>Poetsch 1996*¶ (60)</td>
<td>Germany</td>
<td>28</td>
<td>64</td>
</tr>
</tbody>
</table>

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 Jh 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 Jh consensus regions.
§PCR assays performed using primers for MBR and Jh consensus regions.
||FISH assays performed using Cos Ig10/ PAC210c12 probes.
*FISH assays performed using yeast-artificial-chromosome (YAC)-DNA probes.
G. References


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 \textit{IGH} gene on chromosome 14 (5’-ACCTGAGGAGACGGTGAGC-3’) and a second primer corresponding to the \textit{BCL2} major breakpoint region (MBR) on chromosome 18 (MBR1: 5’-GAGAGTTGCTTTACGTGCCTG-3’) which includes 40-60% of \textit{BCL2} breakpoints involved in t(14;18). Translocation-negative samples were subjected to a second PCR reaction with the \textit{IGH} consensus primer and a second \textit{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 \textit{BCL2} primer site. A 175-base pair segment of the \textit{hemoglobin beta} (\textit{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 \textit{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 \textit{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 Axioscope 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 hemolympathic (HLP) cancer (41) and farming (42) as well as demographic
departnents (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).

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

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\% \, \text{CI}=0.84-3.79) than bcl-2-positive NHL (OR=1.18, 95\% \, \text{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 microarray (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).

F. Acknowledgements

This study was supported in part by the UNC Lineberger Comprehensive Cancer Center (Population Sciences Research Award), the National Institute of Environmental Health Sciences (P30ES10126), the National Institutes of Health National Cancer Institute (R03 CA71617-01), and the Occupational Studies Section, National Cancer Institute. Special thanks to David W. Cowan from the Lineberger Comprehensive Cancer Center
Immunohistochemistry/Hybridization Core Laboratory for guidance with FISH assays;
Stephen Oglesbee, Georgette Dent, and Boyd Yount from UNC-Chapel Hill; Wen-Yi Huang
from NCI; and FARM study collaborators: Ken Cantor, Nat Rothman, Charles Lynch, Jim
Cerhan, and Paige Tolbert.
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‡

<table>
<thead>
<tr>
<th>Exposures</th>
<th>Controls</th>
<th>t(14;18)-positive vs. controls‡</th>
<th>t(14;18)-negative vs. controls‡</th>
<th>t(14;18)-positive vs. t(14;18)-negative§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>OR 95% CI</td>
<td>N</td>
<td>OR 95% CI</td>
</tr>
<tr>
<td>No tobacco use</td>
<td>286</td>
<td>17 1.0</td>
<td>9</td>
<td>1.0</td>
</tr>
<tr>
<td>Used any tobacco</td>
<td>959</td>
<td>64 0.94 0.63 - 1.41</td>
<td>63 1.98 1.09 - 3.59</td>
<td>0.59 0.24-1.44</td>
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<td>Tobacco by product</td>
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<td></td>
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<tr>
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<td>51 0.57 0.33 - 0.98</td>
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<td>0.41 0.13-1.26</td>
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<td>1.54 0.72-3.28</td>
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<td>0.45 0.17-1.32</td>
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<tr>
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<tr>
<td>0-20 pack-yrs</td>
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<td>16 1.0</td>
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<tr>
<td>&gt;0-10</td>
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<td>11 0.85 0.48 - 1.51</td>
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<tr>
<td>&gt;18</td>
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<td>19 1.69 0.97 - 2.93</td>
<td>0.62 0.25-1.51</td>
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<td>6 1.16 0.57 - 2.36</td>
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<td>6 2.12 0.97 - 4.64</td>
<td>3 1.16 0.39 - 3.5</td>
<td>1.75 0.40-7.72</td>
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</table>

