MicroRNA profiling in B cell non-Hodgkin Lymphoma:

Focus on the role of MYC

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The studies described in this thesis were financially supported by:
Graduate School of Medical Sciences
J.K. de Cock Foundation
University Medical Center Groningen

Printing of this thesis was financially supported by:
University of Groningen
Faculty of Medical Sciences, UMCG
Graduate School of Medical Sciences

Cover photo: P.M.E. Eijckeler
Cover: Proefschriftmaken.nl UitgeverijBOXPress
Layout: Proefschriftmaken.nl UitgeverijBOXPress
Printing: Uitgeverij BOXPress, 's-Hertogenbosch

ISBN: 978-90-8891-628-1

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Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
woensdag 29 mei 2013
om 11:00 uur

door

Jan Lukas Robertus
geboren op 14 april 1970
te Sheffield, Engeland

Promotores:	Prof. dr. J.H.M. van den Berg
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Paranimfen: Erwin Geuken
Sandra Hobbelt
Lu Ping Tan

In memory of
Susan Elizabeth Robertus Hussick
Elisabeth Wilhelmina Maria Eijckeler
Reinder Martinus Eijckler

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Chapter 1

Introduction and Scope of thesis

INTRODUCTION

1.1 B-cell non-Hodgkin lymphoma

In the World Health Organization (WHO) tumors of hematopoietic and lymphoid tissues are classified according to their cell type of origin, histology, immunophenotype, clinical characteristics and genetic aberrations. B-cell non-Hodgkin lymphomas (lymphoma (NHL) are derived from mature B-cells and account for approximately 70-90% of lymphoid neoplasms worldwide and 4% of all new cancers each year [1, 2]. The median age at diagnosis is between 60 and 70 years with a slight male predominance [3]. The most common types of NHL are diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL) which together make up more than 60% of all cases [2, 4]. Classification of B-cell NHL is predominantly based on the hypothesis that different B-cell lymphomas reflect the various stages in normal B-cell differentiation, providing a sound biological basis for their classification. This biological basis has been expanded with insights into the underlying molecular mechanisms of B-cell lymphoma development. Chromosomal aberrations and in particular translocations have been shown to be of quintessential importance to lymphomagenesis. For example 90% of FL are characterized by the t(14;18)(q32;q21) translocation and subsequent overexpression of the anti-apoptotic *BCL2* gene located at the chromosome 18q21 breakpoint [5, 6].

1.2 Diffuse large B-cell lymphoma

DLBCL is characterized by a diffuse proliferation of large lymphoid cells with 3 common (centroblastic, immunoblastic and anaplastic) but also many other minor morphological variants. It is defined as a neoplasm of large B-cells with nuclear size equal to or exceeding normal macrophage nuclei or more than twice the size of a normal lymphocyte. According to the Kiel classification which was one of the major foundation stones of the WHO classification, they were thought to be derived from blasts in the germinal center B-cell reaction (centroblasts) or extra-follicular, late or post germinal center blasts (immunoblasts, i.e. precursors of plasma cells). Based on (dis)similarities with normal B-cells, a large variety of cytomorphological variants have been described, including the multilobated and anaplastic variants. Later on, clinical features including the site of presentation became more important, resulting in the description of and more clinical and more clinic-pathologic pathological variants of DLBCL were described. This evolution has led to a very heterogeneous rest group of DLBCL. In the newest WHO classification (2008), DLBCL are divided into various specified DLBCL subtypes and DLBCL not otherwise specified (NOS). Apart from the primary mediastinal large B cell lymphoma and more rare types such as

intravascular large B cell lymphoma, which previously were also considered as subtypes of DLBCL. The specified subtypes of DLBCL include T cell/histiocyte rich large B cell lymphoma (THRLBCL), primary DLBCL of the CNS (PCNSL), testis DLBCL, primary leg type cutaneous DLBCL, leg type (PCLBCL) and EBV positive DLBCL of the elderly.

Clinical aspects

DLBCL accounts for 25% to 30% of all non-Hodgkin lymphoma (NHL) in adults. All specific subtypes such as PCNSL, THRLBCL and PCLBCL are relatively rare. In consequence all DLBCL not belonging to these subgroups make up the vast majority of cases. DLBCL that do not meet any subgroup defining criteria fall in to the biologically heterogeneous category of DLBCL NOS. The median age of DLBCL NOS is between the 6th and 7th decade and there is a slight male predominance [7, 8]. DLBCL most commonly presents as a de novo malignancy but can also present upon progression or transformation of a less aggressive lymphoma such as FL, small lymphocytic lymphoma (CLL), and marginal zone B cell lymphoma. DLBCL presents either nodal or at virtually any extranodal site with the most common extranodal site being gastrointestinal [14]. In 40% of patients, presentation is initially only in extranodal sites. In general, DLBCL is an aggressive, rapidly growing tumor. Fifty percent of patients present with either stage I or II disease. Bone marrow involvement is seen in 11-27% of patients. The International Prognostic Index (IPI) categorizes patients into risk groups using unfavorable prognostic factors such as age above 60 years, poor performance status, Ann Arbor stage II-IV, extranodal involvement in more than two sites, and high serum LDH levels [9]. Although DLBCL is an aggressive lymphoma, it is potentially curable. Since the 1970s, standard therapy has consisted of cyclophosphamide, doxorubicin, vincristine and prednisolone (CHOP) [10]. With the addition of the CD20 monoclonal antibody rituximab (R-CHOP) overall survival rates have dramatically improved. The 5 year survival, which for this disease more or less reflects the cure rate, varies between 30% and 80% depending on IP status and biological heterogeneity [11-13].

Immunophenotype and Genetics

The tumor cells usually express pan B-cell markers such as CD19, CD20, CD22 and CD79a although in some cases one or more of these markers may not be expressed [15, 16]. CD5, usually a marker of T cells, is co-expressed in 5-10% of de novo DLBCL [17]. Gene expression profiling has identified two major prognostically different subgroups of DLBCL. The expression patterns were related to normal counterparts in B-cell development and were characteristic of germinal center B (GCB) cells and (in vitro) activated peripheral B-cells (ABC) [18]. According to the original publication and most subsequent papers, ABC type DLBCL

had a worse prognosis than GCB type [18]. Later on, an immunohistochemical algorithm using a combination of CD10, BCL6 and IRF4/MUM1 has been reported to allow classification DLBCL into GCB-DLBCL and non-GCB-DLBCL (also called ABC-DLBCL) [19]. Not all studies have confirmed the efficacy of this so called Hans algorithm and various other algorithms have been proposed [20-22]. Also after the introduction of R-CHOP, although controversial, ABC-DLBCL still appears to have a poorer prognosis than GCB-DLBCL [23]. DLBCL at immune privileged sites (which mostly are EBV negative), usually have an activated peripheral B-cell type gene expression pattern [24, 25].

The most common translocation in DLBCL involves 3q27 affecting the *BCL6* gene and occurs in 20-40% of DLBCL [26]. *BCL2* translocations occur in 20-30% of cases. *MYC* rearrangements are seen in approximately 10% of DLBCL cases [27-30]. GCB-DLBCL and non-GCB-DLBCL differ in the prevalence of specific chromosomal aberrations. For instance, the t(14;18)(q32;q21) translocation and gains of 12q12 are strongly associated with GCB-DLBCL [31-33]. Characteristic chromosomal aberrations in non-GCB-DLBCL include trisomy 3, gain of 3q and 18q21-q22, loss of 6q21-22 and deletion of the INK4A-ARF locus on chromosome 9 [34, 35]. Ongoing somatic mutations of the immunoglobulin gene somatic (hyper)mutations (SHM), which are caused by activity of Activation Induced Deaminase (AID or AICDA), have been related to these molecular subtypes of DLBCL. Interestingly, while the total amount of SHM is higher in ABC type DLBCL, ongoing SHM are only observed in GCB-DLBCL but are absent in ABC-DLBCL [36]. This likely reflects the AID activity in GCB type DLBCL and the post GCB status of non-GCB-DLBCL. Aberrant SHM somatic hypermutations in *PIM1*, *MYC*, *RHoH/TTF* and *PAX5,* which are also molecular targets of AID*,* are observed in 50% of all DLBCL [37]. Non-GCB-DLBCL show overexpression of many genes of the NF-κB pathway [38, 39]. Also, BLIMP1 is a regulator of plasma cell differentiation [40] and has been suggested to function as a tumor suppressor in the pathogenesis of non-GCB-DLBCL, in fact 25% of non-GCB-DLBCL show inactivating mutations of BLIMP1 [41].

1.3 Burkitt lymphoma

BL is first described in 1958 by Denis P Burkitt [42]. It is an extremely fast growing lymphoma composed of monomorphic medium sized cells with a diffuse monotonous growth pattern and numerous macrophages that ingest apoptotic tumor cells resulting in a characteristic starry sky pattern. From a biological and pathological point of view, and in contrast to DLBCL, BL is a very homogeneous disorder, dominated by a MYC driven, extremely rapid proliferation of germinal center derived blasts.

Clinical aspects

Three clinical variants are recognized; endemic, sporadic and immunodeficiency-associated BL. Each of these three variants varies in clinical presentation, morphology and biology. All BL are potentially curable diseases. Treatment consists of intensive combination chemotherapy regimens with 2 year survival rates of 60-80% with advanced stage disease and up to 100% for patients with low stage disease [43-45]. In particular in children, poor prognostic factors include bone marrow and central nervous system involvement, unresected tumor that is larger than 10 cm in diameter and high LDH serum levels [46].

Endemic BL occurs primarily in equatorial Africa and in Papua New Guinea and represents the most common childhood malignancy in these regions with a peak incidence of 1:10,000, an average age of 4 to 7 years and a male:female ratio of 2:1 [47, 48]. Most cases are characterized by a latency type I Epstein-Barr virus (EBV) infection, i.e a type in which very few viral proteins are expressed by the tumor cells. Endemic BL usually presents as a extranodal mass in the jaw or other facial bones, but also in the abdomen or breasts. Only occasionally does it localize to the bone marrow [48, 49]. Sporadic BL has no geographical predilection and it has an incidence of 1-2% of all lymphomas. It is mainly seen in children with a peak between 5-10 years and in young adults with a median age of 30 years. Sporadic BL represents 30-50% of all childhood lymphomas and has a male: female ratio of 2 or 3:1 [50]. The presentation of sporadic BL is predominantly abdominal with the ileocaecal region as the most frequent site of involvement. Other common sites of involvement are the kidneys, breasts and ovaries [47]. EBV is detected in 30% of sporadic BL cases. The immunodeficiency-associated BL most commonly occurs in patients with human immunodeficiency virus (HIV) and BL may be the initially presenting symptom. Incidence rates vary between 2:1,000 in children and 1:1,000 in adults and EBV is associated with up to 40% of immunodeficiency related BL cases.

Morphology and Immunophenotype

Two morphological variants are recognized in the WHO 2008, the common type and variant with plasmacytoid differentiation. The most common type shows features of very actively proliferating centroblasts within the dark zone of germinal centers. These cells have round nuclei with multiple small nucleoli and a very basophilic cytoplasm. Also immunophenotypically they share features with centroblasts as they show strong expression of surface immunoglobulin IgM, CD20, CD10, CD38, CD77, TCL1 and BCL6, absence or very weak expression of CD44, MUM1/IRF4 and BCL2 protein[51]. Also terminal deoxynucleotidyl transferase (TdT) should be absent. The generation / doubling time of these cells is around 24 hrs and the Ki-67 proliferation index in BL typically approaches 100% [52].

The rare BL with plasmacytoid differentiation often shows eccentric basophilic cytoplasm with a single central nucleolus. Using immunohistochemistry, it has a similar phenotype except for the expression of cytoplasmic IgM. This variant has a strong tendency to present in immunodeficient patients.

Pathogenesis

MYC translocations and the resulting overexpression of MYC is considered to be a hallmark of BL. The most common translocation is t(8;14)(q24;q32) in which the *MYC* gene is translocated to the Ig heavy chain region. Other less common MYC translocations involve the lambda or kappa light chain loci on 22q11 and 2p12, respectively. *MYC* translocations are thought to result from class-switch recombination (CSR) and somatic hypermutation (SHM), two processes that involve double-strand DNA breaks and mediated by amongst other AID [53, 54]. Although being a hallmark of BL, *MYC* translocations are not restricted to BL and approximately 10% of the BL cases lack a detectable *MYC* translocation by fluorescence in situ hybridization (FISH) [55].

MYC translocation result in distinct overexpression of the gene. Most interestingly, while all Burkitt lymphomas have a very high MYC expression, the normal counterparts, i.e. the also rapidly dividing normal germinal center B cells, generally have only very low levels of expression (Dominguez-Sola D, Victora GD, Ying CY, Phan RT, Saito M, Nussenzweig MC,

Dalla-Favera R). The proto-oncogene MYC is required for selection in the germinal center and cyclic reentry. Nat Immunol. 2012 Nov;13(11):1083-91). The effect of MYC overexpression on expression of other genes is a matter of continuous debate. Whereas previous papers claimed an cell type specific effect on gene expression of many genes, the effect being different for B cells than for other cells, recent reports suggest that MYC functions as a non-specific amplifier of gene expression of those genes that are actually actively transcribed [129, 130].

1.4 miRNAs

MicroRNAs (miRNAs) are endogenous small RNAs (21-23 nucleotides) that have emerged as key factors in the posttranscriptional control of as many as one third of all protein coding genes [56]. MiRNAs are located in introns and exons of protein coding genes, in non-protein coding genes or in intergenic regions [57]. Currently approximately 16,000 miRNAs have been identified in different species by cloning and sequence analysis of small RNAs. Of these 16,000 miRNAs roughly 1,700 are found in humans (miRBase release 19; August 2012).

Biogenesis and function

Biogenesis of miRNAs generally starts in the nucleus by transcription of primary transcripts (pri-miRNAs) by RNA polymerase II (RPII). Pri-miRNAs are processed by Drosha (nuclear RNase III) to form hairpin structures of about 70 nucleotides in the nucleus (pre-miRNAs). The resulting pre-miRNAs are transported to the cytoplasm by Exportin5 and processed by Dicer (RNAse type III) into double stranded duplexes of about 22 nucleotides. A single mature strand is generated by unwinding of the duplexes by Helicase. This mature strand is incorporated into the RNA induced silencing complex (RISC) and this complex binds to its target mRNAs [58-60]. Individual miRNA levels in each cell are strictly regulated by the transcription machinery, methylation status and also by regulation of its biogenesis [61].
The first miRNAs identified in animals, lin-4 and let-7, were shown to have crucial roles in developmental timing in C. elegans [127, 128]. Mice deficient for Dicer 1 die around 7.5 days of gestation, indicating the importance of miRNAs and other Dicer regulated pathways during embryogenesis [62].

Numerous cellular processes are under regulatory control of miRNAs, including proliferation, differentiation, apoptosis and cell cycle kinetics [63]. MiRNAs contain a highly conserved ”seed” sequence including nucleotide 2 to 7 at the 5’ end [64]. This seed sequence directs pairing to its specific target mRNAs and results in translational repression and/or degradation of the target mRNA dependent of the degree of homology [65-67]. It is currently thought that the RNA induced silencing complex (RISC) acts in mammals mainly by inhibition of translation, and to a lesser extent by degradation of the target gene transcripts. Both mechanisms ultimately lead to down regulation of the target protein. An important question is whether a specific miRNA has one main target gene in a specific cell type or whether each miRNA has many target genes that might be related to the same cellular pathway. Examples of both can be found in the current literature [68-70]. Another recently reported novel mode of miRNA functioning is facilitating intercellular communication by secreting specific miRNAs into micro-vesicles or exosomes and delivery of these vesicles to other neighboring cells [71].

Deregulation of miRNAs is directly implicated in the development of cancer and many other diseases, including diabetes, cardiac diseases and autoimmune disorders. Several tumor suppressor and oncogenic miRNAs have already been identified [72-74].

1.5 Role of miRNAs in B-cell non-Hodgkin lymphoma

Specific miRNA profiles that characterize different B-cell lymphoma subtypes have been reported [75-82]. Several of these miRNAs have been identified by expression analysis in lymphomas, others by their presence in genomic regions that were previously known to be commonly deleted or amplified, but for which no bona fide tumor suppressor

genes or oncogenes could be identified. One of the first oncogenic miRNAs identified in B-cell lymphoma is miR-155. MiR-155 is highly expressed in germinal center B-cell-derived lymphomas [83-85]. Further evidence for an oncogenic role for miR-155 came from a study demonstrating that E(mu) miR-155 transgenic mice develop polyclonal pre-B-cell proliferations leading to B-cell malignancies at later stages [86]. Myeloproliferative disorders were induced by overexpression of miR-155 in hematopoietic stem cells in a mouse model [87].

A second well known oncogenic miRNA cluster is C13ORF25 also known as the pri-miR-17~92 cluster. The pri-miRNA contains 6 stemloops with 6 dominant miRNAs i.e. miR-17, miR-18a, miR-19b, miR-20a, miR-19b and miR-92. An additional miR-17-3p is transcribed from the 3' end of the stem loop containing miR-17. MiR-17~92 is located in the 13q31 region, which is often amplified in B-cell lymphoma [88, 89]. Overexpression of this cluster in the E(mu) MYC mouse B-cell lymphoma model results in accelerated B-cell lymphoma development [90]. More recently miR-19a and miR-19b have been identified as the key oncogenic components necessary and sufficient for promoting MYC induced lymphomagenesis by repressing apoptosis [91, 92]. Several individual members of this miRNA cluster play an important role in proliferation, tumor angiogenesis and suppression of apoptosis [93-96].

In addition to these oncogenic miRNAs, there are also studies showing the importance of miRNAs with that display tumor suppressor activities in B-cell lymphoma. In CLL, deletion of the 13q14 region targets the primary miRNA transcript of miR-15 and miR-16, resulting in down regulation of these miRNAs in the majority of CLL cases [75] and increased levels of the anti-apoptotic protein BCL2 [97]. Deletion of the miR-15 and miR-16 region in mice causes clonal and autonomously growing indolent B cell lymphoproliferative disorders consistent with the spectrum of CLL observed in humans [98].

MiRNAs in DLBCL

MiRNA profiling has revealed a distinct miRNA expression pattern in DLBCL which is different from other NHL such as BL, CLL and FL [99, 100]. Several studies reported that miRNA signatures can segregate DLBCL cases and cell lines into GCB-DLBCL and ABC-DLBCL subgroups [100-107]. However, only a very limited degree of overlap is seen between the various miRNA predictor sets in the different studies. One miRNA profiling study defined three subgroups based on MYC target gene levels and this clustering was unrelated to GCB-DLBCL and ABC-DLBCL classifications [108]. This may be explained by the fact that MYC regulates expression of a large number (approximately 60) of miRNAs [87, 109-113]. The majority of MYC regulated miRNAs are repressed by MYC, but several miRNAs are induced such as the miR-17~92 cluster[87, 108, 112].

The involvement of several miRNAs in DLBCL was inferred from chromosomal regions with recurrent gains or losses not explained by the presence of oncogenes or tumor suppressor genes. The genomic region including the polycistron miR-17~92 is amplified in 12,5% of GCB-DLBLC [88] but not in non-GCB-DLBCL [114]. Other recurrent numerical genomic aberrations in DLBCL include loci for up to 63 miRNAs including some highly expressed miRNAs such as let-7f, miR-15a/16-1, miR-188, miR-222, miR-223, miR-374, miR-424 and miR-513 [108].

Several miRNAs have been linked to DLBCL lymphomagenesis**.** The oncogenic miR-155 has been shown to be significantly over expressed in DLBCL [73] and more specifically in ABC-DLBCL[115]. MiR-155 directly targets bone morphogenetic protein (BMP)-responsive transcriptional factor SMAD5 and overexpression of miR-155 leads to resistance to growth-inhibitory effects of both TGF-β1 and BMPs in DLBCL[116]. Another interesting role for miR-155 in DLBCL is related to both the NF-κB and the Janus kinase JNK pathways. Both pathways are constitutively active in non-GCB-DLBCL [115, 117]. In DLBCLs that lack inactivating mutations of BLIMP1 epigenetic downregulation of BLIMP1 expression may be caused by let-7b which targets BLIMP1 and is overexpressed in DLBCL relative to normal GCB-cells [118].

MiRNAs such as miR-18a, miR-21, miR-155, miR-181 and miR-222 have been shown to be independent predictors of outcome in patients with DLBCL treated with (R-) CHOP. However, little overlap exists between the various studies with the exception of miR-155 and miR-21 [105, 106, 119, 120]. These disappointing results may be partially explained by the extreme heterogeneity of DLBCL, both at the pathologic, molecular and clinical level, as well by the heterogeneous treatment.

MiRNAs in Burkitt lymphoma

The MYC oncogene may be considered as the driving gene in the pathogenesis of BL. MYC has been shown to enhance expression of the miR-17~92 cluster in BL cases and cell lines [121, 122]. Yet, the relationship between *MYC* and miR-17~92 is not a one way street. Two members of the miR-17~92 cluster; miR-17-5p and miR-20a have been show to target E2F1 which in turn regulates *MYC* and vice versa [87]. MiR-34b and miR-9* are downregulated in *MYC* translocation negative (but high MYC expressing) BL. MiR-34b targets MYC and miR-9* targets E2F1 and as such, both miRNAs may play a role in the regulation of MYC overexpression in such exceptional cases of BL [123]. The complexity of this regulatory network is possibly increased by let-7a that also targets *MYC* and has been shown to revert *MYC* induced growth in BL cell lines [124]. BL shows a distinct miRNA profile that clearly defines it from DLBCL [100]. Another important miRNA in BL pathogenesis is miR-155. In contrast to its normal counterpart, the germinal center B-cell, Burkitt lymphoma lack miR-155 expression [85].

This downregulation is thought to result in high expression of its target activation-induced cytidine deaminase (AID). AID is required for immunoglobulin gene diversification in B lymphocytes and for double strand breaks. Low miR-155 and high AID might thus possibly result in a higher risk of MYC translocation [3,4]

Scope of the thesis

MiRNA expression patterns are deregulated in most cancers. The aim of this thesis is to investigate the expression profiles of miRNAs in various subsets of B-cell NHL. As miRNA expression levels are influenced by the transcription factor MYC, we also focused on the effect of MYC regulated miRNAs in BL.

It is unclear what role the site of presentation plays in the expression of miRNAs in DLBCL. In **chapter 2**, we examined the expression of miRNAs known to be involved in lymphomagenesis and determined if these miRNAs show site specific expression in DLBCL. We in particular focused, with a specific focus on DLBCL that present at immune privileged sites (IP-DLBC), i.e. the central nervous system (CNS) and testis.

In **chapter 3**, we describe our results on the processing of the miR-17~92 cluster, which is involved in many B cell lymphomas. We analyzed and correlated the levels of C13ORF25 and the six members of the miR-17~92 cluster in normal B-cell subsets, 117 NHL cases and in 21 NHL cell lines. We studied the induction of the six members of miR-17~92 by comparing their expression levels to those of their normal B-cell counterparts.

To determine the miRNA profile of B-cell NHL we examined **in chapter 4** the miRNA expression patterns in mantle cell lymphoma, follicular lymphoma, chronic lymphocytic leukemia /lymphoma, and Burkitt lymphoma. We specifically examined the role of MYC on the miRNA expression signature of BL. In **chapter 5**, we studied the role of miRNAs influenced by MYC in BL. We selected differentially expressed MYC regulated miRNAs and examine their effect in a BL cell line. **In chapter 6**, we summarize and discuss results presented in this thesis and discuss possible future perspectives.

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Chapter 2

Specific expression of miR-17-5p and miR-127 in testicular and central nervous system diffuse large B-cell lymphoma

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Mod Pathol. 2009 Apr;22(4):547-55

Abstract

Recent studies have shown that certain non-coding short RNAs, called miRNAs, play an important role in diffuse large B-cell lymphomas. Patients with diffuse large B-cell lymphoma have great diversity in both clinical characteristics, site of presentation and outcome. The aim of our study is to validate the differential expression in germinal center and non-germinal center diffuse large B-cell lymphomas and to study to the extent to which the primary site of differentiation is associated with the miRNA expression profile. We studied 50 de novo diffuse large B-cell lymphoma for the expression of 15 miRNAs (miR-15a, miR-15b, miR-16, miR-17-3p, miR-17-5p, miR-18a, miR-19a, miR-19b, miR-20a, miR-21, miR-92, miR-127, miR-155, miR-181a and miR-221). Apart from 19 nodal cases without extranodal dissemination (stages I and II), we selected three extranodal groups with unambiguous stage I and II disease; 9 cases of primary central nervous system, 11 cases of primary testicular and 11 cases of other primary extranodal diffuse large B-cell lymphomas. All cases were analyzed with qRT-PCR. In situ hybridization for the most differentially expressed miRNAs was performed to show miRNA expression in tumor cells, but not in background cells. MiR-21 and miR-19b showed the highest expression levels. No significant differences were seen between germinal center and activate B-cell center diffuse large B-cell lymphomas in either the total or the nodal group for any of the 15 miRNAs. Two miRNAs showed significant differences in expression levels for diffuse large B-cell lymphoma subgroups according to the site of presentation. MiR-17-5p showed a significant higher expression level in the central nervous system compared with testicular and nodal diffuse large B-cell lymphomas ($P<0.05$). MiR-127 levels were significantly higher in testicular than in central nervous system and in nodal diffuse large B-cell lymphomas ($P<0.05$). We conclude that the location of diffuse large B-cell lymphoma is an important factor in determining the differential miRNA expression pattern .

Introduction

Diffuse large B-cell lymphoma (DLBCL) accounts for approximately 40% of all adult non-Hodgkin lymphomas. DLBCL is a clinically heterogeneous group of lymphomas, which presents at different anatomical sites, either nodal or extranodal. Anatomical sites, such as the testis and central nervous system, show marked differences in genetic aberrations, morphology and clinical behavior [1, 2] . Testicular DLBCL and central nervous system DLBCL both have a different biological and clinical behavior compared with other nodal and extranodal DLBCLs. Gene expression profiling of untreated *de novo* DLBCL has revealed three distinct subgroups. The first with the characteristics of normal germinal center B cells, the second with the characteristics of activated blood memory B-cells and a third group to which no profile could be assigned. The germinal center subgroup has a significantly better prognosis than the other two non-germinal center subgroups [3, 4, 5] . The introduction of rituximab to the standard treatment of patients with DLBCL revealed an increase in complete-response rate and overall survival [6] . More recent studies indicate that the addition of rituximab has improved the progression-free and overall survival rate, specifically for activated B-cell type DLBCL [7, 8] , thus blurring the prognostic differences between DLBCL of germinal center and of activated B-cell origin.