*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>
<thead>
<tr>
<th>Exposures</th>
<th>Controls</th>
<th>bcl-2-positive vs. controls‡</th>
<th>bcl-2-negative vs. controls‡</th>
<th>bcl-2-positive vs. bcl-2-negative §</th>
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<td>N</td>
<td>OR</td>
<td>95% CI</td>
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<td>32</td>
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<td>959</td>
<td>136</td>
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<td>14</td>
<td>0.73</td>
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<td>0.72 - 1.89</td>
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<td>Cigarette pack-years</td>
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<td>0-20 pack-yrs</td>
<td>216</td>
<td>31</td>
<td>1.11</td>
<td>0.75 - 1.63</td>
</tr>
<tr>
<td>20-40 pack-yrs</td>
<td>207</td>
<td>30</td>
<td>1.13</td>
<td>0.79 - 1.61</td>
</tr>
<tr>
<td>40+ pack-yrs</td>
<td>342</td>
<td>50</td>
<td>1.03</td>
<td>0.74 - 1.43</td>
</tr>
<tr>
<td>Cigarettes/day</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>418</td>
<td>49</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>&gt;0-10</td>
<td>176</td>
<td>24</td>
<td>1.21</td>
<td>0.82 - 1.79</td>
</tr>
<tr>
<td>&gt;10-20</td>
<td>363</td>
<td>52</td>
<td>1.09</td>
<td>0.79 - 1.5</td>
</tr>
<tr>
<td>&gt;20</td>
<td>259</td>
<td>39</td>
<td>1.07</td>
<td>0.75 - 1.52</td>
</tr>
<tr>
<td>Product/age started</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cigarettes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;18</td>
<td>332</td>
<td>51</td>
<td>1.14</td>
<td>0.82 - 1.57</td>
</tr>
<tr>
<td>≤18</td>
<td>493</td>
<td>68</td>
<td>1.14</td>
<td>0.84 - 1.53</td>
</tr>
<tr>
<td>Chewing tobacco</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;18</td>
<td>86</td>
<td>11</td>
<td>1.15</td>
<td>0.7 - 1.89</td>
</tr>
<tr>
<td>≤18</td>
<td>29</td>
<td>9</td>
<td>1.69</td>
<td>0.88 - 3.26</td>
</tr>
</tbody>
</table>

*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

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

*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


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


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.


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 initial 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 \textit{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 \textit{CMYC} translocations with partner genes other than \textit{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 initiation.

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


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.

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

<table>
<thead>
<tr>
<th>Exposure</th>
<th>t(14;18)-positive/ bcl-2-positive (n=66)</th>
<th>t(14;18)-negative/ bcl-2-positive (n=40)</th>
<th>t(14;18)-positive/ bcl-2-negative (n=10)</th>
<th>t(14;18)-negative/ bcl-2-negative (n=29)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>OR</td>
<td>95% CI</td>
<td>N</td>
</tr>
<tr>
<td>Used any tobacco</td>
<td>51</td>
<td>1.01</td>
<td>0.56-1.84</td>
<td>35</td>
</tr>
<tr>
<td>Tobacco by product</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cigarettes</td>
<td>43</td>
<td>0.71</td>
<td>0.31-1.62</td>
<td>32</td>
</tr>
<tr>
<td>Pipe</td>
<td>21</td>
<td>1.63</td>
<td>0.91-2.93</td>
<td>10</td>
</tr>
<tr>
<td>Cigar</td>
<td>5</td>
<td>0.52</td>
<td>0.20-1.33</td>
<td>8</td>
</tr>
<tr>
<td>Chew tobacco</td>
<td>12</td>
<td>2.33</td>
<td>1.16-4.71</td>
<td>5</td>
</tr>
<tr>
<td>Snuff</td>
<td>7</td>
<td>1.09</td>
<td>0.46-2.55</td>
<td>2</td>
</tr>
<tr>
<td>No tobacco use</td>
<td>15</td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Cigarettes only</td>
<td>20</td>
<td>0.79</td>
<td>0.40-1.58</td>
<td>17</td>
</tr>
<tr>
<td>Cigarettes and other</td>
<td>23</td>
<td>1.16</td>
<td>0.59-2.27</td>
<td>15</td>
</tr>
<tr>
<td>Other tobacco only</td>
<td>8</td>
<td>1.49</td>
<td>0.60-3.70</td>
<td>3</td>
</tr>
<tr>
<td>0-20 pack-yrs</td>
<td>12</td>
<td>0.86</td>
<td>0.41-1.78</td>
<td>5</td>
</tr>
<tr>
<td>20-40 pack-yrs</td>
<td>11</td>
<td>0.85</td>
<td>0.40-1.79</td>
<td>5</td>
</tr>
<tr>
<td>40+ pack-yrs</td>
<td>17</td>
<td>0.81</td>
<td>0.42-1.55</td>
<td>20</td>
</tr>
<tr>
<td>Cigarettes/day</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>&gt;0-10</td>
<td>9</td>
<td>0.87</td>
<td>0.39-1.93</td>
<td>5</td>
</tr>
<tr>
<td>&gt;10-20</td>
<td>18</td>
<td>0.76</td>
<td>0.40-1.45</td>
<td>8</td>
</tr>
<tr>
<td>&gt;20</td>
<td>14</td>
<td>0.86</td>
<td>0.43-1.72</td>
<td>15</td>
</tr>
<tr>
<td>Product/age started</td>
<td></td>
<td></td>
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<tr>
<td>Cigarettes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;18</td>
<td>17</td>
<td>0.85</td>
<td>0.44-1.63</td>
<td>10</td>
</tr>
<tr>
<td>≤18</td>
<td>26</td>
<td>0.85</td>
<td>0.47-1.53</td>
<td>22</td>
</tr>
<tr>
<td>Chewing tobacco</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;18</td>
<td>7</td>
<td>1.72</td>
<td>0.75-3.95</td>
<td>3</td>
</tr>
<tr>
<td>≤18</td>
<td>5</td>
<td>4.10</td>
<td>1.47-11.43</td>
<td>2</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Exposure</th>
<th>t(14;18)-positive/ bcl-2-positive (n=66)</th>
<th>t(14;18)-negative/ bcl-2-positive (n=40)</th>
<th>t(14;18)-positive/ bcl-2-negative (n=10)</th>
<th>t(14;18)-negative/ bcl-2-negative (n=29)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>OR</td>
<td>95% CI</td>
<td>N</td>
</tr>
<tr>
<td>Farmer</td>
<td>41</td>
<td>1.34</td>
<td>0.79-2.27</td>
<td>21</td>
</tr>
<tr>
<td>Any hair dye</td>
<td>8</td>
<td>2.55</td>
<td>1.15-5.65</td>
<td>8</td>
</tr>
<tr>
<td>Education ≤ 12 years</td>
<td>48</td>
<td>1.18</td>
<td>0.66-2.09</td>
<td>29</td>
</tr>
<tr>
<td>Drank alcohol weekly</td>
<td>28</td>
<td>0.56</td>
<td>0.34-0.94</td>
<td>21</td>
</tr>
<tr>
<td>HLP in first-degree relative</td>
<td>3</td>
<td>1.15</td>
<td>0.35-3.81</td>
<td>2</td>
</tr>
<tr>
<td>HLP in first or second-degree relative</td>
<td>4</td>
<td>0.92</td>
<td>0.32-2.60</td>
<td>4</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Exposures</th>
<th>FL N</th>
<th>OR, 95% CI</th>
<th>DL N</th>
<th>OR, 95% CI</th>
<th>SLL N</th>
<th>OR, 95% CI</th>
<th>BL N</th>
<th>OR, 95% CI</th>
<th>Uncl N</th>
<th>OR, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>No tobacco use</td>
<td>20</td>
<td>1.0</td>
<td>15</td>
<td>1.0</td>
<td>5</td>
<td>1.0</td>
<td>2</td>
<td>1.0</td>
<td>11</td>
<td>1.0</td>
</tr>
<tr>
<td>Used any tobacco</td>
<td>63</td>
<td>0.95 (0.56-1.61)</td>
<td>99</td>
<td>1.91 (1.09-3.36)</td>
<td>28</td>
<td>1.73 (0.66-4.55)</td>
<td>15</td>
<td>2.17 (0.48-9.79)</td>
<td>34</td>
<td>0.89 (0.44-1.78)</td>
</tr>
</tbody>
</table>

**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

<table>
<thead>
<tr>
<th>Product/age started</th>
<th>FL N</th>
<th>OR, 95% CI</th>
<th>DL N</th>
<th>OR, 95% CI</th>
<th>SLL N</th>
<th>OR, 95% CI</th>
<th>BL N</th>
<th>OR, 95% CI</th>
<th>Uncl N</th>
<th>OR, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;18</td>
<td>20</td>
<td>0.91 (0.50-1.67)</td>
<td>36</td>
<td>1.48 (0.88-2.50)</td>
<td>12</td>
<td>1.33 (0.58-3.04)</td>
<td>5</td>
<td>1.45 (0.38-5.58)</td>
<td>11</td>
<td>0.97 (0.42-2.21)</td>
</tr>
<tr>
<td>≤18</td>
<td>36</td>
<td>0.96 (0.56-1.64)</td>
<td>50</td>
<td>1.39 (0.85-2.27)</td>
<td>9</td>
<td>0.65 (2.7-1.59)</td>
<td>8</td>
<td>1.48 (0.43-5.09)</td>
<td>21</td>
<td>1.14 (0.55-2.35)</td>
</tr>
</tbody>
</table>