Recently, a group of 21–23 nucleotide long microRNAs (miRNAs) was discovered. These miRNAs post-transcriptionally downregulate protein expression on the basis of limited sequence complementarity to the 3'-UTR of mRNA transcripts [9] . Many of these miRNAs are involved in cancer by targeting oncogenes or tumor suppressor genes [10, 11, 12, 13] . Certain miRNAs are reported to play a role in B-cell malignancies and in B-cell development; for example, miR-181a overexpression induces a higher percentage B-lineage cells in both tissue culture assays and adult mice cells [12, 14, 15, 16, 17] . One of the first miRNAs linked to DLBCL was miR-155, which is derived from a longer pri-miR-155, also referred to as *BIC*. Both *BIC* and miR-155 have a higher expression level in activated DLBCL when compared with that in germinal center DLBCL [14, 18, 19, 20, 21] . A putative oncogenic role for this miRNA was supported by the spontaneous development of a polyclonal preleukemic B-cell proliferation in an Eμ-miR-155 transgenic mice model [22] . Two other miRNAs, miR-21 and 221, have also been reported to be expressed at higher levels in non-germinal center DLBCL cell lines and cases [21] . Both of these miRNAs target the antiapoptotic *BCL2* gene. MiR-15a and miR-16-1 also target *BCL2,* and miR-16-1 downregulates cell growth and cell cycle progression [19] . These two miRNAs are the most likely targets of the characteristic 13q14 deletion in CLL, and are downregulated in 75% of CLL cases [14, 19, 20] . *BCL6* is a target of miR-127, and this gene has been reported to be downregulated after demethylation of the promoter region

and concurrent higher expression of miR-127. In DLBCL, amplification of 13q31 is associated with the overexpression of the pri-miRNA transcript, *C13ORF25,* containing seven miRNAs, miR-18a, miR-19a, miR-19b, miR-20a, miR-17-3p, miR-17-5p and miR-92 [23, 24, 25] .

Our goal was to determine whether possible differences in expression levels of miRNAs are related to the differences between germinal and non-germinal center DLBCLs, or to the primary site of presentation and biological origin of these lymphomas. To that end, we selected two groups of distinct extranodal DLBCL cases localized in the testis and central nervous system, and compared these groups with other primary extranodal and nodal lymphomas.

Materials and Methods

Patients and materials

Forty-one cases of primary Ann Arbor stage I and II nodal and extranodal DLBCLs were obtained from the tissue bank at the Department of Pathology and Medical Biology, University Medical Center Groningen, Groningen and 10 cases from the Department of Pathology, The Netherlands Cancer Institute, Amsterdam. Each case was reviewed by a pathologist and a hematologist. Formalin-fixed paraffin-embedded tissue sections were stained with hematoxylin and eosin (HE) to determine the percentage of tumor cells. Blocks with a tumor cell percentage of 80% or more were used for RNA isolation. All protocols for obtaining and studying human tissues and cells were approved by the institution's review board for human subject research.

Tissue microarray (TMA)

For immunohistochemistry, a TMA was generated with three 0.6-mm cores per case inserted in a grid pattern into a recipient paraffin block using an MT1 manual tissue arrayer (Beecher Instruments, Sun Prairie, USA). Sections were cut (5 μm) and immunohistochemical staining was performed with mouse antihuman monoclonal antibodies against *CD10* (1:20, 56C6, Novacastra, Newcastle, UK), *MUM1* (1:25, *MUM1*p, DAKO, Copenhagen, Denmark) and *BCL6* (1:20, PG-BP, DAKO) after antigen retrieval. Positive staining was visualized using a peroxidase-labeled second step and staining with diaminobenzidine (DAB). DLBCL cases were classified as germinal center B-cell-like (germinal center) or non-germinal center B-cell-like (non-germinal center) according to the algorithm as described by Hans *et al* [3] . A uniform cutoff level of 30% was chosen for CD10, BCL6 and MUM1.

RNA in situ hybridization (RNA ISH)

RNA *in situ* hybridization was performed as described earlier [18, 26] . miRNA staining was performed using an LNA probe antisense to miR-17-5p and miR-127-3p (Exiqon, Denmark) in three central nervous system DLBCL cases and in three testicular DLBCL cases, according to the manufacturer's protocol.

RNA isolation

RNA isolation was performed using the protocol as described by Specht *et al* [27] . DNAse treatment was performed using a Turbo DNA free kit (Ambion, Woodward, USA) according to the manufacturer's instructions. Efficiency of the DNase procedure was checked using a multiplex PCR with 5 primer sets specific for different genomic DNA loci, and subsequent analysis on a 1.5% agarose gel. No PCR products were seen confirming the effectiveness of the DNAse treatment. RNA concentrations were determined on a NanoDrop® ND-1000 Spectrophotometer (Nano Drop Technologies, Wilmington, Delaware, USA).

qRT-PCR of mature miRNAs and primary miRNA transcripts

MiRNA reverse transcription of 5ng total RNA was performed using Taqman MicroRNA Reverse Transcription Kit and miRNA specific primers in accordance to the instructions supplied by the manufacturer (Applied Biosystems, USA). Superscript II (Invitrogen, USA) and random hexamer primers were used for reverse transcription of *C13ORF25* and *BIC* and *U6* using 200ng of total RNA. Quantification was performed using Taqman MicroRNA and Taqman Gene Expression Assays together with the 7900HT ABI Fast Real-Time PCR system (Applied Biosystems, USA). SYBR green was used for the relative quantification of the pri-miRNA *C13ORF25*. All procedures were in accordance to instructions supplied by the manufacturers. PCR reactions were performed in triplicate, positive and negative controls were included in each run with an input of 0.44ng for miRNAs and 2ng for the other genes. Fluorescence was quantified with the sequence detection system software SDS (version 2.1, Applied Biosystems, USA). Mean cycle threshold values and standard deviations (SD) were calculated for all miRNAs and genes. *U6* was selected from several other validated housekeeping genes (*GAPDH, RP2, HPRT, TBP and B2M*) as it showed a uniform expression level in all samples and a sufficiently low threshold Ct value (median; 22, SD; 1.7). Cases with a Ct value for *U6* > 30 were excluded from further analysis. The amount of target miRNA was normalized relative to the amount of *U6* ($\Delta Ct = \Delta Ct_{miRNA} - \Delta Ct_{U6}$). Relative expression levels were expressed as $2^{-\Delta Ct}$.

Data analysis

To determine significant differences between testicular, CNS, nodal and extranodal non-IP DLBCL a Kruskal-Wallis test was performed with a Dunn's Multiple Comparison Test and a p-value <0.05 was considered significant (GraphPad Prism software, version 3.02). To determine significant differences between germinal and non-germinal center DLBCLs for a specific miRNA, a two-tailed Mann–Whitney *U*-test was used, and a *P*-value <0.05 was considered significant (SPSS, version 14). Univariate analysis was performed with a Pearson's χ^2-test for gender, stage and localization (SPSS, version 14), and a Student's *t*-test was used to compare age between germinal and non-germinal centers. A *P*-value <0.05 was considered to be significant (GraphPad Prism software, version 3.02).

Results

All cases were stained for BCL6, CD10 and MUM1 and classified as germinal or non-germinal centers according to the algorithm described earlier by Hans *et al.*[3] Results for each case are given in Supplementary Table 2. In total, 32% of the cases were positive for BCL6, 26% for CD10 and 52% for MUM1. The nodal DLBCL group contained eight germinal and 11 non-germinal center cases. Eight central nervous system DLBCL cases were classified as non-germinal and one as germinal center DLBCL. For the testicular DLBCL cases, ten cases were classified as non-germinal and one as germinal center. The eleven other extranodal cases were classified as germinal center in four cases and non-germinal center in seven cases. Comparison of the germinal center with the non-germinal center DLBCL groups using a univariate analysis for age, gender, Ann Arbor stage and localization revealed no significant differences (Table 1).

In the total DLBCL group, the expression levels of the individual miRNAs varied greatly (Figure 1). The most abundant miRNA, miR-21, showed a 100-fold higher expression level (median: 28), and miR-19b (median: 8.9) showed a 10-fold higher expression level than did miR-15a, miR-15b, miR-17-3p, miR-17-5p, miR-18a, miR-19a, miR-20a, miR-92a, miR-127, miR-155, miR-181a and miR-221. MiR-16 showed the lowest level of expression in comparison with the other miRNAs (median: 0.01; Table 2). Within the germinal and non-germinal center groups, miR-21 showed the highest median expression level, 0,25 and 0,34, respectively (Table 2).

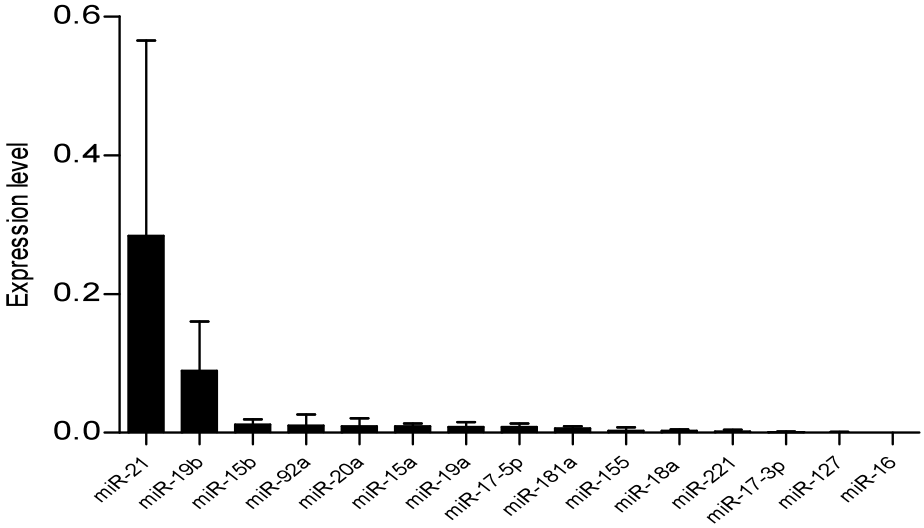

Figure 1. Expression levels of all 15 miRNAs. The median (CI= 95%) expression levels of the 15 selected miRNAs show that miR-21 has a 100 fold higher expression level and miR-19b has a 10 fold higher expression level compared to the other 13 miRNAs**.**

Tabel 1. Overview of 50 DLBCL cases

	Total		**GCB**		**non-GCB**		**p-value***
	n=50	**%**	**n=14**	**%**	**n=36**	**%**	
Age							0.39
Median	59		55		60		
Range	18-87		27-81		18-87		
Gender							0.91
Male	31	63	9	64	20	56%	
Female	19	37	6	36	15	44%	
Stage							0.33
I	35	71	8	53	28	72%	
II	15	29	6	47	8	28%	
Localisation							0.15
Extranodal	31	63	6	47	25	69	
Nodal	19	37	8	53	11	31%	

* Gender, stage and localization were tested using a χ^2 test, age localization were tested using a student-t test.

Tabel 2. Median expression levels of all 15 miRNAs in DLBCL subgroups

	All cases	GCB	non-GCB	Nodal DLBCL	Testicular DLBCL	CNS DLBCL	Extranodal non-IP
	n=50	n=15	n=35	n=19	n=11	n=9	n=11
miR-21	0.284	0.251	0.343	0.270	0.270	0.366	0.465
miR-19b	0.089	0.096	0.087	0.094	0.097	0.086	0.073
miR-15b	0.012	0.012	0.012	0.011	0.009	0.016	0.014
miR-92a	0.010	0.009	0.011	0.008	0.022	0.013	0.010
miR-20a	0.009	0.010	0.009	0.012	0.010	0.008	0.006
miR-15a	0.009	0.009	0.009	0.009	0.015	0.009	0.010
miR-19a	0.008	0.010	0.008	0.008	0.011	0.010	0.008
miR-17-5p	0.008	0.007	0.009	0.006	0.010	0.016	0.006
miR-181a	0.006	0.007	0.006	0.006	0.007	0.005	0.005
miR-155	0.003	0.002	0.003	0.003	0.003	0.006	0.001
miR-18a	0.003	0.003	0.003	0.002	0.003	0.005	0.003
miR-221	0.002	0.002	0.002	0.002	0.001	0.003	0.002
miR-17-3p	0.001	0.001	0.001	0.001	0.001	0.001	0.001
miR-127	0.000	0.000	0.000	0.000	0.001	0.000	0.000
miR-16	0.000	0.000	0.000	0.000	0.000	0.000	0.000

The comparison of all 50 cases showed no significant differences between germinal and non-germinal center DLBCLs for any of the 15 miRNAs. A second analysis was performed using only the nodal group of DLBCL containing eight germinal and eleven non-germinal center cases, which again revealed no significant differences between the expression levels of the 15 miRNAs (Figure 2).

A separate analysis was made of the specific anatomical sites of presentation in testicular, central nervous system, nodal and other extranodal DLBCL cases. MiR-17-5p showed a significantly higher expression level in central nervous system DLBCLs in comparison with that in nodal and testicular DLBCLs (P=0.04; Figure 3A). MiR-127 showed a significantly higher expression level in testicular DLBCL in comparison with that in nodal and central nervous system DLBCLs (P<0.05; Figure 3B). All other miRNAs showed no significant differences in these four DLBCL subgroups.

We subsequently tested the cellular localization of the differentially expressed miRNAs using *in situ* hybridization to confirm that the differential expression was related to higher expression levels in DLBCL cells. RNA-ISH of miR-17-5p in all three central nervous system DLBCL cases (Figure 3c), and that of miR-127 in all three testicular DLBCL cases (Figure 3d) showed an almost restricted staining of tumor cells. Tumor cells showed cytoplasmic and nucleolar staining. MiR-127 has only one proven target, ie, *BCL6.* Correlation

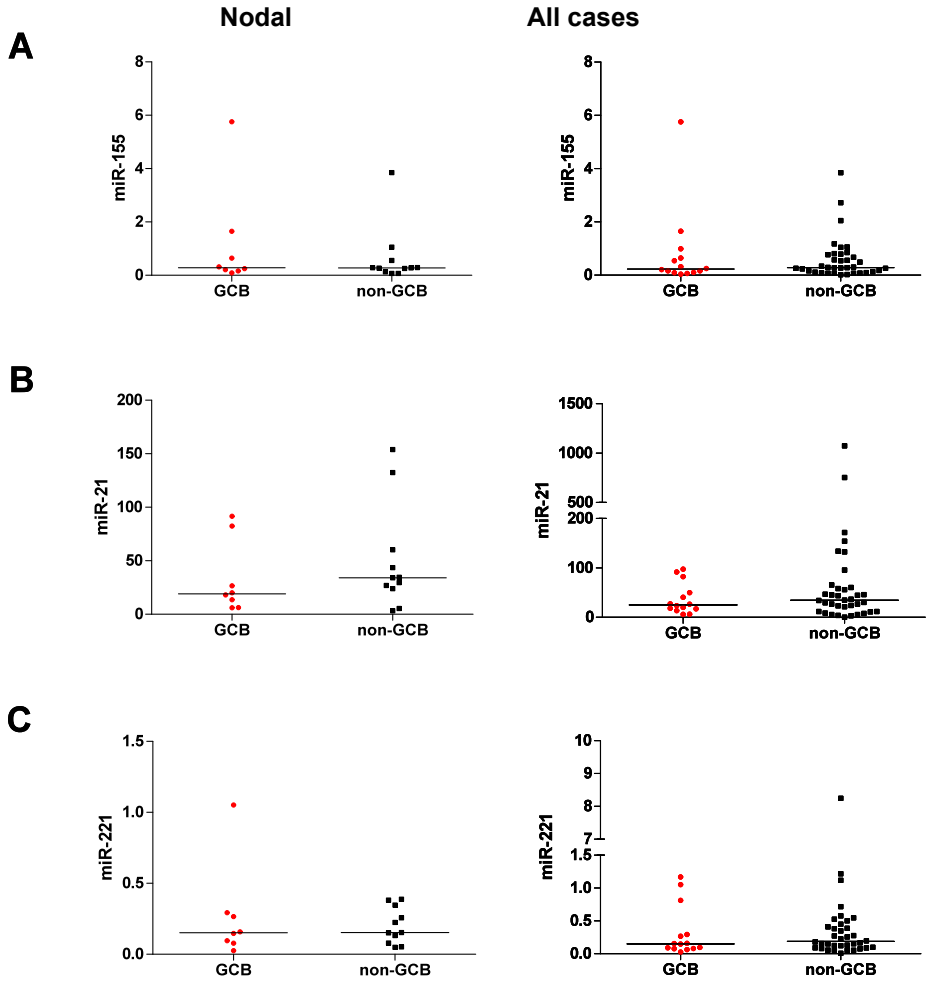

Figure 2. Similar expression levels of miR-155, miR-21 and miR-221 in nodal germinal and non-germinal center diffuse large B-cell lymphomas. The expression levels of miR-155 (a), miR-21 (b) and miR-221 (c) in nodal and in all 50 diffuse large B-cell lymphoma cases; the line indicates the median level. No significant differences are seen between the germinal and non-germinal center groups for any of the three miRNAs either only for the nodal cases or for the total group of diffuse large B-cell lymphoma cases.

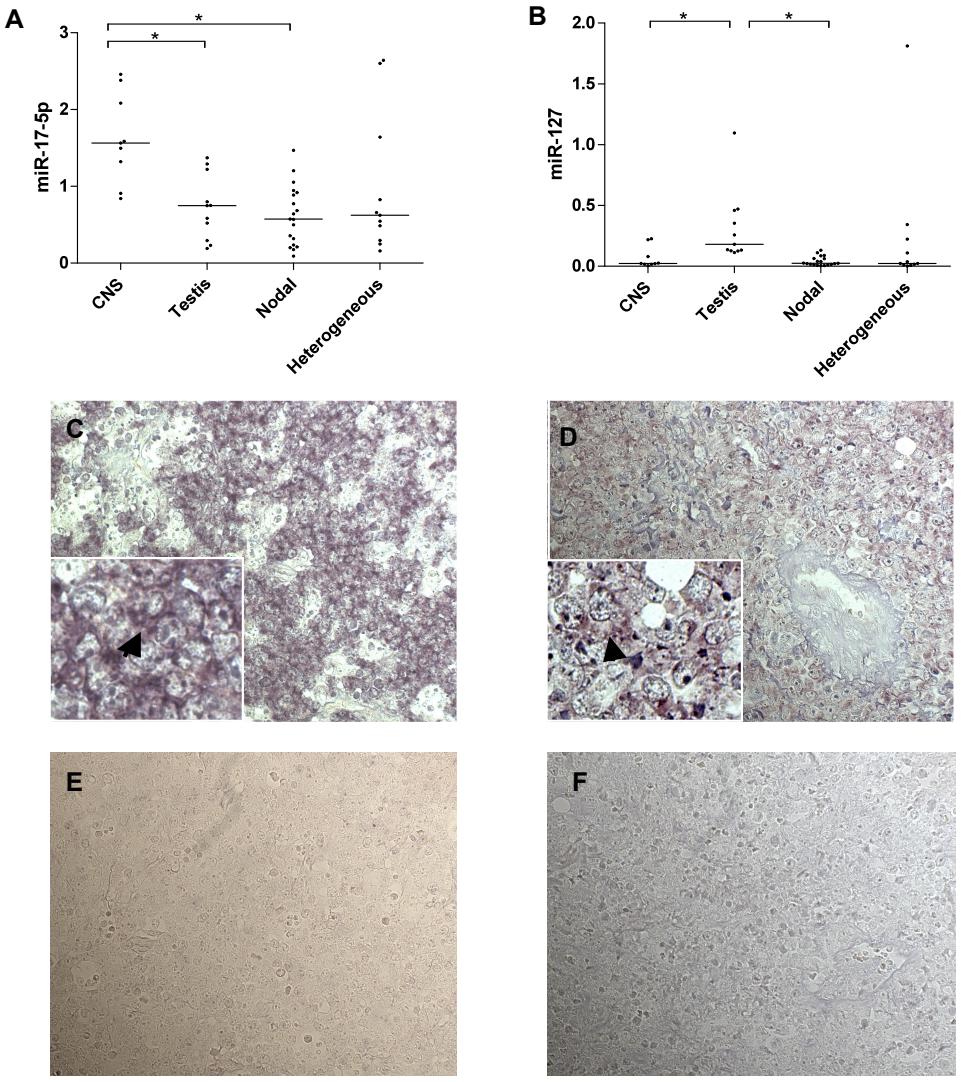

Figure 3. Significant differences in the expression levels for miR-17-5p and miR-127. (A) MiR-17-5p shows a significant difference in median expression levels between the diffuse large B-cell lymphoma groups, with the highest expression in central nervous system diffuse large B-cell lymphoma. (B) MiR-127 shows a higher expression level, specifically for testicular diffuse large B-cell lymphoma (* $p < 0.05$). (C) In situ hybridization for miR-17-5p shows cytoplasmic and nucleolar staining not only in tumor cells of central nervous system diffuse large B-cell lymphoma but also in cells that do not stain for miR-17-5p. (D) The in situ hybridization for miR-127 also shows specific cytoplasmatic and nucleolar staining in tumor cells of testicular diffuse large B-cell lymphoma, but not in the Sertoli cells. (E) A negative DLBCL case that showed no detectable staining for miR-17-5p. (F) A negative DLBCL case that showed no detectable staining for miR-127.

between expression levels of miR-127 and expression levels of *BCL6* using qRT-PCR ($2^{-\Delta C}_{t}$) was significant (*P*=0.027) with an inverse Spearman's coefficient (*r*=−0.3) in the total DLBCL group (data not shown). We also compared the expression levels of miR-127 with the immunohistochemistry results for BCL6 protein expression. When the cases were divided into two groups on the basis of positive BCL6 staining (*n*=15) and negative BCL6 staining (*n*=25), expression levels of miR-127 were lower in the BCL6-positive (median level of 0.004) compared with that in BCL6-negative group (median: 0.07; *P*=0.054) (Figure 4).

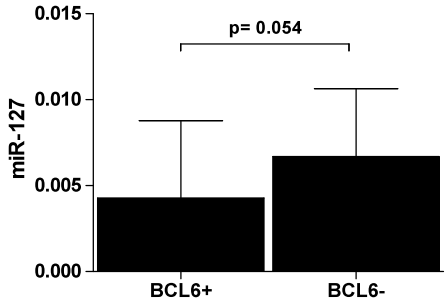

Figure 4. Significant differences in the expression levels of miR-127 in BCL protein expression. MiR-127 shows a significant different expression level (*P*=0.054) between DLBCL with positive IHC staining for BCL6 and DLBCL with negative staining for BCL6.

Discussion

DLBCL is a lymphoma with a widely varying biological and clinical behavior and the different anatomical sites in which the presence of primary DLBCL reflects these heterogeneous aspects. Earlier miRNA studies in DLBCL usually comprised of a heterogeneous group containing cases with mixed sites of presentation. To determine the expression of B-cell-related miRNAs in DLBCL and to examine the differences in miRNA levels in the germinal and non-germinal centers of different subgroups of DLBCL and the relation to the site of presentation, we examined well-defined groups of primary nodal and extranodal DLBCLs. To that end, we selected only Ann Arbor stage I and II cases with nodal, central nervous system or testicular presentation, as well as a heterogeneous group of primary extranodal DLBCL, also only stage I or II. We used paraffin-embedded tissue for our analysis to allow the inclusion of a larger number of cases compared with that in frozen samples. On the basis of recent publications, it is evident that miRNA expression studies result in comparable results for both paraffin and frozen specimens [21, 28].

Consistent with the literature, we found that the testicular DLBCL and central nervous system DLBCL were predominantly non-germinal center [29, 30]. The distribution in the nodal and the heterogeneous other extranodal DLBCL groups was also consistent with earlier reports [2, 4]. Remarkably, no significant differences in expression levels for any of the 15 B-cell related miRNAs were found between the germinal and non-germinal center cases in our total group of DLBCL. In earlier reports, miR-155, miR-21 and miR-221 levels were shown to be higher in non-germinal center DLBCL[18, 20, 21]. A possible explanation for these differences might be that we used specific localizations, ie, primary nodal, testicular, central nervous system and a group of heterogeneous extranodal DLBCL. Earlier reports have used groups of DLBCL with mixed locations and varying Ann Arbor stages. Eis *et al* [20] compared a heterogeneous group with respect to presentation and stage containing only four germinal center and nineteen activated DLBCLs. Kluiver *et al* [18] investigated a group of eighteen DLBCLs from which eleven were extranodal, presenting at eight different anatomical sites. Lawrie *et al* [21] used a heterogeneous group of 35 *de novo* DLBCLs from which 23 cases presented with stage III or IV disease, making a distinction between primary nodal and extranodal almost impossible. Lack of differential expression of these three miRNAs between germinal and non-germinal center DLBCLs in our study cannot be attributed to the inclusion of central nervous system and testicular cases, as analysis of the stage I and II nodal groups and the primary stage I and II heterogeneous extranodal groups also showed no differences for miR-155, miR-21 and miR-221, or for the other 12 miRNAs. This suggests that the differences observed in earlier studies and the lack of differential expression in our stage I and II DLBCL cases may be related to the inclusion of heterogeneous DLBCL cases.

Using qRT-PCR, we found two highly expressed miRNAs, miR-21 and miR-19b, in the DLBCL group. The most abundant, miR-21, showed levels that were 10-fold higher than that in miR-19b and 100-fold higher than that in any of the other 13 miRNAs. Our data are consistent with Lawrie *et al* [21] who also showed high miR-21 levels in DLBCL. Interestingly, miR-21 has been shown to be highly expressed in a variety of different tumors and is associated with the downregulation of BCL2 [31] and PTEN [32]. MiR-19b also showed a high expression with a 10-fold higher expression level than the other 13 miRNAs. MiR-19b is one of the seven miRNAs in the polycistron, *C13ORF25. C13ORF25* is overexpressed in 70% of DLBCLs with 13q31-q32 amplification. Although the miR-17-92 has two homologous clusters, the miR-106a-92 cluster on chromosome X and the miR-106b-25 cluster on chromosome 7, it is the amplification of the 13q31 locus that has been linked to the overexpression of miR-17-92 [24, 33].

MiR-17-5p, another member of the C13ORF25 polycistron, was differentially expressed between the subgroups of DLBCL, showing a significantly higher expression

level in central nervous system DLBCL. C13ORF25 was recently shown to be upregulated by binding of MYC to its promoter region [34]. Mice models have shown that the overexpression of miR-17-92 reduces apoptosis, and further studies have shown that miR-17-5p and miR-20 target the proapoptotic *E2F1* gene [35]. Differences in the levels of miR-19b and miR-17-5p when compared with those in the five other miRNAs of the C13ORF25 cluster may indicate variation in processing efficiency or stability between the nodal, testicular and central nervous system DLBCL subgroups. Interestingly, central nervous system DLBCL has been shown to have a higher expression of MYC than the nodal DLBCL [36]. In central nervous system DLBCL, only the miR-17-5p and miR-20a cluster members were expressed at a significantly higher level, especially in comparison with that in the nodal group. This indicates that both MYC and differences in processing and stability of the mature miRNAs of C13ORF25 may contribute to the higher expression levels of 17-5p in central nervous system DLBCL.

In the testicular DLBCL, miR-127 levels were significantly higher compared with that in the nodal and central nervous system DLBCLs. Earlier studies have shown that the expression of miR-127 was upregulated after treatment with chromatin-modifying drugs, and this induced the downregulation of the proto-oncogene *BCL6*. Transfection of miR-127, precursors in Ramos cells, designed to mimic endogenous miR-127 caused the downregulation of BCL6 protein [10]. We showed a negative correlation between the expression of BCL6 and the expression for miR-127 in DLBCL, which supports the earlier finding that BCL6 is a target for miR-127. BCL6 transcription factor is expressed in germinal centers and is associated with germinal center events [37]. It is one of the major transcriptional regulators of centroblasts and inhibits the differentiation of GC B-cells into plasma and memory cells [38]. In germinal center DLBCL, low miR-127 levels can result in high BCL6 expression levels, and thereby keep the tumor cells in the GC developmental stage, giving a proliferative advantage. The high level of miR-127 in testicular DLBCL can therefore induce the downregulation of BCL6, which is consistent with the non-germinal center phenotype of testicular DLBCL.

Differential expression of specific miRNAs in testicular and central nervous system lymphomas could also be caused by expression in preexistent non-malignant cells, such as Sertoli cells or astrocytes, which might be present in the tumor tissues. We have excluded this possibility by selecting cases with >80% tumor cells and by the application of *in situ* hybridization for the two relevant miRNAs, which revealed a selective expression in tumor cells. Besides cytoplasmic staining, we also observed nucleolar staining. This may be caused by non-specific binding of the probe, although recent reports have suggested mechanisms for miRNA localization in the nucleus [39] and nucleolus [40]. Furthermore, miRNA expression studies on a wide range of normal tissues, including normal brain and testis, using qRT-PCR,

showed that the miR-127 and miR-17-5p expression levels are contrary to those found in testicular and central nervous system DLBCLs [41].

The heterogeneous biology of DLBCL implies that it is essential to select homogeneous groups for pathogenetic studies. In our study, we used strict subsets of DLBCL that were proven to have been derived from three specific sites. By showing significantly different expression levels for miR-17-5p and miR-127 in central nervous system DLBCL and in testicular DLBCL, we support the assumption that each location appears to be a separate biological entity.

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Chapter 3

Marked differences in the miR-17~92 miRNA expression pattern: identification of miR-19b as oncogenic miRNA in non-Hodgkin lymphoma

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In preparation

Abstract

The oncogenic miR-17~92 cluster contains six miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b and miR-92a) that are expressed at variable levels in different normal and malignant cell types. We determined the level of the primary miR-17~92 transcript, *C13ORF25,* and the expression pattern of the six mature miRNAs in three B-cell subsets, 117 non-Hodgkin lymphoma (NHL) cases and 21 NHL cell lines. Within the normal B-cell subsets, significantly higher *C13ORF25* levels were observed for naïve B cells compared to germinal center B cells. In the NHL cases and cell lines, BL showed the highest *C13ORF25* levels. Among mature miRNAs, miR-92a levels were most abundant in the B-cell subsets, in the MCL, BL and CLL cases and all NHL cell lines. In DLBCL cases the miR-19b levels were much higher than the miR-92a levels. Comparison of the levels of the six mature miR-17~92 miRNAs between the NHL cases and their normal B-cell counterparts indicated the highest induction of miR-19b in cases and cell lines of the four NHL subtypes. We conclude that in normal B cells, CLL, MCL and BL cases miR-92a is the most abundant miRNA of the *C13ORF25* transcript, whereas in DLBCL miR-19b showed the highest expression levels. The highest induction in NHL was observed for miR-19b consistent with its known oncogenic role.

Introduction

MicroRNAs (miRNA) are a class of noncoding RNAs that are processed from longer endogenous primary transcripts (pri-miRNA). Each mature miRNA can target multiple protein-coding transcripts based on limited sequence homology, which can lead to a block in translation or to mRNA degradation. Targeting depends on the degree of sequence complementarity between the miRNA and target gene. Especially the seed region, i.e. nucleotide 2-7 at the 5'end of the miRNA, has been reported to be crucial for effective targeting. Several miRNAs are organized into so-called polycistrons that contain multiple miRNA stem loop structures in a single primary transcript. Individual miRNAs within such polycistronic transcripts contain the same or highly similar seed sequences in a proportion of miRNAs. Moreover, shared seed homology can also be observed between miRNAs of different or related polycistronic miRNAs [1]. These miRNA seed family members are thought to target overlapping sets of genes.

Altered expression of miRNAs has been shown in many cancer types and miRNAs are located within genomic regions that show recurrent chromosomal aberrations in cancer [2]. A rapidly increasing number of miRNAs that are involved in many cancer related cellular processes, e.g. cell growth, cell death and angiogenesis, have been identified supporting a role for miRNAs in tumorigenesis [3]. Moreover, several animal models indeed show a crucial role for miRNAs in tumorigenesis [4].

One well-known oncogenic polycistron is the miR-17~92 host gene, also known as *C13ORF25* or Oncomir-1. *C13ORF25* is located at 13q31-32 and contains six miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b and miR-92a). Each of these miRNAs has one or more seed family member in the *C13ORF25* gene or in one of the two other homologous miRNA clusters [5], i.e. the miR-106a~363 cluster on chromosome X and the miR-106b~25 cluster on chromosome 7. Individual miRNAs from the miR-17~92 cluster have been shown to play a role in various cellular processes such as proliferation [6] and angiogenesis [7] and indicate a role in cancer development. Several members of the miR-17~92 cluster are overexpressed in B-cell lymphoma [8, 9] and miR-17~92 has been shown to be the target of the 13q31-32 amplification in diffuse large B-cell lymphoma (DLBCL) [1] and mantle cell lymphoma (MCL)[11]. Further proof that members of the miR-17~92 cluster have oncogenic potential was obtained from studies in a MYC mouse model in which expression of miR-17~92 accelerated lymphomagenesis [12]. Besides overexpression induced by amplification of the 13q31-32 region, *C13ORF25* was also shown to be upregulated by MYC [13]. E2F1, a well known MYC target[14], was targeted by two of the *C13ORF25* miRNAs, i.e. miR-17-5p and miR-20a [3]. This indicated a complex network between MYC, the E2F family, miR-17-5p and

miR-20a [15]. MiR-19a and miR-19b have been shown to be the major oncogenic components in the *Eμ-myc* transgenic mouse model of B-cell lymphoma, this was at least partly due to the repression of the tumor suppressor Phosphatase and tensin homolog (*PTEN*) [16].

The expression of individual members of polycistrons, including *C13ORF25*, has been studied in leukemia cell lines [17] and revealed marked differences between levels of miRNAs derived from the same polycistrons, suggesting variation in processing and/or stability of the individual miRNAs. Remarkably, in solid tumors [4, 9] as well as in lymphoma [4, 18], often only a single miRNA or a subset of the miRNAs of the miR-17~92 cluster are differentially expressed.

In this study, we determined the relative abundance of the six miRNAs of the *C13ORF25* polycistron in a NHL cohort (n=117) including Burkitt lymphoma (BL), chronic lymphocytic leukemia (CLL), MCL and DLBCL. The aim of this study is to analyze the expression patterns and to determine possible differences in these patterns in B-cell lymphoma in comparison to normal B-cell subsets and between NHL subtypes.

Materials and Methods

B-cell subsets.

B cells were purified from human tonsils obtained from children undergoing routine tonsillectomy as previously described[19]. Briefly, mononuclear cells were isolated by Ficoll-Isopaque density gradient centrifugation. Monocytes and T cells were depleted by plastic adherence and sheep red blood cell (SRBC) rosetting, respectively. The total B cell subset was >97% pure as determined by FACS analyses. To sort the different B cell sub populations (naive B cells, memory B cells and GC B cells), cells were stained with FITC-conjugated anti-human IgD, PE-conjugated anti-human CD20, and allophycocyanin-conjugated anti-CD38 and sorted using a FACS aria (BD Biosciences): naive B cells (CD20+IgD+,CD38-), germinal center B cells (CD20+IgD-CD38+) and memory B cells (CD20+IgD-CD38-).

Patient samples.

Formaldehyde Fixed-Paraffin Embedded (FFPE) tissue was obtained from 20 cases of MCL, 19 cases of BL, 50 cases of primary stage I and II nodal and extranodal DLBCL and 28 cases of CLL from the tissue bank at the Department of Pathology, University Medical Center Groningen and the tissue bank at the Department of Pathology, The Netherlands Cancer Institute, Amsterdam (a gift from Dr. D. de Jong). Each case history was reviewed by a hematopathologist and diagnoses were established according to the criteria of the

World Health Organization classification. Only cases containing a tumor cell percentage of >80% were used for RNA isolation and qRT-PCR. All protocols for obtaining and studying human tissues and cells were approved by the institution's review board for human subject research.

Cell lines.

21 cell lines were used for the analysis, including 4 MCL cell lines (HBL-2, JEKO-1, GRANTA-519 and UPN-1), 7 BL cell lines (Raji, CA46, BL65, NAMALWA, DG75, Jiyoye and Ramos), 6 DLBCL cell lines (ROSE, VER, SUDHL6, SUDHL4, OCI-Ly3 and SCHI) and 4 CLL cell lines (EHEB, MEC-1, MEC-2 and JVM3). The mantle cell lines UPN-1 and HBL-2 were obtained from Dr. W. Klapper (Kiel, Germany); JEKO-1, Granta-519 and CLL cell lines JVM-3, MEC-1 and MEC-2 were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Branschweig, Germany). The Burkitt cell lines DG75 and CA46 were obtained from the American type culture collection (ATCC) (LGC standards, Middlesex, UK). The DLBCL cell lines SU-DHL-4 and SU-DHL-6 were obtained from A. Epstein (UCLA, Los Angeles, CA).

Cell lines were propagated in DMEM medium containing 10% FBS (Granta-519), IMDM medium containing 20% (OCI-Ly3) or 10% (MEC-1, MEC-2) FBS or RPMI-1640 medium containing 10% FBS (other cell lines) (Cambrex Biosciences, Walkersville, USA) supplemented with ultraglutamine (2mM), penicillin (100U/ml), streptomycin (0.1 mg/ml; Cambrex Biosciences). Cell lines were cultured at 37°C under an atmosphere containing 5% CO2.

Quantitative RT-PCR.

RNA isolation from FFPE material of DLBCL, CLL, BL and MCL cases was performed as described previously [20]. All samples were DNAse treated using Turbo DNA free kit (Ambion, USA) according to the manufacturer's instructions. Efficiency of the DNase procedure was checked using a multiplex PCR with 5 primer sets specific for different genomic DNA loci and subsequent analysis on a 1.5% agarose gel. No PCR products were seen confirming the effectiveness of the DNAse treatment. RNA concentrations were measured on a NanoDrop® ND-1000 Spectrophotometer (Nano Drop Technologies, Wilmington, Delaware, USA).

For miRNA-specific cDNA synthesis we used 5ng total RNA, the Taqman MicroRNA Reverse Transcription Kit and Taqman miRNA assays for six mature miRNAs of the C13ORF25 cluster. The qPCR reaction was carried out on 0.44ng cDNA using miRNA specific primers in accordance with the instructions supplied by the manufacturer (Applied Biosystems, Foster City, CA, USA). The cDNA synthesis for mRNA was primed with random hexamer primers using Superscript II (Invitrogen, USA) on 200ng of total RNA. SYBR

green (Applied Biosystems) was used for the relative quantification of *C13ORF25*, KSRP with 2ng of cDNA input in a 20µl reaction. PCR reactions were performed in triplicate, positive and negative controls were included in each run. Primer sequences used for PCR were as follows, *C13ORF25* forward primer 5'-TGTGATGTTTTGTTGTGGGTTTG-3'; reverse primer 5'-AGTGCTTTCTTTCCAAATATAGGC-3'. Pri-miR-106~363 forward primer 5'-CAGGGATGAATGGGCAGAG-3'; reverse primer 5'-TGCTTCCTACGTCTGTGTGAACA-3'. KSRP: forward primer 5'-CAGAATACGAATGTGGACAAA-3'; reverse primer; 5'-TCACGTTCCCGGAGGATGT-3'. Quantification was performed using Taqman MicroRNA together with the 7900HT ABI Fast Real-Time PCR system (Applied Biosystems, USA). Fluorescence was quantified with the sequence detection system software SDS (version 2.1, Applied Biosystems, USA). Mean cycle threshold values (Ct) and standard deviations (SD) were calculated for all miRNAs and genes. *U6* was selected as a housekeeping gene to normalize the mRNA and miRNA levels as it showed a uniform expression level in all samples and a sufficiently low threshold Ct value. Cases with a Ct value for *U6* > 30 were regarded to have bad RNA quality and therefore excluded from further analysis. The relative expression levels were expressed as $2^{-\Delta Ct}$.

Given the large number of NHL samples (n=119), qRT-PCR of the NHL cases was performed in separate runs for each of the four subtypes. The comparative threshold cycle method was applied using RNA isolated from a pediatric tonsil obtained during routine tonsillectomy as endogenous reference using the formula $2^{-\Delta\Delta Ct}$ ($\Delta\Delta Ct = (Ct_{sample} - Ct_{U6\ sample}) - (\Delta Ct_{Tonsil} - \Delta Ct_{U6\ Tonsil})$). To allow comparison of the levels of the individual members of the miR-17~92 cluster the sum of the $2^{-\Delta Ct}$ values were set at 100%.

To determine the efficiency of the miRNA qPCR, a 2x serial dilution of cDNA of a cell line with relatively high expression of each of the miRNAs was used in a qPCR reaction. The amplification efficiency was calculated based on the dependence of the Ct value on the cDNA dilution using the following equation: .

Immunohistochemistry. HnRNP A1 was stained using ab50492 (Abcam, Cambridge, UK) with tris/EDTA pre-treatment and a 1:100 antibody dilution followed by detection with labeled GaRPO followed by RaGPO and DAB substrate chromagen solution. Slides were lightly counter-stained with hematoxylin before imaging.

Data analysis.

To determine significant differences in C13ORF25 and individual miRNAs levels within each NHL subtype a Kruskal-Wallis test was performed with a Dunn's Multiple Comparison Test and a p-value <0.05 was considered significant (GraphPad Prism software, version 5.04). To determine the association between the primary transcript and mature miRNA, Pearson

and Spearman's rank correlations together with univariate linear regression were used. A p-value <0.05 was considered significant.

Results

Levels of *C13ORF25* vary in B-cell NHL

C13ORF25 levels were determined by qRT-PCR in normal B-cell subsets, in 117 B-cell NHL samples, including 20 MCL, 19 BL, 50 DLBCL and 28 CLL cases, and in 21 B-cell NHL-derived cell lines. Within the normal B-cell subsets, a significantly higher *C13ORF25* level was observed for naïve B cells compared to GC B cells ($p<0.05$) (Fig. 1A). *C13ORF25* expression within the 117 NHL subtypes revealed marked differences between the four NHL subtypes (Fig. 1B). The levels were the highest in BL and the lowest in CLL (9 fold difference) with marked differences in expression levels between individual cases of each NHL subtype. The BL cases showed a significantly higher *C13ORF25* level compared to CLL ($p<0.001$) and MCL ($p<0.01$). We also observed a significant difference between DLBCL and CLL ($p<0.001$). The overall pattern observed in the NHL cell lines was similar to the NHL cases, but the differences between NHL subtypes were not significant (Fig. 1C) probably due to smaller group sizes. The BL cell lines showed the highest levels of *C13ORF25*, followed by MCL and CLL cell lines, whereas DLBCL cell lines had the lowest levels.

Different levels of the miR-17~92 cluster members in B-cell NHL

We next studied the levels of the six individual members of the *C13ORF25* cluster, i.e. miR-17, miR-18a, miR-19a, miR-20a, miR-19b and miR-92a. Each B-cell subset showed a similar pattern with the highest level observed for miR-92a (up to 60% of the total of all miR-17~92 members together) and the lowest levels for miR-18a and miR-19b (less than 1%) (Fig. 2A). miR-92a levels were significantly higher than the levels of the other miRNAs in all three B-cell subsets ($p< 0.01$). The NHL cases also had significant differences in the levels of each of the six miR-17~92 cluster members. For MCL, BL and CLL, miR-92a was the most abundant miRNA (60-80%) followed by miR-19b (10-30%). In DLBCL, miR-19b was most abundant (~60%), whereas miR-92a levels were much lower (<20%), in the same range as miR-20a and miR-19a.

The cell lines derived from the four NHL subsets showed similar expression patterns, with miR-92a being the most abundant cluster member (45-73%). MiR-19b was the second most abundant in BL and DLBCL (~21%). In MCL, miR-19b and miR-20a levels were similar

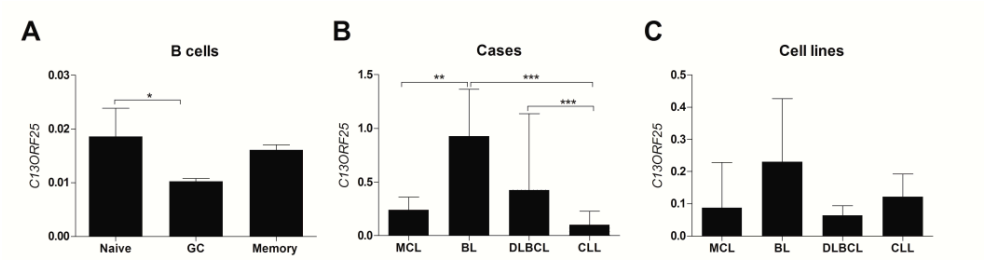

Figure 1. Expression levels of ***C13ORF25*** in normal and malignant B cells. (A) The normal B-cell subsets showed a significant difference in the primary miR-17~92 transcript levels only between naïve and GC B-cell subsets. (B) The B-cell malignancies showed significantly different *C13ORF25* levels that were the highest in BL and the lowest in CLL. *C13ORF25* levels were normalized to an external common calibrator using a comparative threshold cycle method to allow comparison of the levels between the four B-cell malignancies. (C) In the BL cell lines, the levels of *C13ORF25* were significantly higher compared to the DLBCL cell lines. P values were determined using a Kruskal-Wallis test. (*p<0.05, **p<0.01, ***p<0.001).

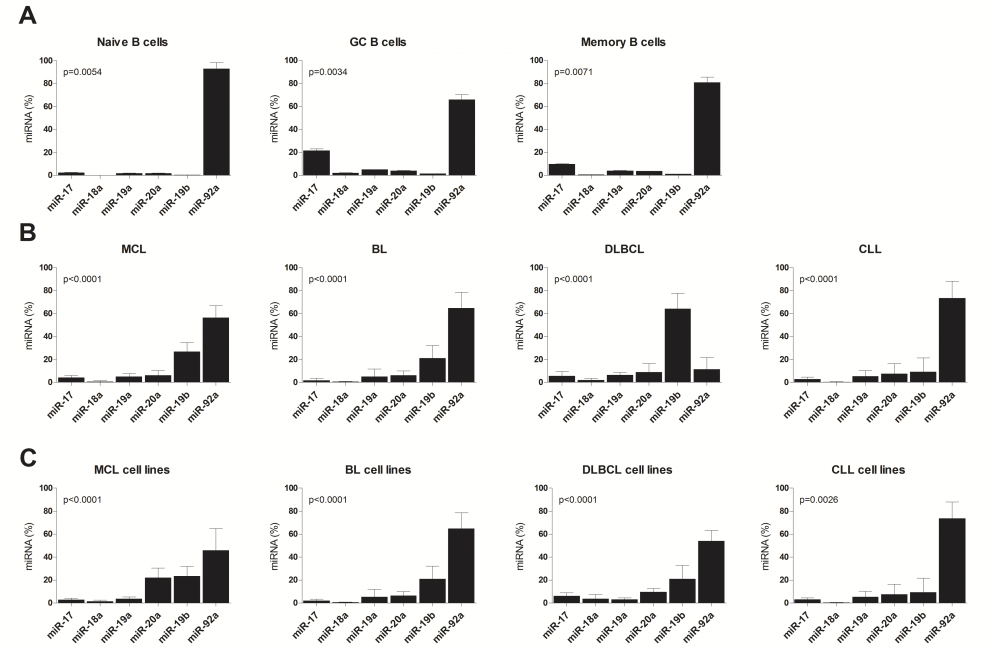

Figure 2. Expression patterns of miR-17~92 miRNAs in B cells and NHL. (A) In the normal B-cell subsets miR-92a levels are significantly higher as compared to the levels of the five other miRNAs. (B) The B-cell malignancies MCL, BL and CLL showed the highest levels of miR-92a, whereas in DLBCL a higher level was observed for miR-19b. (C) The cell lines also showed significant differences within the miR-17~92 cluster with the highest levels for miR-92a. P values were determined using a Kruskal-Wallis test.

(~22%) and were the second/third most abundant miRNAs in MCL. In CLL, four of the five remaining miRNAs showed similar low levels (3-8%).

We next studied the correlation between the levels of the six mature miRNAs and the *C13ORF25* levels for all NHL cases and cell lines. BL cases showed a significant correlation for five of the six miRNAs, i.e. miR-18a (r=0.50, p=0.03), miR-19a (r=0.50, p=0.03), miR-20a (r=0.55, p=0.01), miR-19b (r=0,92; p<0.0001) and miR-92a (r=0.70, p=0.0008) (Fig. 3A). Correlations for the other NHL subtypes were less pronounced, with a significant association observed for miR-92a in MCL, miR-20a in DLBCL, miR-17 and miR-19a in CLL. In the NHL cell lines (Fig. 3B) only two significant associations were observed, which is probably due to the lower number of cell lines studied. Both significant associations were observed in BL cell lines, i.e. miR-19b (r=0.93, p=0.007) and miR-92a (r=0.82, p=0.003).

A possible explanation for the marked differences in expression of individual cluster members might be caused by differences in efficiency of the qRT-PCR procedure. Therefore, we tested the efficiency of the Taqman miRNA assays on serial dilutions of cDNA from the BL-derived CA46 cell line that had a relative high expression level of all six cluster members. The efficiency ranged from 103% to 109% (Supplementary Fig. 1), with the highest efficiency for miR-17 and the lowest efficiency for both miR-18a and miR-92a. Thus, based on these efficiencies it is highly unlikely that the high miR-92a levels are caused by efficiency differences in the qRT-PCR.

A second explanation for the observed differences might be a high or variable expression of the homologous pri-miR-106a~363 transcript that contains two precursor sequences that are highly homologous to the C13ORF25 precursors of miR-92a and miR-19b and result in identical mature miR-92a and miR-19b. To compare both transcripts, qRT-PCR was performed on 7 MCL, 7 BL, 7 DLBCL and 5 CLL cases. In comparison to the high expression levels of *C13ORF25*, the levels of pri-miR-106a~363 were much lower (range 4 to 20 fold) (Supplementary Fig. 2). This indicates that it is unlikely that the high levels of miR-92a and miR-19b in comparison to the other members of the *C13ORF25* cluster can be explained by expression of the homologous pri-miR-106~363 cluster.

A third possibility is that factors influencing the processing efficiency or stability of the individual miRNAs cause differences in mature miRNA levels. KH-type splicing regulatory protein (KSRP) and the RNA binding protein heterogeneous nuclear ribonucleoprotein A1 (HnRNP A1) have been reported to regulate processing of miR-20a and miR-18a, respectively. To identify a possible relation between KSRP levels and the levels of miR-20a, we performed qRT-PCR on 3 MCL, 12 DLBCL, 6 BL, 9 CLL. No relation was observed between KSRP levels and the mature miR-20a levels (data not shown). Immunohistochemical staining of HnRNP A1 in NHL cases showed no difference in expression between tumors with high or low miR-18a levels (data not shown).