**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 lymphocytic 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

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

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 lymphocytic 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

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

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

<table>
<thead>
<tr>
<th>Exposures</th>
<th>Controls</th>
<th>t(14;18)-positive vs. controls‡</th>
<th>t(14;18)-negative vs. controls‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>N OR 95% CI</td>
<td>N OR 95% CI</td>
</tr>
<tr>
<td>Cigarette-years</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>132</td>
<td>13 1.00 0.42 - 1.75</td>
<td>7 1.00</td>
</tr>
<tr>
<td>&gt;0 to 25</td>
<td>238</td>
<td>19 0.85 0.23 - 0.85</td>
<td>11 1.01 0.35 - 2.91</td>
</tr>
<tr>
<td>&gt;25 to 45</td>
<td>331</td>
<td>16 0.44 0.27 - 1.04</td>
<td>25 1.48 0.69 - 3.19</td>
</tr>
<tr>
<td>&gt;45</td>
<td>211</td>
<td>13 0.53 0.27 - 1.04</td>
<td>16 1.46 0.65 - 3.26</td>
</tr>
<tr>
<td>Pipe-years</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>665</td>
<td>38 1.00 0.42 - 1.75</td>
<td>43 1.00</td>
</tr>
<tr>
<td>&gt;0-10</td>
<td>117</td>
<td>11 1.18 0.66 - 2.1</td>
<td>10 0.86 0.47 - 1.58</td>
</tr>
<tr>
<td>&gt;10</td>
<td>139</td>
<td>14 1.61 0.92 - 2.8</td>
<td>9 0.91 0.48 - 1.73</td>
</tr>
<tr>
<td>Cigar-years</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>791</td>
<td>57 1.00 0.42 - 1.75</td>
<td>49 1.00</td>
</tr>
<tr>
<td>&gt;0-25</td>
<td>97</td>
<td>4 0.52 0.21 - 1.28</td>
<td>10 1.28 0.73 - 2.24</td>
</tr>
<tr>
<td>&gt;25</td>
<td>47</td>
<td>3 1.04 0.29 - 3.7</td>
<td>4 2.18 0.88 - 5.39</td>
</tr>
<tr>
<td>Chewing tobacco-years</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>841</td>
<td>51 1.00 0.42 - 1.75</td>
<td>54 1.00</td>
</tr>
<tr>
<td>&gt;0-10</td>
<td>39</td>
<td>4 1.79 0.78 - 4.12</td>
<td>2 0.95 0.26 - 3.49</td>
</tr>
<tr>
<td>&gt;10</td>
<td>64</td>
<td>4 0.77 0.3 - 1.95</td>
<td>6 1.13 0.55 - 2.34</td>
</tr>
<tr>
<td>Snuff-years</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>817</td>
<td>55 1.00 0.42 - 1.75</td>
<td>54 1.00</td>
</tr>
<tr>
<td>&gt;0-20</td>
<td>46</td>
<td>2 0.82 0.28 - 2.41</td>
<td>2 0.79 0.28 - 2.23</td>
</tr>
<tr>
<td>&gt;20</td>
<td>80</td>
<td>6 1.33 0.64 - 2.78</td>
<td>5 0.75 0.32 - 1.78</td>
</tr>
<tr>
<td>Product/age started</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pipe</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;18</td>
<td>228</td>
<td>21 1.31 0.86 - 2</td>
<td>12 0.86 0.5 - 1.47</td>
</tr>
<tr>
<td>≤ 18</td>
<td>65</td>
<td>5 0.90 0.38 - 2.13</td>
<td>8 1.81 0.98 - 3.34</td>
</tr>
<tr>
<td>Cigar</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;18</td>
<td>131</td>
<td>6 0.68 0.33 - 1.4</td>
<td>10 1.23 0.7 - 2.16</td>
</tr>
<tr>
<td>≤ 18</td>
<td>22</td>
<td>1 0.59 0.08 - 4.3</td>
<td>4 3.94 1.78 - 8.71</td>
</tr>
<tr>
<td>Snuff</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;18</td>
<td>95</td>
<td>6 1.36 0.74 - 2.52</td>
<td>2 0.51 0.15 - 1.76</td>
</tr>
<tr>
<td>≤ 18</td>
<td>46</td>
<td>3 0.74 0.24 - 2.29</td>
<td>6 1.46 0.68 - 3.12</td>
</tr>
</tbody>
</table>

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