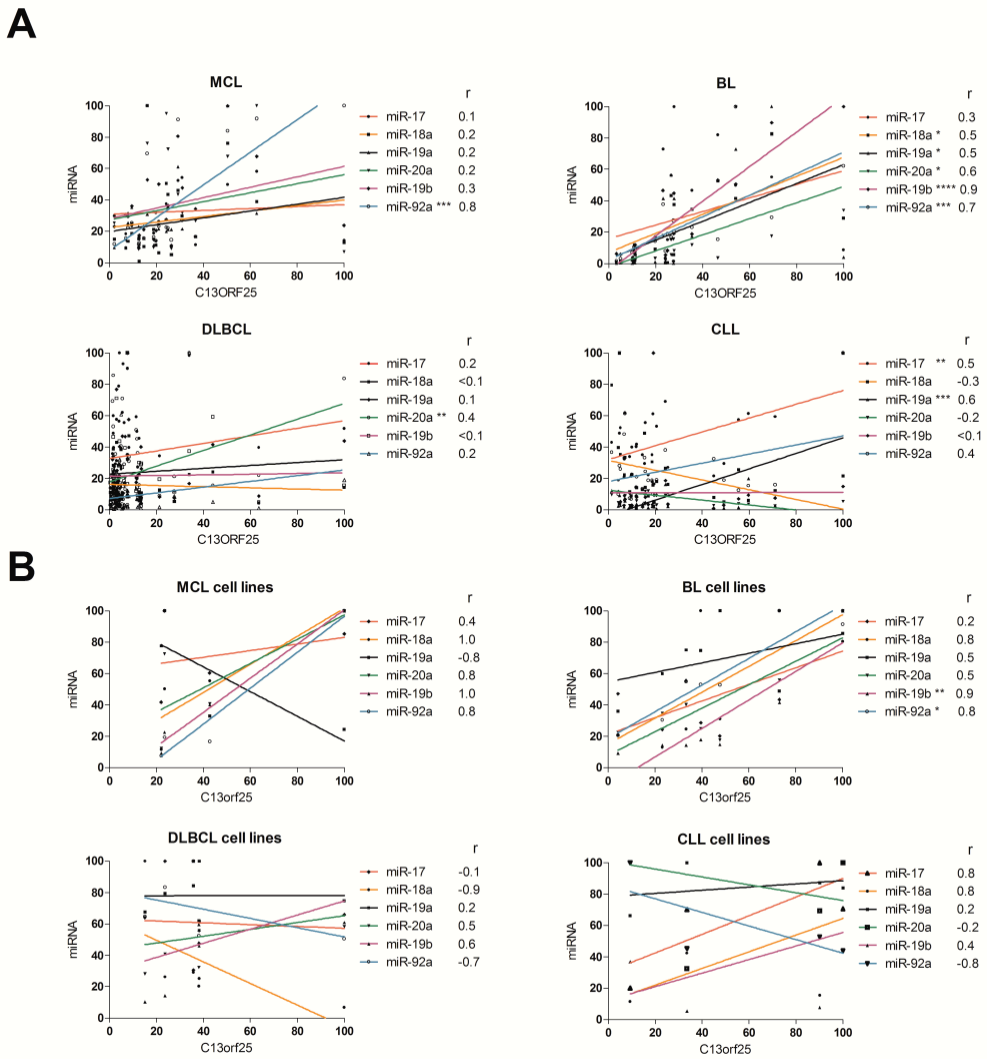

Figure 3. Correlation of ***C13ORF25*** levels with the levels of the six individual miRNAs. (A) In the NHL cases correlations were seen in each of the four NHL subtypes. In MCL and DLBCL, miR-92a showed a significant correlation with *C13ORF25* levels. In BL a significant correlation was seen for five out of the six miRNAs whereas in CLL miR-17 and miR-19a levels showed significant correlations with *C13ORF25* levels. (B) In the NHL cell lines only two significant correlations were observed, i.e. for miR-19b and miR-92a in the BL cell lines. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Fold induction levels in NHL subtypes

To determine whether the levels of the members of the miR-17~92 cluster are deregulated in NHL we studied the fold induction of the miR-17~92 cluster members for each NHL subtype in relation to their normal B-cell counterparts (Fig. 4). The fold induction of all miR-17~92 cluster members as compared to their normal counter parts were increased albeit at variable levels. The strongest fold increases were observed for miR-19b in all four NHL subtypes with fold inductions varying from 10 for CLL compared to memory B cells to 415 for MCL compared to naïve B cells. For the cell lines the fold increase ranged from 746-fold for CLL compared to memory B cells, to 1344-fold for DLBCL compared to GC B cells. For the five other members of the miR-17~92 cluster the fold increase in comparison to normal B cells ranged from 1 to 21 fold for the NHL cases and from 5 to 643 fold for the cell lines. Despite the marked high levels of miR-92a, there was no apparent fold increase as compared to normal B cell subsets. Thus, miR-19b is the most pronounced upregulated member of the miR-17~92 cluster in NHL.

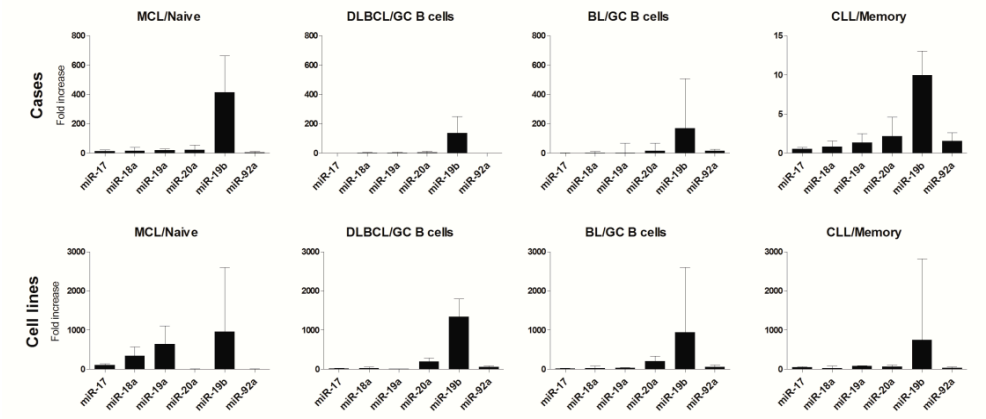

Figure 4. Fold induction levels in NHL subtypes. Levels of the mature miRNAs in the NHL cases are given relative to the levels of the mature miRNAs in their normal B-cell counterparts. The cases all showed a significant fold increase in the expression levels of miR-19b ($p<0.001$). The cell lines also showed a significant fold increase in the expression of miR-19b ($p<0.05$). P values were determined using a Kruskal-Wallis test.

Discussion

In this study we analyzed the expression pattern of the 6 miRNAs that are processed from the noncoding *C13ORF25* transcript in NHL. We showed that normal B cells and each of the four NHL subtypes have a distinct expression pattern for the six miRNAs. MiR-92a was the most abundant miRNA in the normal B-cell subsets and three of the four NHL subtypes, whereas miR-19b was the most abundant cluster member in DLBCL. In comparison to their normal B cell counterparts, all NHL subtypes showed the strongest fold-increase for the oncogenic miR-19b.

MYC and 13q31-32 amplification are two well-known mechanisms to induce *C13ORF25* in NHL [21, 3, 10, 22]. We observed significantly different *C13ORF25* levels in each of the four NHL subtypes. BL cases showed the highest *C13ORF25* levels, which is consistent with the genetic hallmark of BL; a translocation of the MYC locus to one of the immunoglobulin loci. The variations in *C13ORF25* levels observed between and within each NHL subtype are most likely caused by differences in MYC levels and/or the presence of 13q31-32 amplifications in individual cases.

The most abundant miR-17~92 cluster members in B cells and NHL were miR-92a and miR-19b. The miR-106a~363 cluster contains miRNAs that are identical to miR-19b and miR-92a derived from the miR-17~92 cluster [23]. In the NHL cases the level of *C13ORF25* was always much higher than the level of pri-miR-106a~363. Thus, despite the presence of a homologue cluster that also contains miR-19b and miR-92a, it is unlikely that the higher miR-92a and miR-19b levels in comparison to the four other *C13ORF25* members can be explained by co-expression of the *pri-miR-106a~363* cluster. We next examined the correlation between the primary miRNA transcript and the mature miRNAs to assess possible differences in the biogenesis or stability of individual miRNAs. BL showed a good correlation between C13ORF25 and five of the six cluster members, in the other NHL subtypes the correlation was less obvious. A poor correlation between primary transcript and mature miRNAs has been reported for miR-138 in murine brain and murine neuroblastoma cell line N2A [24], let-7 in neural cell specification [25] and miR-143 and miR-145 in colorectal adenocarcionoma [26]. These studies indicate that the levels of primary transcripts do not necessarily correlate with the levels of mature miRNAs.

Two RNA binding proteins, i.e. KSRP and hnRNP A1, have been shown to be involved in biogenesis of certain members of the miR-17~92 cluster. HnRNP A1 facilitates processing of miRNA-18a [27] and KSRP enhances biogenesis of a group of miRNAs including miR-20a and miR-106a [28, 29]. We observed no difference in hnRNP A1 staining intensity between tumors with high and low miR-18a levels, indicating that differences in miR-18a levels are not likely

due to differences in hnRNP A1 expression. This might, in part, be caused by the overall low miR-18a levels observed in NHL. Since there was no antibody available that showed a good staining pattern on FFPE tissue sections, we analyzed KRSP by qRT-PCR. KRSP levels showed no relationship to miR-20a expression on a selection of the NHL cases with variable miR-20a levels. There are currently no known factors that affect biogenesis of miR-19a, miR-19b or miR-92a. Chaulk et al showed that the tertiary structure of the miR-17~92 transcript *in vitro* is organized in such a way that the miR-92a and miR-19b stem-loops are internalized making them less accessible and thus less efficiently processed [30]. It would be interesting to analyze the structure of this primary transcript in B cells or in NHL cell lines to establish if in this cell type the miR-92a and miR-19b stem-loops are more accessible for processing. Moreover, it may be speculated that despite lower pri-miR-106a~363 levels, accessibility of miR-19b and miR-92a is much better in this transcript, and that a substantial proportion of the mature miR-19b and miR-92a miRNAs are derived from this transcript despite its lower levels.

We observed that miR-92a was the most abundant miRNA in the B-cell subsets, the MCL, BL and CLL cases and in the NHL cell lines. MiR-19b usually was the second most abundant miRNA in the NHL cases and cell lines. In contrast to the MCL, BL and CLL cases, the DLBCL cases showed the highest levels for miR-19b. Analysis of the individual DLBCL cases showed that in 49 of the 50 cases the miR-19b levels were indeed higher than the miR-92a levels (Supplementary Fig. 3). MiR-92a has been shown to be involved in carcinogenesis by suppression of angiogenesis by targeting Integrin α5 (ITGA5) [31] and by affecting cellular proliferation in colon and hepatocellular carcinoma cell lines [9]. MiR-92a is overexpressed in a wide variety of cancers, but a role in B-cell lymphomagenesis is less evident. MiR-92a is usually not among the consistently overexpressed miRNAs in profiling studies in NHL [32-34]. He et al. demonstrated that overexpression of a truncated miR-17~19b cluster, thus without miR-92a, cooperates with MYC to promote lymphomagenesis in mouse models [12]. This indicates that miR-92a is not essential for the oncogenic effect of the miR-17~92 cluster. We observed no specific induction of miR-92a in comparison to their normal B cell counterparts, despite the high miR-92a levels observed in the majority of the NHL cases. Thus, it is unlikely that miR-92a plays a main role in NHL lymphomagenesis.

Finally, we assessed the putative oncogenic changes of the mature miRNA levels in relation to their normal counterparts. For all four NHL subtypes, miR-19b showed the highest fold increase ranging from 10- to ~1300-fold. It is unclear why only miR-19b and not the closely related miR-19a is induced in the NHL cases and cell lines. This might indicate that targets specific for miR-19b play a role in lymphomagenesis. MiR-19b, together with miR-19a, accelerated the MYC-induced lymphomagenesis in transgenic mice models [16, 34]. MiR-19a and miR-19b have also been shown to be significantly upregulated in Cyclin D1-

positive MCL patients [35]. Proven targets of miR-19b include protein kinase, AMP-activated, alpha 1 catalytic subunit (Prkaa1), protein phosphatase 2 (PP2a), Bcl-2-like protein 11 (Bim) [36], pro-angiogenic protein FGFR2 [37] and the tumor suppressor gene *PTEN* [38]. PTEN negatively regulates the phosphatidylinosiol-3-kinase (PI3K) pathway [38] and activation of the PI3K pathway is a key element for the malignant transformation of MYC expressing germinal center B cells in a BL mouse model [39]. Overall, there is strong evidence that miR-19a and miR-19b are important oncogenic miRNAs in NHL pathogenesis. The mechanism of specific upregulation of miR-19b and not the other members of the miR-17-92 cluster in NHL compared to normal B cells is not yet known.

In conclusion, we showed that miR-92a is the most abundant miRNA of the miR-17~92 cluster, in normal B cells, CLL, MCL and BL cases. In DLBCL, miR-19b showed the highest levels, and miR-92a was the second most abundant miRNA. Despite high miR-92a levels, we observed the highest fold induction for miR-19b in all four NHL subtypes in comparison to their normal B-cell counterparts, consistent with its known oncogenic role.

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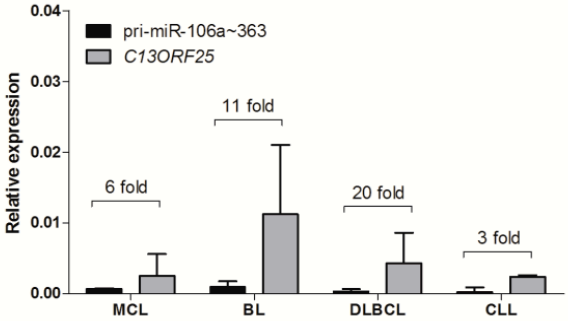
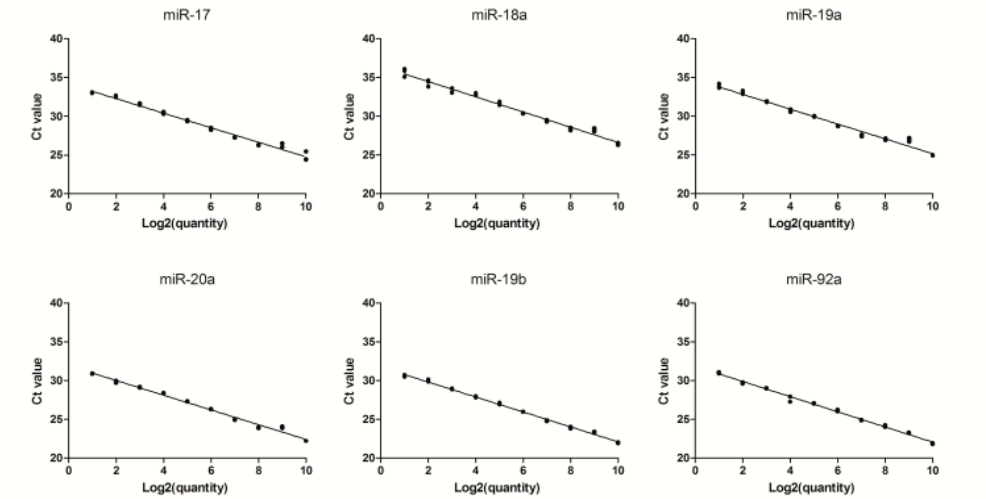
Supplementary Figures

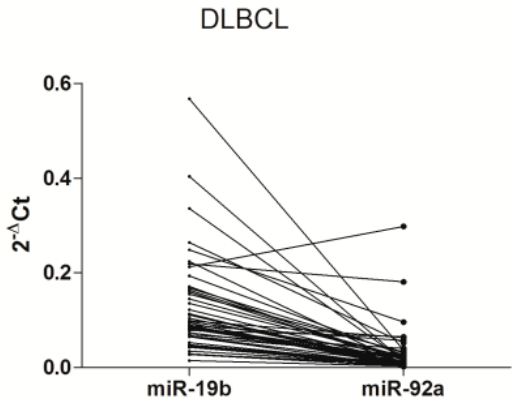


SUPPLEMENTARY FIGURE 1. Relative expression of C13ORF25 and pri-miR-106a~363. In the four NHL subtypes C13ORF25 levels were higher than the levels of the homolog pri-miR-106a~363 cluster.



SUPPLEMENTARY FIGURE 2. Efficiency of qRT-PCR procedure. Using a sample with relative high levels of all six cluster members the PCR efficiency was determined. The efficiencies ranged from 103% for miR-92a to 109% for miR-17.

	Ct value (Log2=10)	Efficiency (%)
miR-17	24.8	109
miR-18a	26.4	103
miR-19a	25.0	106
miR-20a	22.2	107
miR-19b	22.0	105
miR-92a	21.8	103



SUPPLEMENTARY FIGURE 3. Expression levels of miR-19b and miR-92a in individual DLBCL cases. To determine if the relative high expression of miR-19b in contrast to miR-92a is due to extreme outliers, the levels of both miRNAs were compared separately for each DLBCL case. Only one DLBCL case showed a higher level of miR-92a compared to miR-19b.

Chapter 4

MiRNA profiling in B cell non-Hodgkin lymphoma: a MYC-related miRNA profile characterizes Burkitt lymphoma

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Br J Haematol. 2010 Jun;149(6):896-9

Abstract

Recent studies have indicated the importance of miRNAs in B cell maturation and also in the development of B cell lymphoma. To obtain insight into the role of miRNAs in B cell lymphoma we determined the miRNA expression profile of four normal B cell subsets, mantle cell lymphoma (MCL), follicular lymphoma (FL), paediatric Burkitt lymphoma (BL) and chronic lymphocytic lymphoma (CLL). Unsupervised clustering revealed two main clusters, i.e. one for normal B cell subsets and one for the four lymphoma samples. In the B cell subsets, 23 differentially expressed miRNAs were identified with the most specific patterns for GC B cells and plasma cells. 73 miRNAs were differentially expressed between the four NHL, with the most discriminating pattern for BL. Since BL are characterized by high MYC expression and frequently have 13q31 amplification, we determined differential expression of 49 of the 60 miRNAs known to be regulated by MYC. A significant down- or upregulation was observed for 39 (80%) of these miRNAs in the BL compared to the other NHL. High expression of the MYC inducible miR-17-92 cluster members was strongly associated with 13q31 amplification. In conclusion, the most characteristic miRNA profile was observed for BL and included many MYC regulated miRNAs, consistent with the presence of a t(8;14) and a high MYC expression in all BL cases.

Introduction

Several studies have indicated the importance of miRNAs in B cell maturation and in the development of B cell lymphomas. The oncogene *MYC* plays an important role in B cell lymphomagenesis, particularly in Burkitt lymphoma (BL). Several recent publications have shown that *MYC* regulates expression of up to 60 miRNAs (Tables I and SI). The impact of the translocation and overexpression of *MYC* on the miRNA profile in BL has not yet been explored. We determined the miRNA expression profile of paediatric t(8;14) positive and high *MYC* expressing BL in comparison to *MYC* translocation negative mantle cell lymphoma (MCL), follicular lymphoma (FL) and chronic lymphocytic leukaemia (CLL). As a control we included normal B cell subsets obtained from hyperplastic tonsils.

Table I. Known *MYC* regulated miRNAs and differential expression between BL *versus* CLL, MCL and FL.

miRNA	Significant differences between BL and other NHL				
	Flagged	***P*-value**	Corrected ***P*-value**	Regulation	Fold change
			Induced		
miR-7	Yes	0·0264	0·0340	Up	2
miR-17-3p	Yes	ns			
miR-17-5p	Yes	0·0264	0·0340	Up	2
miR-18a	Yes	0·0004	0·0011	Up	4
miR-19a	Yes	0·0138	0·0188	Up	3
miR-19b	Yes	ns			
miR-20a	Yes	0·0298	0·0375	Up	2
miR-20b	Yes	ns			
miR-92	Yes	ns			
miR-106a	Yes	0·0105	0·0161	Up	2
miR-128b	Yes	ns			
miR-130b	Yes	0·0038	0·0064	Up	3
miR-206	Yes	ns			
miR-213	No				
miR-370	Yes	0·0138	0·0188	Up	2
miR-422a	No				
miR-468	No				
			Repressed		
let-7e	Yes	0·0002	0·0010	Down	4
miR-15a	Yes	0·0038	0·0064	Down	3
miR-16	Yes	0·0017	0·0035	Down	2
miR-22	Yes	0·0138	0·0188	Down	2

Table I. Known *MYC* regulated miRNAs and differential expression between BL *versus* CLL, MCL and FL.

miRNA	Significant differences between BL and other NHL				
	Flagged	***P*-value**	Corrected ***P*-value**	Regulation	Fold change
miR-23a	Yes	0·0005	0·0013	Down	2
miR-23b	Yes	0·0038	0·0064	Down	2
miR-24	Yes	0·0002	0·0010	Down	3
miR-26a	Yes	0·0002	0·0010	Down	7
miR-26b	Yes	0·0002	0·0010	Down	4
miR-29a	Yes	0·0002	0·0010	Down	9
miR-29b	Yes	0·0002	0·0010	Down	8
miR-29c	Yes	0·0002	0·0010	Down	16
miR-30c	Yes	0·0059	0·0094	Down	2
miR-30e-3p	Yes	0·0051	0·0083	Down	3
miR-30e-5p	Yes	0·0002	0·0010	Down	6
miR-34a	Yes	0·0006	0·0015	Down	2
miR-99b	Yes	0·0014	0·0031	Down	3
miR-101a	Yes	0·0003	0·0010	Down	6
miR-125b	Yes	0·0004	0·0012	Down	2
miR-134	No				
miR-139	Yes	0·0004	0·0011	Down	7
miR-140	Yes	0·0121	0·0179	Down	3
miR-142-3p	Yes	0·0032	0·0061	Down	4
miR-144	Yes	ns			
miR-146a	Yes	0·0004	0·0012	Down	5
miR-150	Yes	0·0002	0·0010	Down	45
miR-195	Yes	0·0002	0·0010	Down	5
miR-207	No				
miR-210	Yes	ns			
miR-215	Yes	ns			
miR-223	Yes	0·0009	0·0020	Down	4
miR-342	Yes	0·0002	0·0010	Down	3
miR-451	Yes	ns			
miR-466	No				
miR-467	No				
miR-489	No				
miR-494	Yes	0·0020	0·0039	Up	3
LET-7A	Yes	0·0002	0·0010	Down	3
miR-124	No				
miR-148	Yes	ns			
miR-155	Yes	0·0003	0·0010	Down	8
miR-196	Yes	0·0012	0·0027	Down	4
miR-346	No				

Results

Hierarchical clustering showed that the B cell subsets and the non-Hodgkin lymphomas (NHLs) formed two distinct sub-clusters (Fig 1). Unsupervised clustering of the 23 miRNAs significantly differentially expressed between the four B cell subsets (>4-fold) revealed one cluster for the naïve and memory B cells and two additional clusters for the germinal centre (GC) B cells and plasma cells. These results were consistent with previously published data (Appendix S1). 76 miRNAs were differentially expressed (>4-fold) between the four NHL subtypes. Most differences were observed between BL and the other NHLs (CLL *n* = 58, FL *n* = 32 and MCL *n* = 36 miRNAs). Unsupervised hierarchical clustering analysis revealed a unique miRNA profile in BL (Appendix S2). In contrast, a maximum of eight miRNAs were differently expressed between MCL, FL and CLL. A list of validated target genes of the differentially expressed miRNAs is presented in Table SII. Comparing each malignancy to its normal counterpart (MCL with naïve, FL and BL with GC B cells and CLL with memory cells), 54–77 miRNAs were differentially expressed (Figure S1).

Of the 60 reported MYC regulated miRNAs (Table I), 50 were expressed in at least three of the NHL samples and therefore considered to be evaluable. A significant difference between the high *MYC* BL and the other low *MYC* lymphoma samples was observed for 39 miRNAs. The miRNA pattern in BL is consistent with the expected MYC regulation. CLL showed an almost inversed pattern. MCL and FL showed intermediate pattern. *, indicates miRNAs with a proven MYC binding site in the promoter region.

MYC expression has been shown to be a dominant factor in the regulation of many miRNAs. In the present series of lymphomas, quantitative reverse transcription polymerase chain reaction (qRT-PCR) showed a much higher expression in BL than all other NHL (Fig 1C). Comparison of the *MYC*-high BL to all other *MYC*-low lymphomas revealed 122 differentially expressed miRNAs (Table SIII), including 39 of the 50 evaluable (78%) known targets of *MYC* (10 miRNAs were not expressed in our cases) (Fig 1D and Table I). The expression level of these 39 miRNAs was always consistent with the expected up- or downregulation, with most miRNAs being downregulated. This indicates a dominant *MYC*-induced miRNA profile in primary BL. This signature included 39 of the known *MYC*-regulated miRNAs. The 83 other miRNAs were also differentially expressed between *MYC*-high BL and *MYC*-low NHL samples. At present, there are no data supporting a direct *MYC*-dependent regulation for those 83 miRNAs. A *MYC*-dependent miRNA signature has also been suggested for diffuse large B cell lymphoma based on a differential expression of *MYC* targets [1].

Four BL cases (B4, B5, B6 and B7) and two MCL cases (M2 and M3) harboured a 13q31.3 amplification (determined by fluorescence *in situ* hybridization, results not shown).

These six cases showed high expression of the miR-17-92 cluster (Fig 1D). Both in BL and MCL, this association was independent of the *MYC* expression level (Fig 1C). In line with the findings of Tagawa *et al* (2007) [2] this indicates that genomic amplification and not *MYC* overexpression is instrumental in the expression of the miR-17-92 cluster.

Discussion

The miR-17-92 cluster is positively regulated by *MYC* and acts with *MYC* to accelerate tumour development [3]. MiR-17 and miR-20, two members of this cluster, promote cell cycle progression via E2F1 [4]. One interesting MYC-repressed gene is *MIRLET7A*. As MYC is a direct target of *MIRLET7A* this suggests a positive stimulatory loop for MYC [5]. Induction of *MIRLET7A* in the Namalwa BL cell line resulted in reduced MYC expression and reduced proliferation, whereas downregulation of MYC resulted in increased expression of *MIRLET7A*. In addition to a significant downregulation of *MIRLET7A*, we also observed a significant downregulation for *MIRLET7E* in BL compared to the three other NHL subtypes. MiR-150 was also significantly downregulated in BL and targets *MYB*, which has an essential role in haematopoietic and lymphoid development and apoptosis [6]. Overexpression of miR-150 in BL cells resulted in reduced MYB levels and increased apoptosis [7]. Interestingly miR-15a has recently also been shown to repress the *MYB* oncogene in leukemic cell lines [8] . Low expression of miR-150 in BL compared to the other three NHL subtypes and of miR-15a in comparison to CLL might thus result in enhanced MYB levels in BL. Ectopic expression of miR-26a in BL cell lines impaired cell cycle progression via its target *EZH2*, a member of the polycomb-group of genes [9]. The low expression of miR-26a in BL is thus consistent with the previously observed high expression of EZH2 in BL. Concurrent with previous reports, we found downregulation of miR-155 in BL. Although it is unclear which target gene(s) promote tumorigenesis in lymphomas, loss of the miR-155 dependent regulatory control of activation-induced cytidine deaminase (AID) expression, by the introduction of a point mutation in the miR-155 binding site, resulted in increased levels of AID and a higher frequency of *MYC* translocations [10]. This is consistent with low miR-155 levels in *MYC*/8q24 translocation-positive BL. Finally, several other MYC-regulated miRNAs have been implicated in B cell lymphoma and were found to be downregulated in BL e.g. miR-23a/b targeting glutaminase, miR-125b targeting IRF4 and PRDM1/BLIMP1, miR-146a targeting IRAK1 and TRAF6 and miR-223 targeting LMO2 (Table SII).

To conclude, this study showed a very characteristic *MYC*-induced miRNA expression profile in BL. This indicates that MYC regulates BL cell fate in a direct mode at the transcriptional level and indirectly at the translational level by influencing the miRNA profile. Our data support previous studies showing *MYC*-induced miRNA changes in cell lines and animal models.

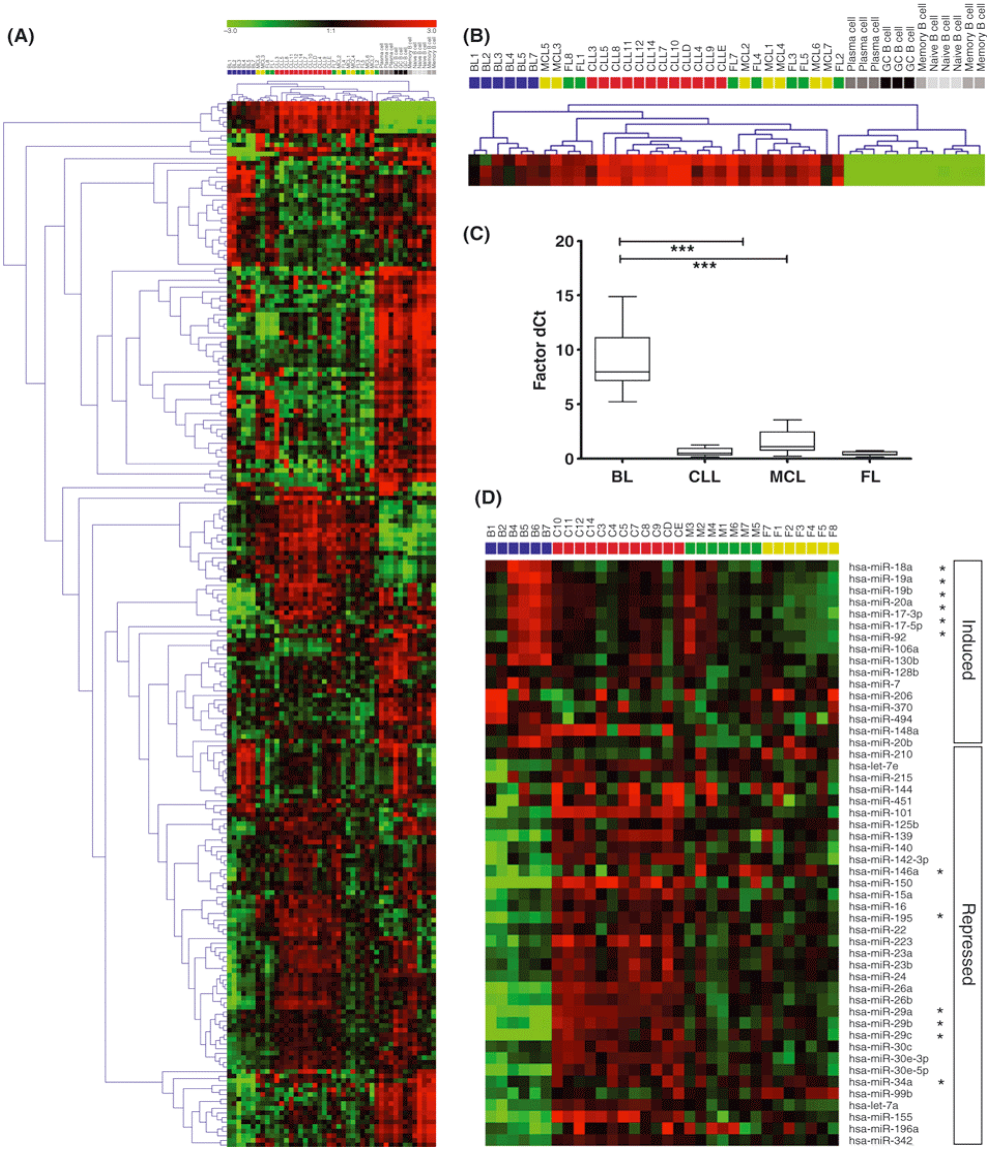

Figure 1. Heatmap of the unsupervised hierarchical clustering of all miRNAs and miRNAs that are MYC-regulated. MiRNA profiles were generated using Agilent Oligo Microarray Kit (G4470A; Agilent, Santa Clara, CA, USA) and the protocol provided by the manufacturer. In total, 256 out of the 556 human miRNAs were expressed in at least three out of the 44 samples. **(A)** Heatmap of 256 miRNAs expressed in at least three samples. **(B)** Enlargement of the clustering tree. With the exception of one FL sample (#FL2) all normal B cell samples cluster separately from the lymphoma samples. **(C)** Box plot of the MYC expression levels in BL, CLL MCL determined by qRT-PCR. High MYC expression levels were observed in all six MYC translocation-positive BL, whereas the MYC levels were much lower in MCL, CLL and FL.). **(D)** Heatmap of the MYC induced miRNAs; MYC induced and repressed miRNAs are indicated on the right-hand side.

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Supplementary figures

Appendix S1: B cell subsets

Approach

Naïve, germinal center (GC), memory and plasma cells were purified from 3 human tonsils obtained from children undergoing routine tonsillectomy resulting in a total of 12 B cell samples. Briefly, mononuclear cells were isolated by Ficoll-Isopaque density gradient centrifugation. Monocytes and T cells were depleted by plastic adherence and SRBC rosetting, respectively. The total B cells fraction was >97% pure as determined by FACS analysis. B cells were stained with FITC-conjugated anti-human IgD, PE-conjugated anti-human CD20, and allophycocyanin-conjugated anti-CD38 and sorted using a FACS aria (BD Biosciences) in: naive B cells (IgD+,CD38-), GC B cells (IgD-CD38+), plasma cells (IgD-,CD38++) and memory B cells (IgD-,CD38-).

Microarray analysis was performed using the G4470A Agilent Oligo Microarray Kit (G4470A; Agilent, Santa Clara, USA) and the protocol provided by the company Data were analyzed using GeneSpring GX10.0 software (Agilent, Santa Clara, USA) following the manufacturers recommendations. Raw data were normalized using a shift to the 75th percentile, without a baseline transformation. Unsupervised clustering analysis of miRNA expression data was performed using Genesis. After median centring of the miRNA expression data over all samples, complete similarity metrics were calculated and clustering was performed using Euclidean distance and average linkage. One way ANOVA with a post hoc Tukey Honestly Significantly Different (HSD) test and Benjamini Hochberg false discovery rate (FDR) correction were performed to identify significantly differentially expressed miRNAs. Differences in miRNAs were considered statistically significant if the P value was less than 0.05. To identify biologically relevant miRNAs a fold change in expression level of at least 4.0 was taken as a cut off.

Results

Out of the 127 miRNAs expressed in the four mature B cell subsets isolated from hyperplastic tonsils, 23 were >4 fold differentially expressed between the different populations (Table 1). Only a single miRNA, i.e. miR-146a, was differentially expressed between naïve and memory B cells using ANOVA and a four fold difference as selection criteria. Relaxing the criteria to only ANOVA did not reveal any additional miRNAs. Unsupervised clustering of the 23 differentially expressed miRNAs revealed one cluster for the naïve and memory B cells and

two additional clusters for the GC and plasma cells (Fig. 1). These data were consistently with previously published studies also showing a rather similar miRNA expression pattern in naïve and memory cells as compared to centroblasts or germinal center B cells (Lawrie *et al*, 2007;Malumbres *et al*, 2008;Basso et al., 2009;Tan et al., 2009;Zhang *et al*., 2009). Heatmaps generated for the miRNAs differentially expressed in these studies using our microarray data data revealed similar miRNA expression patterns (Figures 2, 3 and 4) [1-3].

Table 1. Overview of the 23 miRNAs differentially expressed in B cell subsets.

	Fold	Naïve B cells	Memory cells	Plasma cells
Naïve B cells				
up	>16			
	8-16			
	4-8		miR-146a	
down	4-8			
	8-16			
	>16			
GC B cells				
up	>16	miR-148a		
	8-16	miR-15a, miR-93, miR-130b, miR-193	miR-148a	miR-28, miR-93, miR-151, miR-222
	4-8	miR-22, miR-28, miR-146a, miR-301, miR-331, miR-425-5p, miR-454-3p	miR-15b, miR-28, miR-93, miR-106a, miR-130b, miR-301, miR-331, miR-454-3p, miR-425-5p	miR-7, miR-17-5p, miR-106a, miR-221, miR-425-5p
down	4-8	miR-768-3p, miR-768-5p	miR-150, miR-768-3p	miR-193b
	8-16	miR-150		miR-22, miR-148a
	>16			
Plasma cells				
up	>16	miR-22, miR-148a, miR-193b	miR-22, miR-148a, miR-193b	
	8-16	miR-130b		
	4-8	miR-146a	miR-130b, miR-331, miR-365	
down	4-8	miR-342, miR-151, miR-768-5p	miR-342, miR-221	
	8-16	miR-150, miR-221, miR-222	miR-222	
	>16		miR-150	

Five of the miRNAs, i.e. miR-146a, miR-150, miR-193b, miR-221 and miR-222, differentially expressed between the four normal B cell subsets, also show significant differences between the four lymphoma subsets (Supplementary file 2).

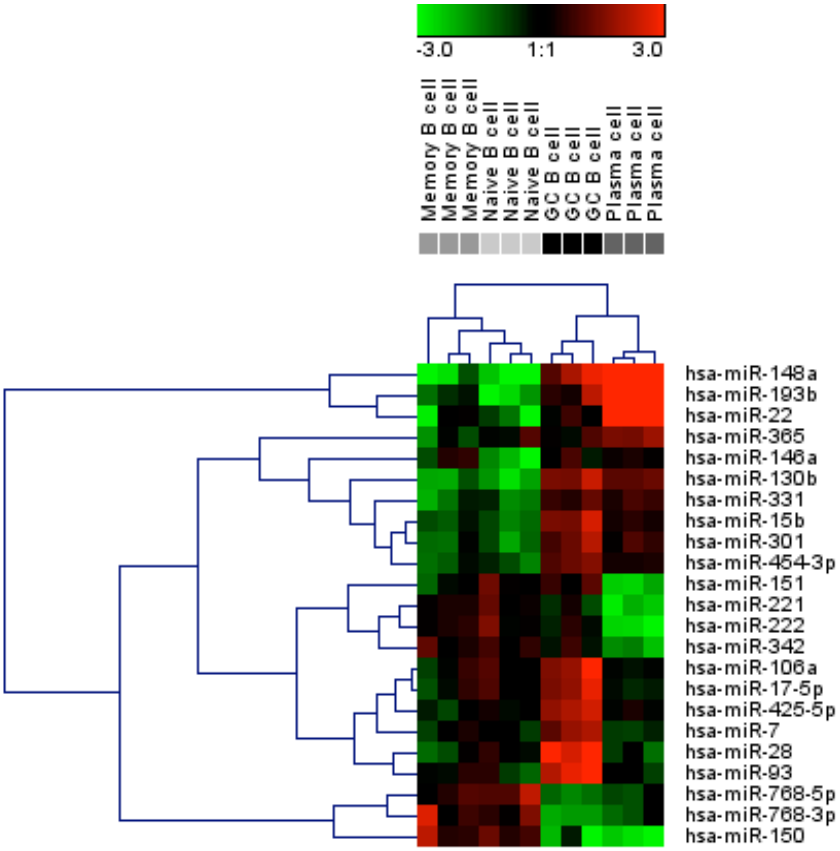

Figure 1. Heatmap of the unsupervised hierarchical clustering of the miRNAs differentially expressed in naïve, GC, memory and plasma cells. Both naïve and memory B cells show a similar expression pattern, whereas GC B cells and plasma cells each show a more distinct profile.

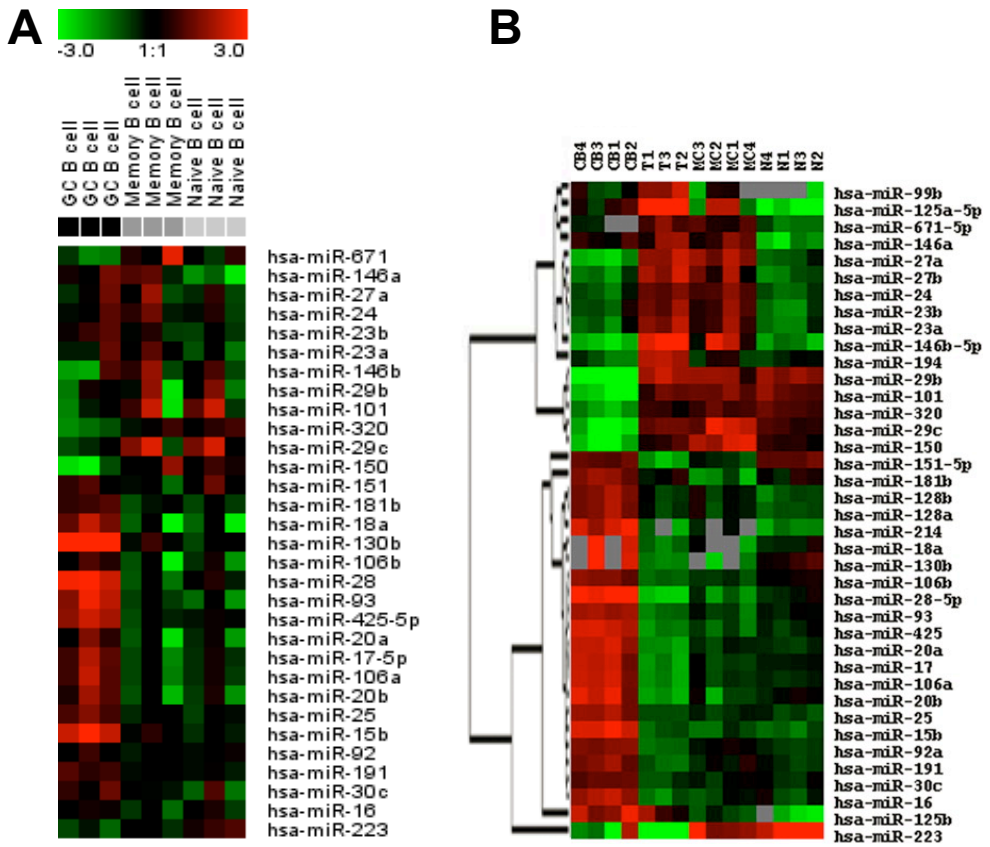

Figure 2. Expression pattern of the classifier miRNA subset from Malubres et al., (2008) in our data set. A, Heatmap of our data for the miRNAs described by Malubres et al. .**B,** Heatmap of Malubres et al. To allow a better comparison only the three B cell subsets analyzed in both studies are shown, i.e. GC B, memory and naïve in our study and centroblasts, memory cells and naïve cells in the study by Malubres et al.

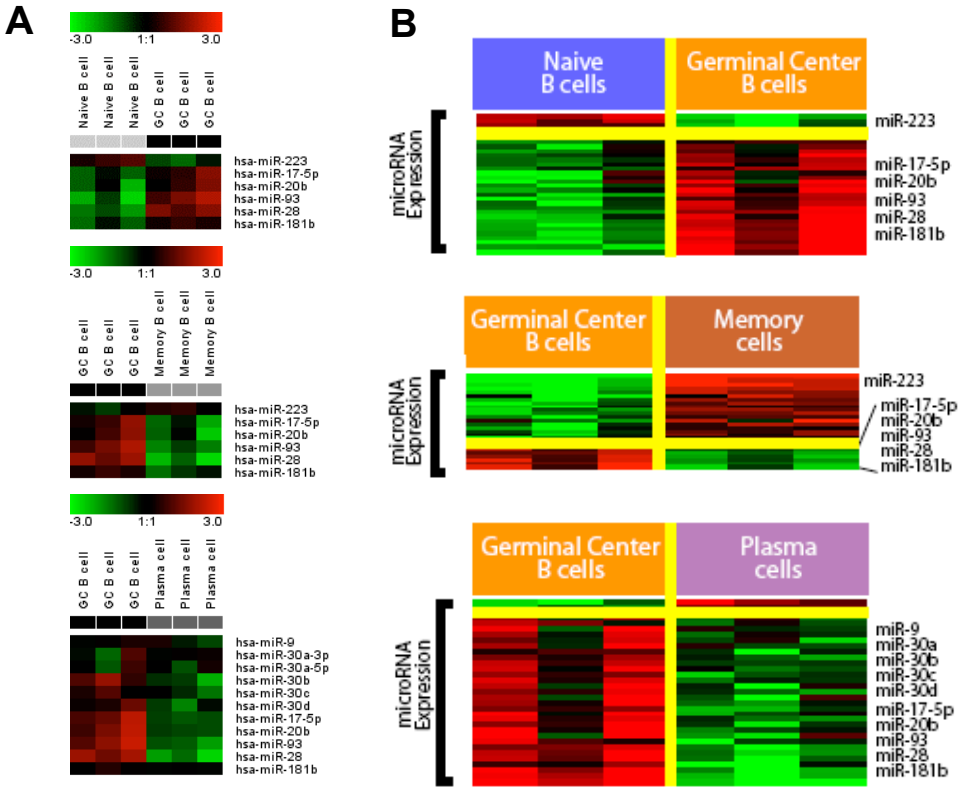

Figure 3. Comparison to Zhang et al., (2009). A, heatmap of our data for the miRNAs described by Zhang et al, **B**, data of Zhang et al. Since the online data are not available yet we only show the heatmap for the miRNAs specifically mentioned in the paper of Zhang et al.

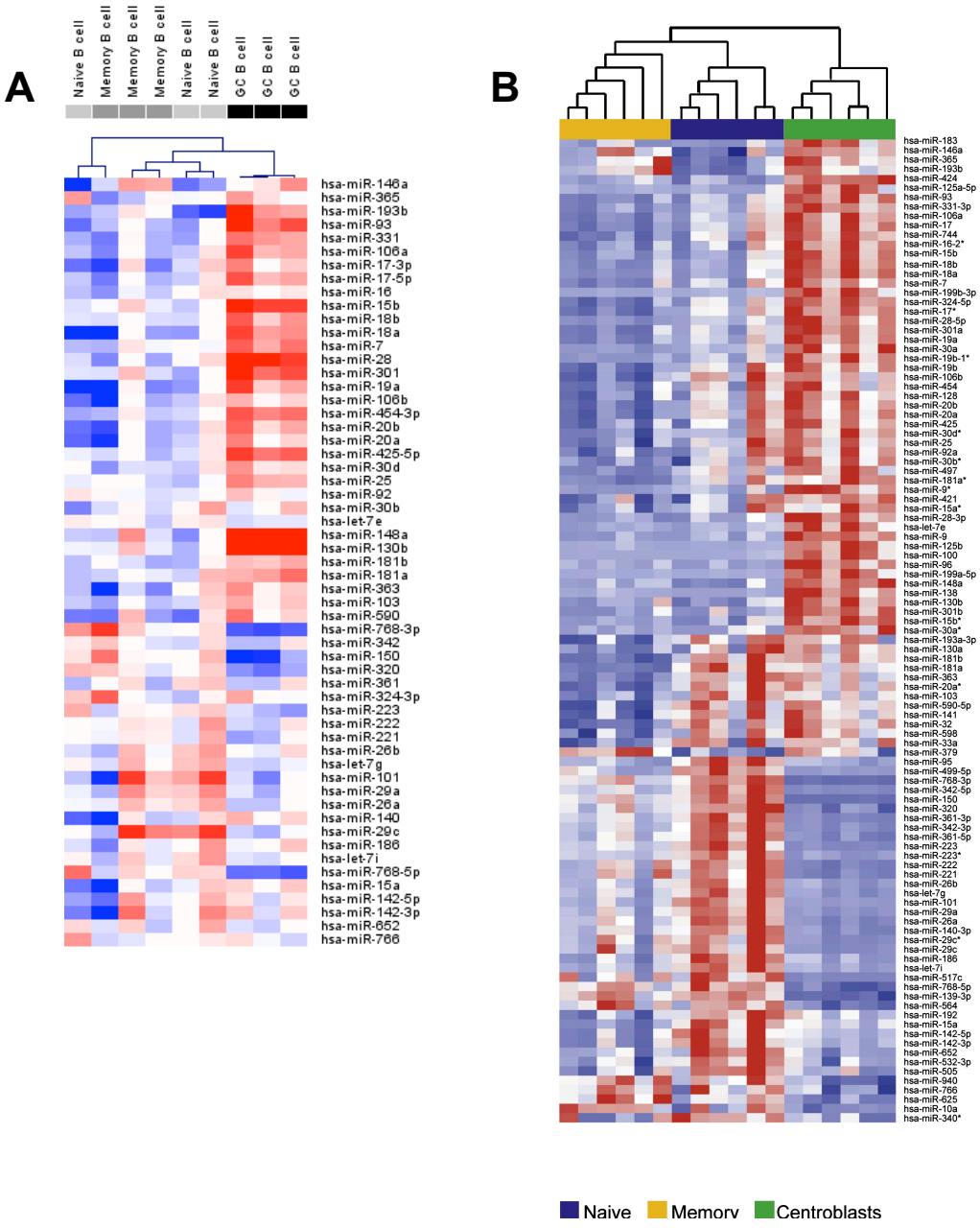

Figure 4. Comparison to Basso et al., (2009). A, Heatmap of our data for the miRNAs described by Basso et al. **B,** Heatmap of Basso et al. To allow a better comparison the three B cell subsets analyzed in both studies are shown, i.e. GC B, memory and naïve in our study and centroblasts, memory cells and naïve cells in the study by Basso et al.

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Appendix S2: NHL subsets

Approach

Specimens of frozen lymph nodes of 32 patients were collected from the UMCG tissue bank. Each individual diagnosis was reviewed by an experienced haematopathologist according to the classification of the WHO. The lymphoma samples were selected to have at least 60% tumor cells and each case was selected by strict definition according to the WHO classification. The samples were derived from 6 patients with BL (all paediatric patients with an abdominal mass, phenotype: CD10+, BCL2- and EBV- and carrying a *MYC*/8q24 breakpoint as assessed by fluorescence in situ hybridization), 7 patients with FL (all nodal, CD10+, CD5-, BCL2+ and grade 1 or 2), 7 patients with MCL (all nodal, CD5+ and cyclinD1+) and 12 patients with CLL (all nodal, CD5+ and CD23+, and cyclinD1-; 8 cases were ZAP70+ and 4 ZAP70-).

Microarray analysis was performed using the G4470A Agilent Oligo Microarray Kit (G4470A; Agilent, Santa Clara, USA) and the protocol provided by the company Data were analyzed using GeneSpring GX10.0 software (Agilent, Santa Clara, USA) following the manufacturers recommendations. Raw data were normalized using a shift to the 75th percentile, without a baseline transformation. Unsupervised clustering analysis of miRNA expression data was performed using Genesis. After median centring of the miRNA expression data over all samples, complete similarity metrics were calculated and clustering was performed using Euclidean distance and average linkage. Mann Whitney test was performed with Benjamini Hochberg FDR. Differences in miRNAs were considered statistically significant if the P value was less than 0.05. To identify biologically relevant miRNAs a fold change in expression level of at least 4.0 was taken as a cut off.

Results

76 miRNAs were >four fold differentially expressed between the four NHL subtypes (Table 1). Unsupervised clustering of these miRNAs demonstrated an inversed pattern for BL and CLL. The pattern for FL and MCL was similar but less distinctive as compared to the BL and CLL (Fig. 1). The highest numbers of differentially expressed miRNAs were observed between BL and the other three NHL, i.e. 52 with CLL, 32 with MCL and 30 with FL. In contrast only five to eight miRNAs were significantly differently expressed in comparisons between MCL, FL and CLL. Analysis of a predictor subset for BL vs CLL reported by Zhang et al [1], revealed that 11 of the 20 miRNAs were evaluable in our NHL samples (the other 9 were not flagged as present). Unsupervised clustering of these miRNAs in the presently studied cases of BL and CLL revealed a distinct clustering pattern discriminating between BL and CLL (Figure 2).

Table 1. Overview of the 76 miRNAs differentially expressed in the four NHL

	Fold	MCL	FL	CLL
BL				
up	>16			miR-629, miR-663
	8-16	miR-560, miR-629, miR-663	miR-629, miR-663	miR-18a, miR-560
	4-8	miR-18a*, miR-136, miR-373*, miR-376a, miR-492, miR-498, miR-557, miR-584	miR-17, miR-18a, miR-18a*, miR-19a, miR-20a, miR-20b, miR-373*, miR-557, miR-560	miR-181a, miR-181a*, miR-181b, miR-182, miR-188, miR-193b, miR-373*, miR-452, miR-492, miR-498, miR-513, miR-557, miR-584, miR-601, miR-630
down	4-8	let-7B, let-7G, miR-10b, miR-26a, miR-29a, miR-139, miR-195, miR-29b, miR-30e-5p, miR-196a, miR-455, miR-497	miR-26a, miR-29a, miR-31, miR-29b, miR-99b, miR-125a, miR-139, miR-146a, miR-155, miR-196b, miR-222, miR-455, miR-497, miR-768-3p, miR-768-5p	let-7C, let-7C, let-7E, let-7F, miR-7a, miR-15a, miR-26b, miR-99a, miR-142-3p, miR-195, miR-196a, miR-200c, miR-221, miR-222, miR-223, miR-335, miR-338, miR-361, miR-660
	8-16	miR-29c, miR-146a, miR-338, miR-363, miR-768-3p, miR-768-5p	miR-29c, miR-138, miR-582	let-7G, miR-10b, miR-26a, miR-29a, miR-30e-5p, miR-101, miR-139, miR-497, miR-768-3p
	>16	miR-14, miR-150, miR-200c	miR-150	miR-29c, miR-150, miR-155, miR- 768-5p
FL				
up	>16			
	8-16			miR-182
	4-8	miR-136, miR-582		miR-138, miR-582
down	4-8			miR-101, miR-150, miR-335
	8-16	miR-363		
	>16	miR-141, miR-200c		
CLL				
up	>16	miR-335		
	8-16			
	4-8	miR-150, miR-155		
down	4-8	miR-181c, miR-200c, miR-363		
	8-16	miR-141		
	>16	miR-182		

Five of the miRNAs, i.e. miRNAs, i.e. miR-146a, miR-150, miR-193b, miR-221 and miR-222, differentially expressed between the four B cell lymphoma subtypes are also differentially expressed between the four normal B cell subsets (Supplementary file 1).

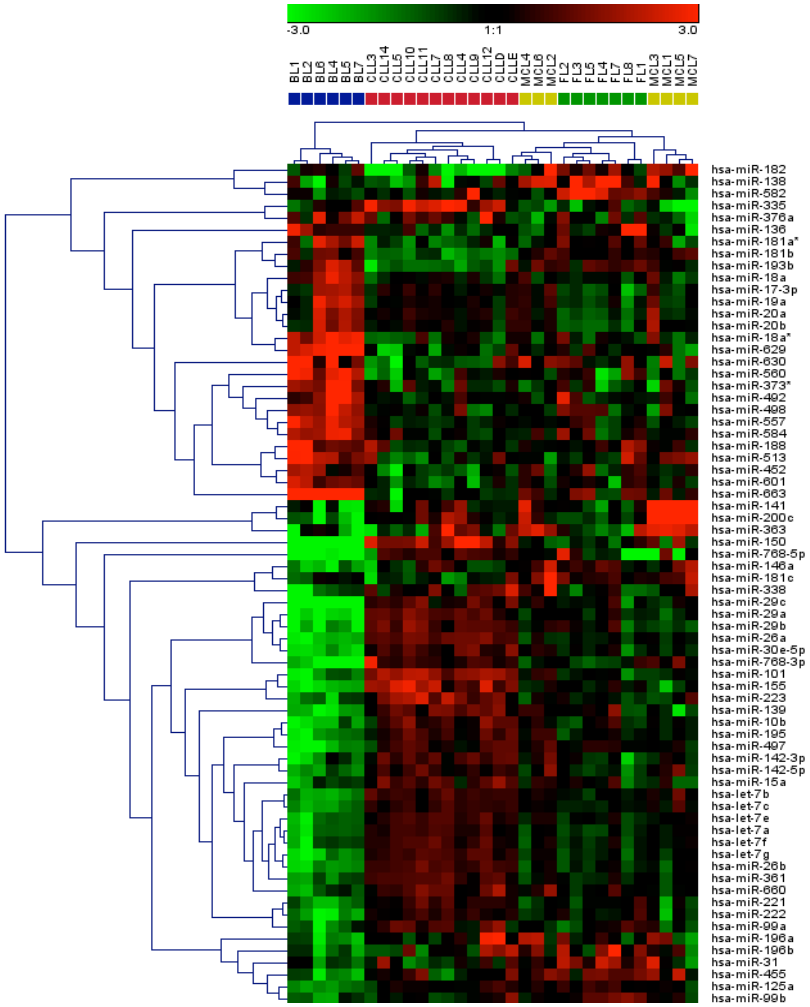

Figure 1. Heatmap generated by unsupervised hierarchical clustering of the 76 miRNAs differentially expressed between MCL, BL, FL and CLL. Most distinct inverse patterns are observed for BL and CLL. FL and MCL display a less specific, intermediate expression pattern.

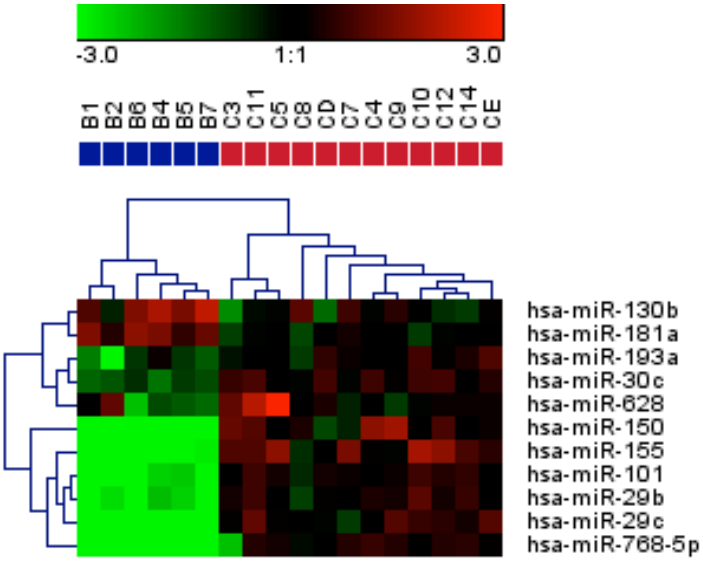

Figure 2. Comparison of our profiling data with the previously published predictor miRNA set of Zhang et al., (2009). Since the total data set is not available yet we only show the heatmap for the miRNAs specifically mentioned in the paper of Zhang et al. The heatmap contains 11 out of the 20 miRNA predictor set reported by Zhang et al separating BL from CLL. The other nine miRNAs were not flagged as present. The Heatmap indicates that BL and CLL can be separated based on the predictor set of Zhang et al.

References

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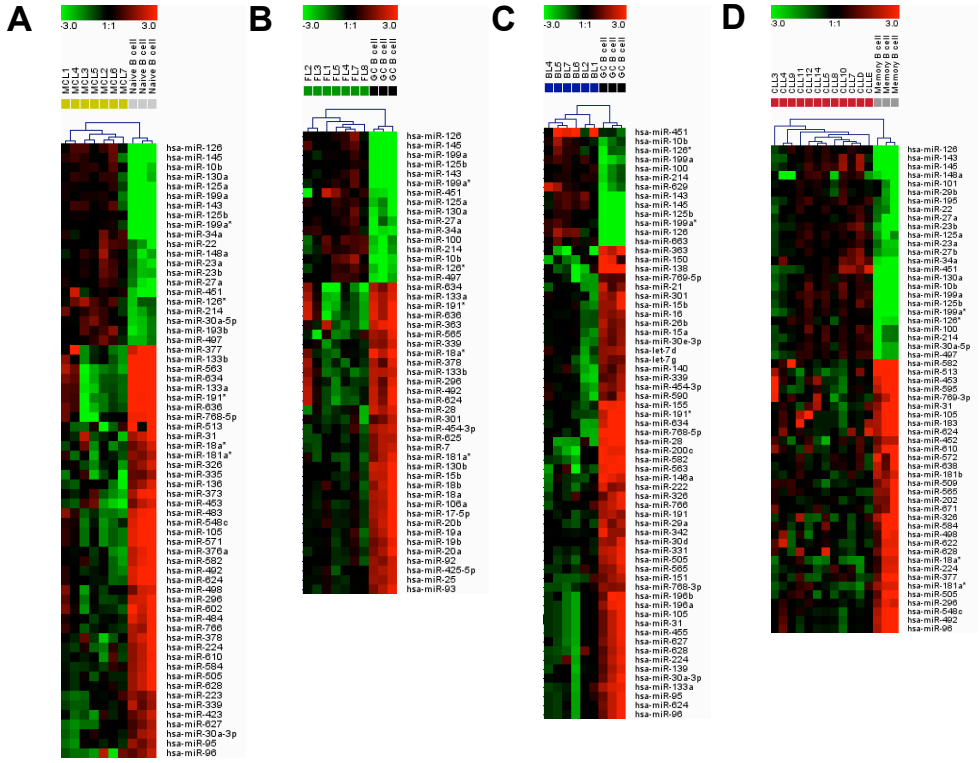

Figure S1. Heatmap of the miRNAs differentially expressed (> 4 fold) between the lymphomas and their postulated normal counterparts. A, Heatmap of the 77 miRNAs differentially expressed between MCL and naïve B cells. **B**, Heatmap of the 69 miRNAs differentially expressed between FL and GC B cells. **C**, Heatmap of the 54 differentially expressed miRNAs between BL and GC B cells. **D**, Heatmap of the 70 miRNAs differentially expressed between CLL and memory B cells.

Table S1. Overview of known c-Myc regulated miRNAs and differential expression between BL versus CLL, MCL and FL**.**

c-Myc Regulated miRNAs				Significant differences between BL and other NHL				
induced miRNA	species	ref	BS	flagged	p-value	corrected p-value	regulation	fold change
miR-7	HSA & MMU	3		yes	0.0264	0.0340	up	2
miR-17-3p	HSA	3	yes	yes	ns			
miR-17-5p	HSA & MMU	1,2,3,4	yes	yes	0.0264	0.0340	up	2
miR-18a	HSA & MMU	1,4	yes	yes	0.0004	0.0011	up	4
miR-19a	HSA	1,3	yes	yes	0.0138	0.0188	up	3
miR-19b	HSA & MMU	1,4	yes	yes	ns			
miR-20a	HSA & MMU	1,3,4	yes	yes	0.0298	0.0375	up	2
miR-20b	HSA	3		yes	ns			
miR-92	HSA & MMU	1,3,4	yes	yes	ns			
miR-106a	HSA & MMU	3,4		yes	0.0105	0.0161	up	2
miR-128b	HSA	3		yes	ns			
miR-130b	MMU	4		yes	0.0038	0.0064	up	3
miR-206	MMU	3		yes	ns			
miR-213	HSA	3		no				
miR-370	MMU	3		yes	0.0138	0.0188	up	2
miR-422a	HSA	3		no				
miR-468	MMU	3		no				
Repressed miRNA								
let-7e	HSA	3		yes	0.0002	0.0010	down	4
miR-15a	HSA & MMU	3		yes	0.0038	0.0064	down	3
miR-16	HSA & MMU	3,4		yes	0.0017	0.0035	down	2
miR-22	HSA & MMU	3		yes	0.0138	0.0188	down	2
miR-23a	HSA	3,5		yes	0.0005	0.0013	down	2
miR-23b	HSA	5		yes	0.0038	0.0064	down	2
miR-24	HSA	3		yes	0.0002	0.0010	down	3
miR-26a	HSA & MMU	3,4		yes	0.0002	0.0010	down	7
miR-26b	HSA	3		yes	0.0002	0.0010	down	4
miR-29a	HSA & MMU	3	yes	yes	0.0002	0.0010	down	9
miR-29b	HSA	3	yes	yes	0.0002	0.0010	down	8
miR-29c	HSA & MMU	3	yes	yes	0.0002	0.0010	down	16
miR-30c	HSA	3		yes	0.0059	0.0094	down	2
miR-30e-3p	HSA	3		yes	0.0051	0.0083	down	3
miR-30e-5p	HSA	3		yes	0.0002	0.0010	down	6
miR-34a	HSA & MMU	3	yes	yes	0.0006	0.0015	down	2
miR-99b	HSA	3		yes	0.0014	0.0031	down	3
miR-101a	MMU	3		yes	0.0003	0.0010	down	6
miR-125b	HSA	3	no	yes	0.0004	0.0012	down	2
miR-134	MMU	4		no				
miR-139	MMU	3		yes	0.0004	0.0011	down	7
miR-140	MMU	3		yes	0.0121	0.0179	down	3
miR-142-3p	MMU	3		yes	0.0032	0.0061	down	4
miR-144	MMU	3		yes	ns			
miR-146a	HSA & MMU	3	yes	yes	0.0004	0.0012	down	5
miR-150	HSA & MMU	3	no	yes	0.0002	0.0010	down	45
miR-195	HSA & MMU	3	yes	yes	0.0002	0.0010	down	5
miR-207	MMU	4		no				
miR-210	HSA	3		yes	ns			
miR-215	MMU	3		yes	ns			
miR-223	HSA	3		yes	0.0009	0.0020	down	4
miR-342	MMU	3		yes	0.0002	0.0010	down	3
miR-451	HSA	3		yes	ns			
miR-466	MMU	3		no				
miR-467	MMU	3		no				
miR-489	MMU	4		no				
miR-494	MMU	4		yes	0.0020	0.0039	up	3
let-7a	HSA	2		yes	0.0002	0.0010	down	3
miR-124	HSA	2		no				
miR-148	HSA	2		yes	ns			
miR-155	HSA	2		yes	0.0003	0.0010	down	8
miR-196	HSA	2		yes	0.0012	0.0027	down	4
miR-346	HSA	2		no				

Table SII. Overview of validated target genes for differentially expressed miRNAs

miRNA	Target genes	References
hsa-let-7b	BCL7A, CDC25A, CyclinD1, -D3, - A, Cdk4, HMGA2, MTPN	Huang et al, 2007, Wang et al, 2007, Sampson et al, 2007, Schultz et al, 2008, Motoyama et al, 2008
hsa-miR-7	EGFR, IRS-2	Kefas et al, 2008
hsa-miR-10b	HOXA10, HOXD10, RHOC, MEIS1	Ma et al, 2007, Garzon et al, 2008
hsa-miR-15a	BCL2, CCND1, WNT3A	Cimmino et al, 2005, Bonci et al, 2008
hsa-miR-15b	BCL2	Xia et al, 2008
hsa-miR-18a	GR, CCN2, ESR1	Uchida et al, 2008, Ohgawara et al, 2009, Liu et al, 2009
hsa-miR-17-5p	BCL2L11/BIM, E2F1-3, PTEN, CDKN1A/p21, RBL2	O'Donnell et al, 2005, Novotny et al, 2007, Wang et al, 2007, Ivanovska et al, 2008, Xiao et al, 2008
hsa-miR-23a/b	Glutaminase	Gao et al, 2009
hsa-miR-26a	EZH2, SMAD1	Sander et al, 2008, Wong et al, 2008, Luzi et al, 2008
hsa-miR-29a	BACE1, CDC42, NEF, p85α	Hariharan et al, 2005, Hebert et al, 2008, He et al, 2008, Park et al, 2009, Ahluwalia et al 2008
hsa-miR-29b	CDC42 Mcl-1, NEF, p85α	Hariharan et al, 2005, Mott et al, 2007, Park et al, 2009
hsa-miR-29c	CDC42, p85 α	Park et al, 2009
hsa-miR-93	E2F1, CDKN1A/p21	Petrocca et al, 2008, Mendell et al, 2008
hsa-miR-101	ATXN-, COX-2, EZH2	Lee et al, 2008, Strillacci, 2009, Friedman 2009
hsa-miR-106a	RB1, IL10	Volinia et al, 2006, Sharma et al, 2009
hsa-miR-125b	IRF4, PRDM1/BLIMP1	Malumbres et al, 2009
hsa-miR-130b	MECP2	Burmistrova et al, 2007
hsa-miR-138	h-TERT	Mitomo et al, 2008
hsa-miR-141	ATGF beta2, EMT, E-Cadherin, SIP1, ZEB1, ZFHX1B, ZEB2	Katoh et al, 2008, Nakada et al, 2008
hsa-miR-146a	BRCA-1, BRACA-2, CXCR4, IRAK-1, IFNγ, iNOS, LMP-1, NFκB, PTC1, ROCK1, TRAF6	Taganov et al, 2006, Labbaye et al, 2008, Dai et al, 2008
hsa-miR-148a	DNMT3b, TGIF2	Duursma et al, 2008, Lujambio et al, 2008
hsa-miR-150	MYB, P2X7	Zhou et al, 2008, Lu et al, 2008, Xiao et al, 2008, Barroga et al, 2008
hsa-miR-155	AID, RhoA, AGTR1, FGF7, ZNF537, ZIC3, IκBκε, AGTR1, BACH1, Fos, Pu.1, TP53INP1	Kong et al, 2008, Gibcus et al, 2009, Gottwein et al, 2007, Tili et al, 2007, Yin et al, 2008, Vigorito et al, 2007, Gironella et al, 2007
hsa-miR-181c	PCAF	Liu et al, 2007
hsa-miR-182	ADCY6, JAK2	Guqlielmelli et al, 2007, Xu et al, 2007
hsa-miR-195	NGN3, NeuroD, NKX2	Joqlekar et al, 2007, Mellios et al, 2008
hsa-miR-196a	ANXA1, HOX, HOXb8	Mansfield et al, 2004, Debernardi et al, 2007, Luthra et al, 2008, Kim et al 2009
hsa-miR-196b	HOXB8a	Cohen et al, 2008
hsa-miR-200c	E-cadherin, ZEB1, ZEB2, TGFβ2	Hurteau et al, 2006 -2007, Burk et al, 2008, Nakada et al, 2008, Park et al, 2008

miRNA	Target genes	References
hsa-miR-221	HOXB5, ERα, CDKN1B/p27, c-Kit, CDKN1C/p57	Visione et al, 2007, Kim et al, 2008, Zhao et al, 2008, Miller et al, 2008, Fornari et al, 2008
hsa-miR-222	Era, MYC, CDKN1B/p27, c-KIT, p57	Fell et al,i 2005, Poliseno et al, 2006, Visione et al, 2007, Zhao et al, 2008, Sun et al, 2008, Miller et al, 2008, Urbich et al, 2008, Felicetti et al, 2008, Medina et al, 2008

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Table SIII. Overview of differentially expressed miRNAs between Myc high BL and all other Myc low lymphomas

MiRNA	corrected p-value	p-value	regulation	fold change
hsa-let-7a	0,0019	0,0002	down	3,1
hsa-let-7b	0,0019	0,0002	down	4,4
hsa-let-7c	0,0019	0,0002	down	3,8
hsa-let-7d	0,0031	0,0007	down	2,8
hsa-let-7e	0,0019	0,0002	down	3,6
hsa-let-7f	0,0045	0,0012	down	2,9
hsa-let-7g	0,0019	0,0002	down	5,2
hsa-let-7i	0,0045	0,0012	down	3,0
hsa-miR-100	0,0083	0,0028	down	1,8
hsa-miR-101	0,0020	0,0003	down	5,8
hsa-miR-103	0,0104	0,0038	down	1,4
hsa-miR-106a	0,0231	0,0105	up	2,4
hsa-miR-10b	0,0020	0,0004	down	5,4
hsa-miR-125a	0,0019	0,0002	down	3,5
hsa-miR-125b	0,0022	0,0004	down	2,0
hsa-miR-126	0,0040	0,0010	down	2,9
hsa-miR-130a	0,0031	0,0007	down	2,8
hsa-miR-130b	0,0104	0,0038	up	2,9
hsa-miR-136	0,0133	0,0051	up	2,6
hsa-miR-139	0,0020	0,0004	down	6,7
hsa-miR-140	0,0256	0,0121	down	2,5
hsa-miR-141	0,0282	0,0138	down	5,9
hsa-miR-142-3p	0,0096	0,0032	down	3,5
hsa-miR-142-5p	0,0031	0,0007	down	3,3
hsa-miR-143	0,0145	0,0059	down	2,1
hsa-miR-145	0,0031	0,0007	down	2,8
hsa-miR-146a	0,0022	0,0004	down	4,7
hsa-miR-148b	0,0020	0,0003	down	2,4
hsa-miR-150	0,0019	0,0002	down	45,2
hsa-miR-155	0,0020	0,0003	down	8,2
hsa-miR-15a	0,0104	0,0038	down	2,7
hsa-miR-16	0,0057	0,0017	down	2,0
hsa-miR-17-5p	0,0498	0,0264	up	2,2
hsa-miR-181a*	0,0231	0,0105	up	3,2
hsa-miR-181b	0,0399	0,0205	up	2,1
hsa-miR-186	0,0208	0,0091	down	2,0
hsa-miR-188	0,0065	0,0020	up	3,4
hsa-miR-18a	0,0020	0,0004	up	3,9
hsa-miR-18b	0,0050	0,0014	up	2,9
hsa-miR-191	0,0022	0,0004	down	2,6
hsa-miR-193a	0,0399	0,0205	down	2,3
hsa-miR-193b	0,0282	0,0138	up	2,9
hsa-miR-195	0,0019	0,0002	down	4,6
hsa-miR-196a	0,0045	0,0012	down	4,0
hsa-miR-196b	0,0189	0,0079	down	3,2
hsa-miR-197	0,0358	0,0180	down	1,7
hsa-miR-198	0,0019	0,0002	up	3,4
hsa-miR-199a	0,0020	0,0004	down	3,0
hsa-miR-19a	0,0282	0,0138	up	3,1
hsa-miR-200c	0,0022	0,0004	down	8,0
hsa-miR-21	0,0208	0,0091	down	2,1
hsa-miR-212	0,0399	0,0205	up	2,0
hsa-miR-214	0,0104	0,0038	down	1,9
hsa-miR-22	0,0282	0,0138	down	1,8
hsa-miR-221	0,0031	0,0007	down	3,1
hsa-miR-222	0,0020	0,0004	down	4,4
hsa-miR-223	0,0034	0,0009	down	3,7
hsa-miR-23a	0,0025	0,0005	down	2,2
hsa-miR-23b	0,0104	0,0038	down	2,0
hsa-miR-24	0,0019	0,0002	down	2,5
hsa-miR-26a	0,0019	0,0002	down	7,0
hsa-miR-26b	0,0019	0,0002	down	4,5
hsa-miR-27a	0,0028	0,0006	down	2,2
hsa-miR-27b	0,0231	0,0105	down	1,8
hsa-miR-28	0,0145	0,0059	down	2,9
hsa-miR-296	0,0208	0,0091	up	1,6
hsa-miR-29a	0,0019	0,0002	down	9,4
hsa-miR-29b	0,0019	0,0002	down	7,6
hsa-miR-29c	0,0019	0,0002	down	16,2
hsa-miR-30a-3p	0,0073	0,0024	down	2,7
hsa-miR-30a-5p	0,0117	0,0044	down	2,1
hsa-miR-30b	0,0019	0,0002	down	2,8
hsa-miR-30c	0,0145	0,0059	down	1,9
hsa-miR-30d	0,0019	0,0002	down	2,1

Chapter 5

MYC regulated miRNAs play a role in the high proliferative state of Burkitt lymphoma cells

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In preparation

Abstract

MYC is an oncogenic transcription factor involved in multiple cellular processes by regulating the expression of more than 50 microRNAs (miRNAs). Several studies showed that MYC contributes to the miRNA expression profile of B-cell non-Hodgkin lymphoma (NHL). In this study we analyzed the functional role of 11 miRNAs differentially expressed between MYC-high Burkitt lymphoma (BL) and MYC-low chronic lymphocytic leukemia (CLL) as determined in our previous miRNA profiling (Robertus et al, 2010). Differential expression was confirmed on the same patient set as used for the original microarray study and on a new patient cohort. Overexpression of the ten MYC-repressed miRNAs in ST486 BL cells revealed for 6 miRNAs a more than 50% decrease in the percentage of green fluorescent protein (GFP)+ cells in a period of 18 days indicating tumor suppressive activity. One MYC repressed miRNA, i.e. miR-155, induced a more than 50% increase in GFP+ cells upon overexpression indicating a growth advantage. Inhibition of the miR-17~92 cluster, including the only MYC-induced miRNA, miR-18, revealed no significant effect on cell growth. Our findings show that overexpression of only one of the high number of known MYC regulated miRNAs, is sufficient to induce a marked effect on cell growth.

Introduction

B-cell non-Hodgkin lymphoma (NHL) accounts for approximately 90% of all lymphoid malignancies, with an annual incidence of approximately 19 cases per 100,000 individuals [1]. Burkitt lymphoma (BL) represents approximately 1-2% of all lymphoma cases and most frequently affects young children [2]. It is a homogeneous, fast growing lymphoma subtype, characterized by a chromosomal translocation between the *MYC* gene and one of the immunoglobulin heavy or light chain gene regions. These translocations result in overexpression of MYC [3] and a biological and pathological behavior that is consistent with a MYC driven proliferation of germinal center derived B cells [3].

MYC is an oncogenic transcription factor involved in cell growth, proliferation and maintenance of stem cell properties [4, 5]. The importance of MYC action was recently underlined by the identification of approximately 7,000 MYC binding sites in BL cell lines [6-8]. Besides regulation of a high number of protein-coding genes, MYC also regulates the expression of multiple microRNAs (miRNAs). MiRNAs are short (21-23 nucleotides) non-coding RNA molecules that mediate posttranscriptional silencing of their target genes [9]. MiRNAs participate in many important cellular processes like cell cycle regulation, apoptosis and proliferation [10, 11] and deregulated miRNA expression has been observed in various lymphomas [12]. There are approximately 60 know MYC regulated miRNAs of which the vast majority is repressed by MYC. A small subset, including the oncogenic miR-17~92 cluster are induced by MYC [13, 14]. Two members of this cluster, miR-19a and miR-19b, have been identified as the main contributors of tumorigenesis in a B cell lymphoma mouse model [15, 16]. Two other members of the miR-17-92 cluster, i.e. miR-17 and miR-20a, have been shown to target E2F1, a pro apoptotic member of the E2F family [14]. E2F1 itself is also a target of MYC suggesting a regulatory feedback loop [14].

In a previous study [18], we identified a clear MYC induced miRNA expression profile in BL as compared to MYC low lymphoma including chronic lymphocytic leukemia (CLL), mantle cell lymphoma and follicular lymphoma. In this study we further examined the role of 11 MYC regulated miRNAs in BL cells, including one up- and ten downregulated miRNAs.

Materials and Methods

Tissue samples

Germinal center (GC) (IgD-CD38+) B cells were purified from 3 human tonsils obtained from children undergoing routine tonsillectomy as previously described [18]. Specimens of frozen lymph nodes of 6 BL and 12 CLL patients used in the previous miRNA profiling study [18] were used to confirm the differential expression patterns of 11 selected miRNAs (see below). For the independent validation set, paraffin embedded patient material was used of 7 additional BL cases and 15 additional CLL cases. Each individual diagnosis was reviewed by an experienced hematopathologist according to the classification of the 2008 WHO [19]. The BL samples were derived from pediatric patients with an abdominal mass (phenotype: CD10+, BCL2-, EBV- and *MYC*/8q24 breakpoint positive as assessed by fluorescence in situ hybridization). The CLL samples were retrieved from patients with nodal presentation (phenotype: CD5+, CD23+, cyclinD1-). All protocols for obtaining human tissue samples were performed in accordance to the guidelines from the Institutional review board or Medical Ethical committee of the University Medical Center Groningen.

Cell lines

The ST486 cell line was cultured at 37ºC under an atmosphere containing 5% CO_2 in RPMI-1640 medium (Cambrex Biosciences, Walkersville, USA) supplemented with ultraglutamine (2mM), penicillin (100U/ml), streptomycin (0.1mg/ml; Cambrex Biosciences), and 20% fetal calf serum (Cambrex Biosciences). ST486 was purchased from ATCC. Phoenix-ampho cells were cultured in DMEM supplemented with 2mM ultraglutamine, 100 U/ml penicillin/ streptomycin and 10% fetal bovine serum (Cambrex Biosciences).

miRNA and miRNA sponge overexpression

MiRNAs were cloned as previously described [20]. Briefly, primers with 5' Xho1 and 3' EcoR1 restriction sites were designed to amplify the miRNA precursor and flanking sequence (Supplementary Table 1). MiRNA genes were amplified from DNA of a healthy control and cloned into the retroviral vector MXW-PGK-IRES-GFP. The miR-17~92 sponges were generated previously [21].

Retroviral transductions

To generate retroviral particles, Phoenix-Ampho packaging cells were $CaPO_4$ transfected using 37,5µg retroviral vector in T75 flasks. Viral particles were harvested after two days

and concentrated with Retro-X concentrator (*Clontech*, *Saint-Germain*-en-*Laye*, *France*) according to the manufacturer's protocol. Target cells were transduced with the virus by spinning at 2,000rpm for 2hrs with a final polybrene concentration of 4µg/ml.

GFP competition assay

After infection of ST486 cells, GFP expression was monitored tri-weekly for a period of three weeks. Data were acquired on a FACS Calibur flow cytometer (BD PharMingen) and analyzed using FlowJo software (version 7.6, Treestar, Ashland, OR). The GFP percentage determined at the first day of measurement (3 days post infection) was set to 1. Each GFP competition assay was performed at least twice. GFP+ cells were sorted ~2 weeks after infection using a MoFlo sorter (Dako cytomation) to monitor the induction of the overexpressed miRNA.

RNA isolation procedure and quality check

Total RNA was isolated from the purified B cell subsets, frozen tissue samples and GFP sorted cells using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The integrity of the RNA was checked on a 1% agarose gel. Only good quality RNA samples were used for subsequent analysis. RNA from FFPE tissue was extracted as reported previously by Specht et al [60]. Samples were DNAse treated using Turbo DNA free kit (Ambion, USA) according to the manufacturer's instructions. Efficiency of the DNase procedure was checked using a multiplex PCR with 5 primer sets specific for different genomic DNA loci and subsequent analysis on a 1.5% agarose gel. No PCR products were seen confirming the effectiveness of the DNAse treatment. RNA concentrations were measured on a NanoDrop® ND-1000 Spectrophotometer (Nano Drop Technologies, Wilmington, Delaware, USA).

Quantitative (q)RT-PCR

For miRNA-specific cDNA synthesis we used 5ng total RNA, the Taqman MicroRNA Reverse Transcription Kit and Taqman miRNA assays (let-7a, miR-18a, miR-26a, miR-26b, miR-29a, miR-29b, miR-29c, miR-101, miR-142, miR-150, miR-155). The qPCR reaction was carried out on 0.44ng cDNA using miRNA specific primers in accordance with the instructions supplied by the manufacturer (Applied Biosystems, Foster City, CA, USA). *RNU49* was selected as a housekeeping gene to normalize the miRNA levels as it showed the most uniform expression level in all samples and appropriate threshold Ct values.

To determine the *MYC* transcript levels, qRT-PCR was performed using a Taqman gene expression assay (Hs00153408, Applied Biosystems, Foster City, CA, USA) according to the instructions of the manufacturer. Random hexamers were used to prime the cDNA

synthesis using 300ng of total RNA and Superscript II (First-Strand cDNA Synthesis System, Invitrogen, Carlsbad, CA, USA). The PCR reactions were performed in triplicate in a volume of 20 µl with 1× qPCR master mix (Eurogentec, Liège, Belgium) and 2ng cDNA. For frozen patient material the MYC expression levels were normalized to *RPII* for RNA isolated from frozen tissue and to *U6* for RNA isolated from paraffin embedded material.

All reactions were performed on an ABI7900HT Sequence Detection System device (Applied Biosystems) using the standard program (10 min at 95°C, followed by 40 cycles of 15s at 95°C and 60s at 55°C). Relative expression levels were calculated using the delta Ct method [ΔCt= Ct(gene of interest) - Ct(housekeeping gene)]. The standard deviation (SD) of the ΔCt [SD(Ct)] was calculated using the formula [$SD(\Delta Ct)=SD(\text{gene of interest})^2$ + $SD(\text{housekeeping gene})^2$]. The factor difference was calculated as $2^{-\Delta Ct}$.

Statistical analysis

Selection of significant differential expressed miRNAs between BL and CLL samples was determined using a Kruskal-Wallis test with Bonferroni multiple testing correction using Genespring GX 12.0 software (Agilent, Santa Clara, CA) and a fold difference cut-off of four. Unsupervised clustering analysis of the 11 resulting miRNAs was performed using Genesis (Sturn et al., 2002). After median centering of the miRNA expression data over all samples, complete similarity metrics were calculated and clustering was performed using Euclidean distance and average linkage.

Significant differences in the expression levels within the qRT-PCR data of the array cases including the GC B cells, was established with a Mann-Whitney test and a Dunn's multiple comparisons as a post-test. The qRT-PCR data of the validation cases were analyzed with a Kruskal-Wallis test (Prism 6.0, GraphPad Software Inc., San Diego, CA). P values of less than 0.05 were considered statistically significant.

Validated target genes search

A list of validated target genes was compiled utilizing miRecords [22] (release date November 25, 2010) and an extensive literature search identifying published experimentally validated candidate miRNA target genes. Validation was defined as downregulation in a luciferase reporter assay, or downregulation at the protein level shown by Western blot or FACS.

Results

Selection of MYC regulated miRNAs

Selection of MYC regulated miRNAs was based on our previously published miRNA profiling study of NHL [18]. We focused specifically on the comparison between BL and CLL, since these two NHL subtypes showed the most extreme differences of the MYC regulated miRNAs. Ten of the 11 selected miRNAs showed a significant higher expression level in BL with differences in expression ranging from 4.3 to 95 fold. One miRNA, i.e. miR-18a, showed a higher level (4 fold) in BL compared to CLL. The germinal center B cells (GCB) showed an intermediate expression pattern for the ten MYC repressed miRNAs whereas the level of the MYC induced miR-18a in GC B cells was similar to the level observed in BL cases (Fig. 1).

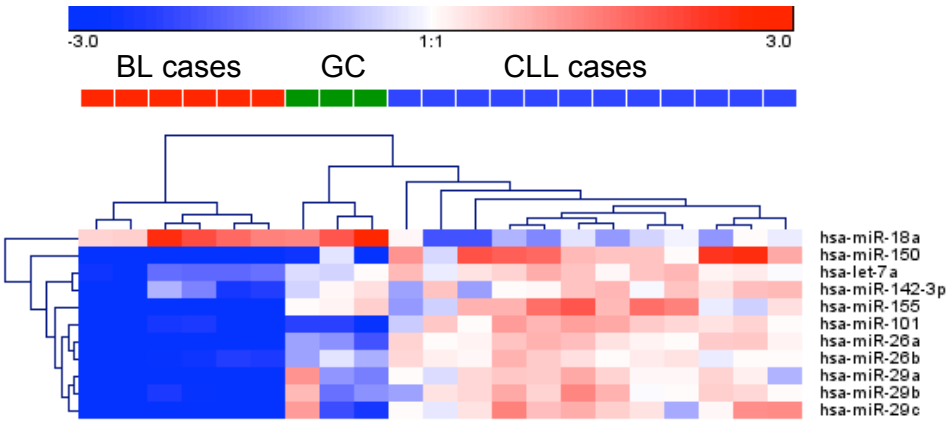

Fig. 1. Heatmap of the 11 miRNAs selected for functional studies. Heatmap of 11 miRNAs in six BL and 12 CLL samples and three GCB cell subsets of the microarray profiling data of our previous profiling study (Robertus et al., 2010) [24]. Unsupervised clustering shows a clear distinction between BL and CLL, with the GCB cells clustering together with the CLL cases.

qRT-PCR validation of candidate miRNAs

To validate the expression levels of the eleven miRNAs, qRT-PCR was performed using the same cases as used for the miRNA expression profiling study [18] (Fig. 2A). As expected, MYC levels were significantly higher in BL compared to CLL cases. Eight out of ten MYC repressed miRNAs showed significant differences in expression between BL and CLL. MiR-142-3p

and Let-7a also showed the expected trend but differences were not significant. MiR-18a showed significantly higher expression levels in BL compared to CLL.

In a second independent patient cohort consisting of 7 BL and 15 CLL cases. MYC levels were also significantly higher in BL compared to CLL cases. A significantly lower expression was observed of all ten MYC-repressed miRNAs and significantly increased levels of miR-18a in BL cases compared to CLL cases (Fig. 2B). Thus, differential expression of all MYC regulated miRNAs showed the expected expression pattern in MYC-high BL compared to MYC-low CLL.

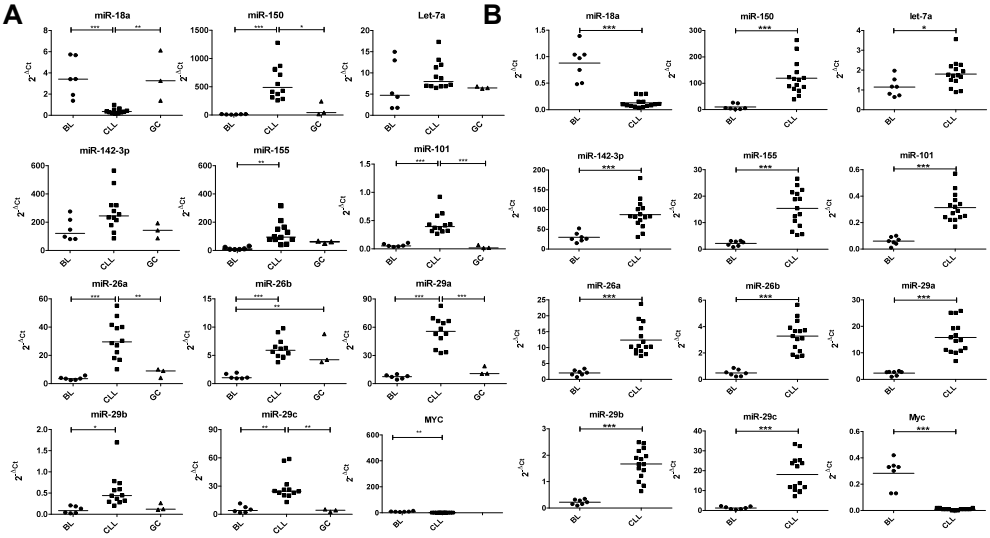

Fig. 2. Validation of differentially expression patterns of the 11 selected miRNAs. A. Nine of the selected miRNAs showed a significant difference of expression between the MYC-high BL cases and the MYC-low CLL cases used for the profiling study. The MYC repressed miR-18a showed a significant higher expression in BL compared to CLL. MYC levels were significantly higher expressed in BL compared to CLL. **B.** Seven independent BL cases and 15 independent CLL cases showed significantly different miRNA expression levels between BL cases and CLL cases for all 11 miRNAs. MYC levels were significantly higher expressed in BL compared to CLL.

MYC regulated miRNAs affect BL cell growth

To determine the effect of the MYC repressed miRNAs, we stably overexpressed the ten selected MYC repressed miRNAs in the EBV negative BL-derived cell line ST486. Overexpression of the miRNAs was validated on RNA isolated from FACS sorted GFP positive

cells. Of the ten miRNAs analyzed for overexpression, miR-150, miR-26a/b, miR-29a, let-7a and miR-155 showed overexpression ranging from 8 to 3000 fold (Fig. 3a). MiR-29c showed only a slight overexpression compared to empty vector control and no overexpression was observed for miR-29b, miR-101 and miR-142-3p. It should be noted that the endogenous Ct values were very low for miR-142-3p, which might make it difficult to further enhance expression of this miRNA.

Next, we determined the effect of overexpression of the ten MYC repressed miRNAs on growth of ST486 cells in a GFP competition assay (Fig. 3b). For six miRNAs the GFP percentage decreased more than 50% within 3 weeks. For miR-150 a 50% reduction in GFP+ cells took less then 2 days, for miR-26a and miR-26b ~6 days and for miR-29a, miR-29b

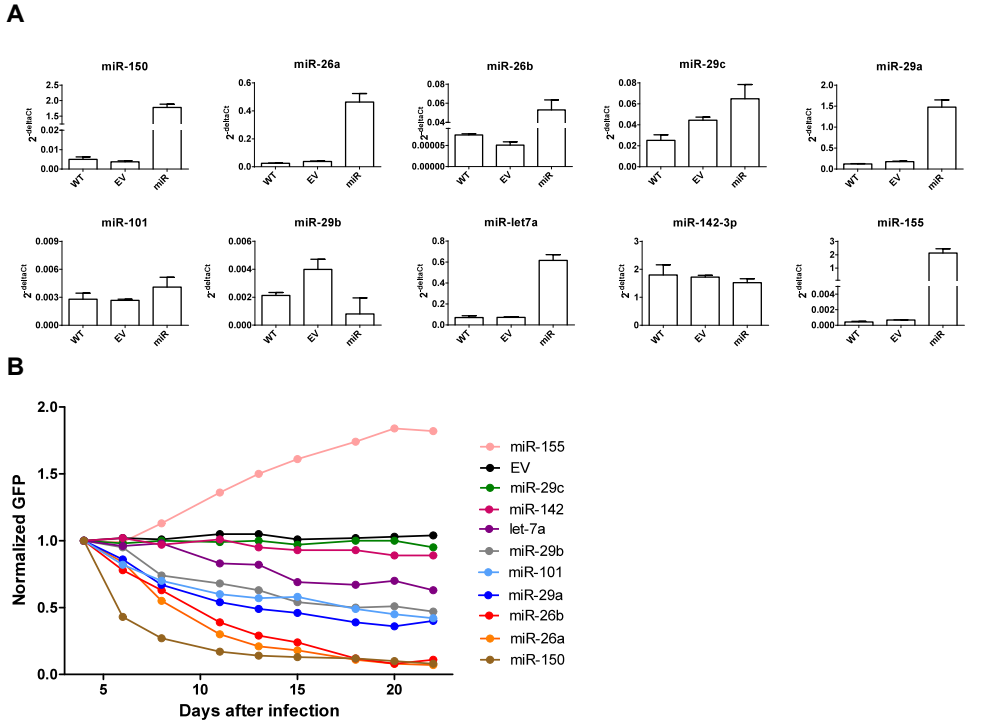

Fig 3. Overexpression and effect on growth of the 10 MYC repressed miRNAs. A. Quantification of overexpression by qRT-PCR of the ten MYC repressed miRNAs in the BL ST486 cell revealed a clear induction with fold changes ranging from 8 to 3,000 as compared to wild type and empty vector. **B.** GFP competition assay in ST486 cells for the ten MYC repressed miRNAs. The empty vector control showed a consistent percentage of GFP+ cells, as expected. Six miRNAs showed a more than 50% decrease in GFP+ cells, whereas one miRNA, miR-155 showed more than 80% increase in GFP+ ST486 cells. Two miRNAs showed no effect on proliferation and one miRNA, let-7a showed a minor decrease in GFP+ cells.

and miR-101 ~14 days. Let-7a showed a slight decrease of 20% in GFP+ cells in 3 weeks. MiR-155 was the only miRNA that showed an increase in GFP+ cells over time with a more than 80% increase in GFP+ cells in three weeks. MiR-142 and miR-29c showed no clear changes in the percentage of GFP+ cells as was the case for the empty vector control. Thus, seven out of ten MYC repressed miRNAs affected the growth of ST486 cells, six miRNAs decreased cell growth and one miRNA increased cell growth.

miR-17~92 inhibition only moderately affects cell growth

Our microarray data indicated that besides in addition to miR-18, also the other members of the miR-17~92 cluster showed higher expression levels in BL compared to CLL. This did not reach significance, probably due to large differences between individual samples (Fig. 4A). Therefore, we decided to determine the effect of inhibition of the entire miR-17~92 cluster on BL cell growth. To this end we used previously generated miRNA sponges that contain three binding sites for each miRNA seed family member present in the miR-17~92 cluster [23]. Overexpression of two miR-17~92 sponges that differ in the order of the miRNA binding sites and in the spacer sequences between the binding sites resulted in a consistent minor decrease in cell growth (~20% decrease in percentage of GFP+ cells). The empty vector control and the control with scrambled miRNA seed binding regions did not show any decrease in the percentage of GFP+ cells (Fig. 4b).

Experimentally validated target genes of the miRNA showing a phenotype

A list of validated target genes was compiled for the seven miRNAs that showed an effect on ST486 cell proliferation using miRecords in combination with a literature search. The resulting target gene list is shown in Supplementary table 2. This search revealed 8 targets for miR-26a, 5 targets for miR-26b, 14 targets for miR-29a, 19 targets for miR-29b, 24 target for miR-101, 12 targets for miR-150 and 173 targets for miR-155.

Based on a more than 50% change in GFP+ cells, miR-150, miR-26a/b and miR-155 were selected as the potentially most interesting miRNAs. Target genes of these four miRNAs with potential interest to B cell lymphoma, proliferation or tumorigenesis, included amongst others; Apoptotic BCL2-asscociated athanogen 4 (BAG4), Pleiomorphic adenoma gene 1 (PLAG1), Phosphatase and tensin homologue (PTEN), Enhancer of zeste homolog 2 (EZH2) and transcription factor E2F7 (E2F7) for miR-26a/b; c-myb, Fms-like tyrosine kinase 3 (FLT3), Early growth response 2 (EGR2), Mucin 4, cell surface associated (MUC4), V-AKT murine thymoma viral oncogene homolog 2 (AKT2), Dyskeratosis congeita 1 (DKC1), Purinergic receptor P2X, Ligand-gated ion channel (P2RX7) and Zinc finger E-box binding homeobox 1 (ZEB1) for miR-150; and Tumor protein p53-inducible nuclear protein

1 (Tp53INP1), Histione deacetylase 4 (HDAC4), Transforming growth factor beta regulator 1 (TBRG1) and Phosphatidylinositol, 3-kinase regulatory subunit alpha (PIK3R1) for miR-155.

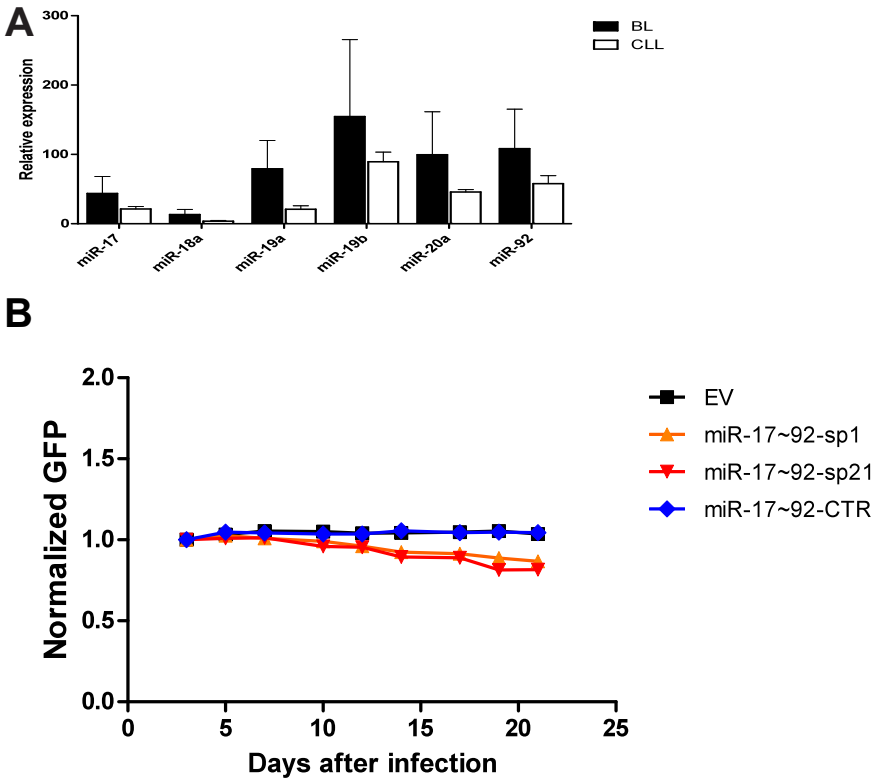

Fig 4. Inhibition of miR-17~92 in ST486 cells. A. Expression levels of all members of the miR-17~92 cluster based on the profiling data. Each miRNA showed a higher level of expression in BL cases compared to CLL cases, but the difference was significant only for miR-18a. **B.** GFP competition assay in ST486 cells infected with the combi-sponges against the miR-17~92 cluster. Simultaneous inhibition of the entire miR-17~92 cluster and other seed family members resulted in a 20% decrease in GFP+ cells. An empty vector and a combi-sponge vector with seed scrambled miRNA binding sites were used as negative controls.

Discussion

In recent studies MYC has been shown to play an important role in influencing the expression pattern of miRNAs in B-cell NHL. In this study we showed a functional effect of 7 MYC repressed miRNAs. Six miRNAs repressed ST486 cell growth and one miRNA enhanced ST486 cell growth.

MYC repressed miRNAs miR-150, miR-26a/b, miR-29a and miR-155 showed a clear overexpression in sorted GFP+ cells and also showed a clear effect on cell growth. Let-7a showed overexpression but no clear effect on cell growth. MiR-101 and miR-29b showed no overexpression but did show a negative effect on cell growth. As the Ct values for miR-101 and miR-29b were high it might be that the lack of detectable overexpression is caused by ineffective miRNA production of the construct or production of miR-101 isoforms that cannot be detected by the miR-101 assay. The latter explanation might be more likely for these two miRNAs given their effect on ST486 cell growth. MiR-142-3p and miR-29c showed no clear overexpression and also no change in cell growth. Thus no conclusions can be drawn for these two miRNAs. For miR-142-3p the endogenous levels are already very high and it is very well possible that miR-142-3p needs to be repressed in order to evaluate any possible effect on cell proliferation. For three of the ten miRNAs i.e. miR-26b, miR-29c and miR-142-3p, qRT-PCR assays were used that target a miRNA isoform that is according to the miRBASE [62] not the most abundant isoform.

The MYC-induced miRNA, miR-18a, showed higher expression levels in BL as compared to CLL. In addition, higher expression was also observed for the other members of the miR-17-92 cluster. Overexpression of sponge constructs containing all six members of the miR-17~92 clusters showed a ~20% decrease in ST486 cell growth. It has been shown that the miR-17~92 cluster can cooperate with MYC in mouse models of MYC induced B-cell lymphomagenesis. In particular miR-19 was shown to be critically involved [6, 7]. Although we observed a specific consistent minor effect with both miR-17~92 sponges it is at present unclear whether this effect is the maximal influence of the miR-17~92 cluster on BL cell growth whether this limited effect is due to incomplete inhibition of the highly expressed miR-17~92 cluster.

Of the six miRNAs that induced a clear decrease in ST486 cell proliferation, the miR-150 effect was most prominent. This is consistent with previous data [24, 25] showing that miR-150 has tumor suppressive activity in a mouse model using MYC transduced B cell lymphoma cell lines and in NK and NK/T-cell lymphoma lines. MiR-150 has 12 experimentally validated target genes, including 8 genes related to cell growth, lymphoma or tumorigenesis. One of these target genes is c-Myb. C-Myb is a known proto-oncogene with elevated expression

levels in acute myeloid and lymphoid leukemia [17, 26]. Knock down of miR-150 and c-Myb both have a pronounced affect on B-cell differentiation, indicating that c-Myb is a main target in defining the miR-150 phenotype [27]. Moreover, we recently showed an inversed correlation between miRNA-150 and c-Myb protein expression on normal tonsils [28]. In human B-cell leukemia lines, knockdown of c-Myb expression suppressed proliferation and colony formation. In AML, miR-150 functions as a tumor suppressor by repression of FLT3 and c-Myb. Both genes are involved in the HOXA9/MEIS1/FLT3/Myb/Lin28 signaling network [29]. Moreover, MYC is a known target of c-Myb [30] suggesting a possible feedback loop with miR-150. Another validated miR-150 target is early growth response 2 (EGR2) [31]. This zinc-finger transcription factor is a member of the early growth response gene (EGR) family. EGR2 is a pro apoptotic gene and plays a role in B cell development [32] and is also involved in the development of T and B cells [33]. Mucin 4, cell surface associated (MUC4) suppresses growth and malignant behavior of pancreatic cancer cells [34]. V-AKT murine thymoma viral oncogene homolog 2 (AKT2) is overexpressed in B-cell lymphoma and this overexpression is associated with increased invasion and metastasis [35]. Dyskeratosis congeita 1 (DKC1*)* is a predominantly nucleolar protein that is essential for the formation of pseudo-uridine in RNA and the telomerase RNA subunit hTR [36, 37]. Overexpression of DKC1 has been observed in solid tumors, and the silencing of DKC1 can reduce telomerase activity and rRNA pseudouridylation [38]. Zinc finger E-box binding homeobox 1 (ZEB1) is associated with adverse clinical presentation and clinical outcome in large B-cell lymphoma (LBCL) [39]. It may very well be that the high ZEB1 levels are caused by the MYC-induced repression of miR-150. The poorer prognosis in of ZEB1 positive DLBCL may thus be consistent with the poorer prognosis observed for MYC positive B cell lymphoma.

MiR-26a/b induced a more than 50% decrease in GFP positive cells in 21 days. One of the validated miR-26a/b target genes, BAG-4 has been shown to bind to anti-apoptotic protein Bcl-2 [40]. Another validated target is PLAG1. PLAG1 is a transcription factor [41] and a oncogene first discovered in studies of pleomorphic adenomas of the salivary glands [42]. Overexpression of PLAG1 was observed in CLL cells, compared to normal peripheral B cells [43]. This is not consistent with our findings that miR-26a levels are higher in CLL as compared to normal GC B-cells and BL. These differences are possibly caused by differences in control sample, being normal pheripheral B cells or BL. MiR-26a also targets oncogene EZH2 [44] and EZH2 has been shown to induce MYC expression, which might represent another feedback loop [45]. Van Kemenade et al (2001) [46] showed high expression of EZH2 in aggressive B cell lymphoma, in particular BL. This is consistent with our findings of low miR-26a expression levels in BL. The miR-26 target gene PTEN is a known tumor suppressor gene and its downregulation has been shown to play a role in MYC activation in T-ALL [47].

Overexpression of miR-155 showed a marked increase in the percentage of GFP positive ST486 cells. MiR-155 has more than 100 validated target genes, including several genes that have been linked to the pathogenesis of B cell lymphoma. MiR-155 is a well know oncomiR that has been shown to have increased expression levels in various B-cell lymphoma such as Hodgkin lymphoma, primary mediastinal B-cell lymphoma and DLBCL [48]. However, in BL miR-155 expression levels were strongly downregulated [49]. Targeting of AID by miR-155 [50, 51] may explain the pathogenetic relevance of this unexpected downregulation of miR-155 in BL. AID is involved in somatic hypermutation and class-switching of the immunoglobulin genes. Enhanced expression of AID results in the formation of chromosomal translocations involving the MYC locus [52]. As MYC translocations are the hallmark of BL, it can be speculated that low levels of miR-155 are a prerequisite for the initiation phase of Burkitt lymphomagenesis, and that the tumor cells remain at that stage of maturation with ongoing AID expression. Another mechanism of enhanced AID expression might be achieved via induction of BCL6 which results in repression of miR-155 [53]. MiR-155 targets the proapoptotic gene Tp53INP1 in gastric MALT lymphoma [54]. Via targeting HDAC4, miR-155 indirectly results in enhanced levels of BCL6 and its target genes [55]. Unpublished data by Slezak et al showed that inhibition of the protein coding isoform of TBRG1 phenocopies the miR-155 induced effects in ST486 cells, which indicates that this gene may play an important role in the observed miR-155 phenotype. These findings are consistent with the postulated role of TBRG1 as a growth inhibitory protein [56]. Another target of miR-155 is Phosphatidylinositol, 3-kinase regulatory subunit alpha (PIK3R1) [57]. PIK3R1 is required for normal functioning of PTEN. Loss of PTEN function leads to activation of AKT which in turn drives cell survival and proliferation [58, 59].

In conclusion, we have shown that MYC repressed miRNAs miR-150 and miR-26a/b strongly inhibit cell growth whereas miR-155 overexpression results in a growth advantage for BL. As MYC regulates the expression of many protein coding genes and miRNA genes it is remarkable that the modulation of a single MYC regulated miRNAs has such a profound effect on cell growth.

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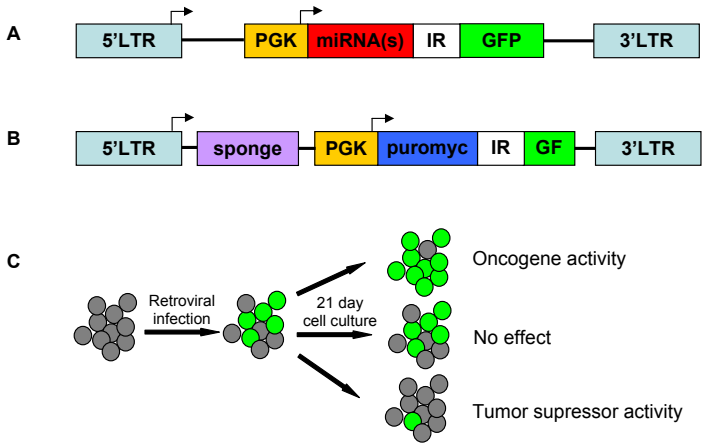
Supplementary Figure



Supplementary figure 1. Schematic overview of the viral vectors used for miRNA and sponge overexpression. A. Retroviral miRNA overexpression construct. PGK = phosphoglycerate kinase promotor, miRNA(s) = miRNA gene, IRES = internal ribosome entry site, GFP = green fluorescent protein. **B.** Retroviral combi-sponge overexpression construct. Sponge = sponge with multiple binding sites (MBS) for multiple miRNAs, PGK = phosphoglycerate kinase promotor, IRES = internal ribosome entry site, GFP = green fluorescent protein. **C.** Schematic representation of the GFP competition assay. The GFP percentage was determined tri-weekly for three weeks. Depending on the change in GFP percentage the miRNA can be considered to have oncogenic activity, no effect or tumor suppressor activity.

Supplementary Table 1. Primer sequences used for cloning the miRNA stem-loop regions

miRNA	Forward primer (5'-3')	Reverse primer (5'-3')
miR-26a-1	catgctacctcgagcctgcccactgctgaccc	ctcatgcagaattcctgtagtcctcagtcaccc
miR-26b	gtgcgaagctcgagatacatgtggaatgtcagagg	gttagcacgaattcgcacaggaaggagactggg
miR-29a	gttggctagctcgaggggatgaatgtaattgtacagg	gatggaattcgtgaaattcaacttagaaactgg
miR-29b-2	cctctctactcgagatgttgccttgtgcagccc	ccacgtatgaattcctctactgtcacctctccc
miR-29-c	ggtgctagctcgagttgggtgtcgattgtcatgg	gttagcacgaattctcagttacagaagttagtaggg
miR-101	cttgctacctcgagccttctttttcttctgcctcc	ctcacgatgaattcagaagcagaataactctccc
miR-142-3p	gtagcagactcgaggtagaggaggcaagtctgg	gtcacgatgaattcaggcctttcaggcatctgg
Let-7a-1	cttgcacctcgagtgcatagattatgcatgtagcc	ctcacgatgaattcaagaagcaaaaggtttcccc
miR-150	gttggctagctcgagcagtttctgcgactcaggg	gatggaattcaacaggacaggacacacgg
miR-155	cttggctagctcgagtgtcactccagctttataacc	catggaattcacagattgaaaaatgataaagcc

Supplementary Table 2. Overview of the experimentally validated target genes

miR-150	miR-26a	miR-26b	miR-29a	miR-29b	miR-101	miR-155					
AKT2	CCNE1	ALS2CR2	BACE1	BACE1	ACP1	ADAM10	CREB1	IGJ	MPP5	SKI	ZNF273
BCAP	E2F7	CHFR	CD276	Cav2	ATM	AGTR1	CSF1R	IKBKE	MRPL18	SLA	ZNF28
c-myb	EZH2	PTP4A1	CDC42	CDC42	CASP3	AICDA	CSNK1A1	IL13RA1	MRPS27	SLC33A1	ZNF611
CXcr4	PLAG1	SERBP1	CDK6	CDK6	DAD1	ANAPC16	CTLA-4	IL17RB	MYBL1)	SLC35F2	ZNF652
DKC1	PTEN	TAF12	col2a1	COL1A1	DICER1	APAF1	CTLA4	INPP5D	MYD88	SMAD1	ZNF83
EGR2	SERBP1		COL3A1	col2a1	EIF2C4	ARID2	CUX1	INPP5F	MYO10	SMAD2	
FLT	SMAD1		CXXC6	COL3A1	EIF3S1	ARL15	CYP2U1	INTS6	MYO1D	SMAD5	
gag-pol	TGFBR2		DNMT3A	CXXC6	EZH2	ARL6IP5	CYR61	IRAK3	MYST3	SMARCA4	
MUC4			DNMT3B	DNAJB11	HRAS	ARMC2	DCUN1D2	ITK	NARS	SOCS1	
NOTCH3			IGF-1R	DNMT3A	ICOS	ARNT	DET1	JARID2	NIK	SOX6	
P2X7			MCL1	DNMT3B	ITGB3	BACH1	DHX40	KBTBD2	Nup153	SPI1	
ZEB1			PIK3R1	IGF-1R	KLK6	BCL2	E2F2	KGF	PAK2	TAB 2	
			PTEN	Insig1	LIN28	BCORL1	EIF2C4	KIAA0430	PCDH9	TAF5L	
			SPARC	MCL1	MCL1	C10orf26	eNOS	KIAA1274)	PDCD4	TBC1D14	
				MMP-2	MED28	C16orf62	ETS1	KLHL5	PHC2	TBCA	
				PIK3R1	MOR	C3orf18	EXOSC2	LCORL	PHF14	TBRG1	
				SFPQ	MYCN	C5orf44	FADD	LDOC1	PHF17	TCF12	
				SP1	NIRF	CACNA1C	FAM135A	LEDGF/p75	PICALM	TLE4	
				TCL1A	PRDM1	CARD11	FAM177A1	LNX2	PIK3R1	TM6SF1	
					PTGS2	CARHSP1	FAM199X	LRIF3	PKN2	TNPO3	
					RAVER2	CASP3	FAM91A1	LRRC59	POLE3	TOMM20	
					RTCD1	CCDC41	FGF7	LSM14A	PRKAR1A	TP53INP1	
					SMOX	CCND1	FOXO3	MAP3K10	PSIP1	TRAK1	
					κB-Ras2	CDC40	GABARAPL1	MAP3K14	RAB11FIP2	TRIM32	
						CDC73	GATM	MAP3K7IP2	RAC1	TRIP13	
						CEBPB	GCET2	MASTL	RAPGEF2	TSGA14	
						CHD9	GCFC1	MATR3	RHEB	TSPAN14	
						CIAPIN1	GNAS	MBNL3	RHOA	UBQLN1	
						CKAP5	GOLT1B	MCM8	RIPK1	VPS18	
						CLUAP1	HBP1	MeCP2	RNF123	WEE1	
							HDAC4	MED13L)	SAP30L	WHSC1L1	
							HIF1A	MEF2A	SATB1	WWC1	
							HIVEP2	MEIS1	SDCBP	ZKSCAN5	
							HOMEZ	MITF	SECISBP2	ZNF248	
								MORC3	SHIP	ZNF254	

Chapter 6

Summary and Discussion
Future Perspectives

Summary and Discussion

Most non-Hodgkin lymphoma (NHL) subtypes are characterized by specific chromosomal aberrations and aberrant gene expression patterns, some of which have been shown to be particularly relevant to their pathogenesis. In the past decade research on microRNAs (miRNAs) has further contributed to the insight into the pathogenesis of NHL, but the picture is far from complete. MiRNAs are a group of regulatory small RNAs that belong to the non-coding RNA family. MiRNAs can post-trancriptionally regulate the expression of their target genes, which include oncogenes and tumor suppressor genes, and as such contribute to tumorigenesis. Studies on miRNAs might therefore further improve our understanding of the molecular abnormalities that underlie the pathogenesis of lymphomas and cancer in general.

This thesis focused on the role of miRNAs in the pathogenesis of lymphomas, in particular that of diffuse large B cell lymphoma (DLBCL, chapter 2 and 3) and Burkitt lymphoma (BL, chapter 3, 4 and 5).

Patients with DLBCL have a great diversity in both clinical presentation and outcome. About 4% of DLBCL present at immune privileged (IP) extranodal sites such as the testis and the central nervous system (CNS). In spite of their low incidence, these lymphomas form a very specific subset of DLBCL with amongst others, a distinct pattern of dissemination, a distinct ABC type gene expression, very high levels of AID mediated mutations, and frequent loss of HLA class I and II expression. The aim of our study presented in chapter 2 was to identify miRNAs that are specifically expressed in testicular and CNS DLBCL. We examined the expression of 15 miRNAs known to play a role in B-cell pathogenesis in 50 cases of Ann Arbor stage I and II primary DLBCL. The DLBCLs consisted of testicular, CNS, other primary extranodal non-IP and nodal DLBCL. Our analysis revealed that miR-21 and miR-19b showed the highest expression levels in the total DLBCL group and also in each of the four subgroups. Previous studies have examined the miRNA profile between GCB and non GCB DLBCL but to date no clear consensus miRNA pattern that discriminates between these two DLBCL subtypes has been found [1]. We found no significant differences between GCB and non-GCB DLBCL when we examined the combined IP, non-IP and nodal group and also not when we specifically focused on the nodal DLBCL subgroup. The lack of a consistent miRNA pattern discriminating between GCB and non-GCB may in part be related to the heterogeneous nature of the DLBCL group and the fact that most studies used different in- and exclusion criteria.

By comparing testicular and CNS IP DLBCL to non-IP extranodal and nodal DLBCL we found that 2 of the 15 miRNAs showed significant differences. One of these miRNAs

was miR-17, which showed a significant higher expression level in CNS DLBCL compared to testicular DLBCL and nodal DLBCL. These findings are in line with results published by Fischer et al. who identified miR-17 as one of the miRNAs differentially expressed when CNS DLBCL was compared to nodal DLBCL [2]. In a recent study by Baraniskin et al [3] 3 miRNAs, i.e. miR-19, miR-21 and miR-92, were identified as markers for primary CNS DLBCL. We also analyzed these miRNAs, but did not find significant differences. These differences between studies may be caused by different sample preparation, the use of a different housekeeping gene or the relative smaller sample size used in their study. MiR-17 and one of its seed family members, i.e. miR-20a, target transforming growth factor beta induced receptor type II (TGFBRII) and this results in reduced transforming growth factor-β (TGFβ) signaling [4]. The second miRNA we identified was miR-127, which was significantly higher expressed in testicular DLBCL when compared to CNS and nodal DLBCL. A relevant miR-127 target in relation to DLBCL biology is BCL6. Consistent with this, we observed an inverse correlation between BCL6 and miR-127 levels in DLBCL. Interestingly, miR-127 also targets B-lymphocyte-induced maturation protein 1 (BLIMP-1) and X-box binding protein 1 (XBP-1) [5]. MiR-127 has also been shown to be overexpressed in EBV positive BL compared to EBV negative BL and GC B-cells and to be differentially expressed during B-cell development [5]. The differences in expression of miR-127 and miR-17 may reflect the different biological identities of testicular DLBCL and CNS DLBCL.

In chapter 3 we investigated the expression levels of the primary miR-17~92 transcript, C13ORF25, and the expression pattern of the six mature miRNAs in 117 non-Hodgkin lymphoma (NHL) cases, 21 NHL cell lines and three B-cell subsets. Within the normal B-cell subsets, a significantly higher C13ORF25 level was observed in naïve B cells compared to GC B cells. In the NHL cases and cell lines, BL consistently showed the highest C13ORF25 levels. Of the mature miRNAs, miR-92 levels were most abundant in the B cell subsets, all NHL cell lines and in the MCL, BL and CLL cases. In the DLBCL cases, the miR-19b levels were higher than the miR-92 levels. The high miR-19b expression levels observed in this series of DLBCL is consistent with the high expression levels observed in IP, non-IP and nodal DLBCL studied in chapter 2. Comparison of the miRNA levels between the NHL and their normal counterparts showed a consistent strong induction of miR-19b in all cases and cell lines of all four NHL subtypes. The induction of miR-19b is consistent with its known oncogenic role [6, 7]. The marked increase of miR-19b is suggestive of an increased stability and/or increase in processing efficiency in B-cell NHL as compared to normal B cells. This is supported by a recent study of Ji et al [8] who also suggested that C13ORF25 miRNAs are affected by transcriptional mechanisms and varying processing efficiencies.

To gain a more complete insight into the role of miRNAs in B cell lymphoma we examined the miRNA expression profiles of four normal B cell subsets, MCL, FL, pediatric BL and CLL (Chapter four). Unsupervised clustering revealed two main clusters, i.e. one for the normal B cell subsets and one for the four lymphoma samples. In the normal B cell subsets 23 differentially expressed miRNAs were identified, with GC B cells and plasma cells showing the most specific patterns. 73 miRNAs were differentially expressed between the four lymphoma subtypes. Remarkably, most of these miRNAs were specifically deregulated in BL. Within this study, BL wasis the only NHL subtype characterized by high MYC expression. Consistent with this, 39 of the 49 known MYC regulated miRNAs were differentially expressed between BL and the other NHL subtypes. The strong influence exerted by MYC on the miRNA expression profile in BL suggests that MYC regulates cellular processes directly at the transcriptional level of both protein coding genes and miRNAs and indirectly by the regulatory effects of the miRNAs. Interestingly, a study by Ji et al [8] showed that the promotor region of C13ORF25 is not only bound by MYC but also by inhibitory members of the MYC family suggesting a more intricate relationship between MYC and miR-17~92. Several NHL carry an amplification of 13q31 encompassing the primary miR-17~92 transcripts (C13ORF25). We studied the influence of this amplification on the expression of miRNA members of this cluster. High expression of miR-17~92 cluster members was strongly associated with 13q31 amplification in our series of B cell NHL. Our data are consistent with the results published by Schiffman et al [9], who found miR-17 expression to be correlated with 13q31 gain in pediatric BL and Tagawa et al [10] who demonstrated a higher expression of the miR-17~92 cluster in t(8;14) positive BL and DLBCL cases with 13q31 amplification as compared to t(8;14) cases without this amplification. Also, Rinaldi et al [11] showed 13q31amplification and higher expression of miR-17~92 in human mantle cell lymphoma.

In chapter 5 we investigated the role of eleven MYC regulated miRNAs that were differentially expressed between BL and CLL as based on the expression profiling studies performed in chapter 4. The levels of ten of these miRNAs were low in the MYC-high BL cases, whereas the level of one miRNA was high in BL compared to the levels in MYC-low CLL samples. Differences in expression were validated on the same set as used for the original microarray study and confirmed on an independent set of BL and CLL cases. Overexpression of the MYC repressed miRNAs in BL revealed for six miRNAs a 50% or more decrease in the percentage of miRNA over expressing cells (based on co-expression of GFP) in a period of 18 days. This indicated that most MYC repressed miRNAs strongly influence the growth of BL cells. One MYC repressed miRNA, i.e. miR-155, showed a more than 50% increase in GFP+ cells upon overexpression indicating a strong growth advantage. It is remarkable that of the high number of known MYC regulated genes and miRNAs, overexpression of a single

MYC regulated miRNA can already have a strong influence on cell growth of a BL cell line. Recent studies [12, 13] have shown that MYC is most likely not associated with repression of gene transcription but rather acts as an amplifier of active gene transcription. Genes that were previously described as being repressed by MYC are most likely genes that are not induced by MYC or induced less than average compared to other actively transcribed genes. This may also be the case for the previously identified MYC repressed miRNAs, including those that have been studied by us. Although these miRNAs may not be actively repressed by MYC, low expression is clearly beneficial for the high proliferative state of BL cells.

In conclusion, we identified two miRNAs that characterize the testicular and CNS DLBCL cases. We further showed marked differences in the expression pattern of the oncogenic miRNA cluster, miR-17~92 with miR-92 being the most abundant miRNA in most cases, and miR-19b being the most significantly induced miRNA as compared to their normal counterparts. We further established specific miRNA expression profiles in different NHL subtypes and showed that the profile of BL is strongly defined by MYC. Finally, we identified seven MYC regulated miRNAs that have a remarkably strong impact on growth of BL cells.

Future Perspectives

Since the initial discovery of miRNAs 10 years ago it has become clear that miRNAs play an important role in the regulation of gene expression. In fact, miRNAs are predicted to regulate the expression of up to 60% of all protein coding genes. MiRNAs also play an important role in most human diseases including cancer. Many miRNAs are located in genomic regions that are either amplified or deleted in human cancers and miRNA expression studies have shown that most human cancers exhibit aberrant miRNA expression. In this thesis we focus on one type of cancer in particular, namely B-cell NHL.

Analysis of miRNA expression for the identification of differences between specific types of NHL is a topic with great clinical potential. Various miRNAs have already been identified as individual markers or as part of a specific profile for certain B-cell NHL, such as DLBCL, CLL, MCL and FL and also in differentiating BL from DLBCL [14]. In the most recent WHO classification of tumors of hematopoietic and lymphoid tissues [15], the largest category of DLBCL is DLBCL not otherwise specified (NOS). This includes all cases of DLBCL-NOS) that do not belong to a specific subtype or other category of DLBCL as defined the WHO 2007. It is in this category of DLBCL where that miRNA expression profiling may play an important role by further defining various subgroups in the future. The importance of miRNAs as diagnostic biomarkers may lay in the more subtle context of the differential diagnosis of uncertain histopathological cases. It is possible that miRNAs could play a role in the identification of so called grey zone lymphoma such as B-cell lymphoma with features intermediate between

diffuse large B-cell lymphoma and Burkitt lymphoma and B-cell lymphoma with features intermediate between primary mediastinal large B-cell lymphoma (PMBL) and classical Hodgkin lymphoma (HL). As grey zone lymphomas have an aggressive clinical course and poor outcome, identification of miRNAs that can clearly distinguish these grey zone lymphomas would be clinically advantageous. To date, no clear miRNAs markers have been found for grey zone lymphoma and as such this offers an interesting direction for practical application of miRNA expression studies.

To further define miRNA expression profiles in DLBCL we examined 14 DLBCL cases comprised of nodal, IP and one case of extranodal DLBCL NOS. MYC status was determined using FISH and GCB/non-GCB status was defined using the algorithm described by Hans et al [16]. Unsupervised hierarchical clustering of the 350 miRNAs that were expressed revealed a clear clustering of two groups of patients (Fig 1A). Localization, MYC status or GCB/non-GCB status showed no correlation with this clustering pattern. A student t-test performed between the cases that grouped in the two clusters revealed 75 significantly differentially expressed miRNAs (Fig 1B). We subsequently examined a separate set of cases previously published by Montes-Moreno et al [1]. A similar miRNA expression pattern was observed using a subset of the 75 miRNAs that were present on their array. Although further analysis of our experimental data still needs to be performed, these initial results point towards possible miRNA defined subgroups of DLBCL that to our knowledge has not yet been described.

Recent technical advances in sequencing technology of mRNA has greatly enhanced the detection of alternative splice variants, alternative poly-A usage and sequence variants of transcripts. Similarly, small RNA sequencing technology has facilitated identification of novel miRNAs, sequence variants introduced by ADAR editing and changes in prevalence of isoforms. Combined with techniques such as Ago2-RIP-Chip and variants thereof, can now be used to reliably identify miRNA target sequences in a genome-wide high throughput procedure. In a recent study by Deng et al (2011) [17], a specific regulation of a selection of transcript isoforms by miRNAs has been observed. Similarly, we also observed a specific regulation of the protein coding isoforms of TBRG1 by miR-155. This indicates that miRNA-target gene regulation is a complex process regulated by multiple factors.

The expansion of our knowledge on the miRNA expression pattern in B-cell NHL has opened novel pathways for clinical applications. Specific miRNA expression patterns have been used as a method for predicting therapy response, detection of minimal residual disease and survival. Detection of such miRNAs in plasma, serum and CNS fluid offers a unique opportunity for minimally invasive monitoring methods. Four miRNAs, i.e. miR-15a, miR-16-1, miR-29c and miR-155, were downregulated, and miR-34a was upregulated in serum of DLBCL patients [18]. In a study by Ohyashiki et al [19] miR-92a plasma levels were

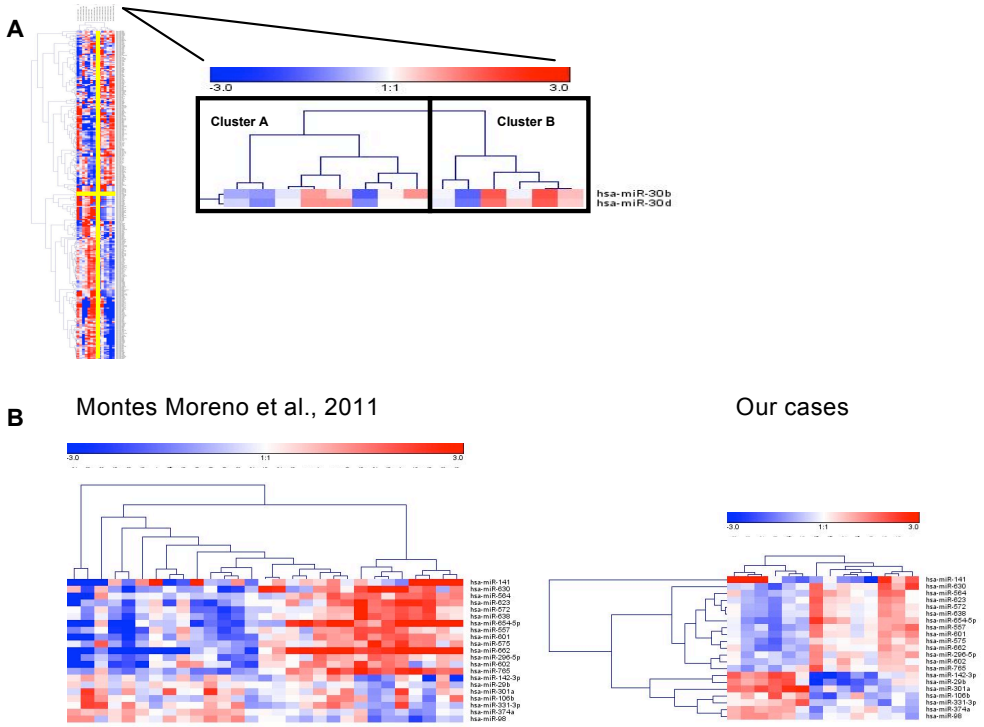


Figure. 1. Expression profiles of DLBCL cases **A.** Unsupervised clustering of 14 DLBCL cases revealed two distinct clusters A and B. No correlation was found with parameters such as location and MYC status. **B.** A Mann Whitney test revealed 74 significantly differentially expressed miRNAs between cluster A and B. 21 of the 74 miRNAs were available in a seperate dataset published by Montes Moreno containing 29 DLBCL cases. Supervised clustering of the same 29 cases using the same order of miRNAs as depicted in our cases showed a similar but less distinct expression pattern.

found to be downregulated in DLBCL, FL and T cell NHL compared to healthy subjects. In chapter three we found miR-19b as the most abundant miRNA in DLBCL cases and we also observed relatively high levels of miR-92a. Differences in results may be caused by patient selection. In our study we used only Ann Arbor stage I and II primary DLBCLs. In the study by Ohyashiki et al, plasma levels of miR-92a were measured in DLBCL patients in complete remission, relapse patients and also patients with Ann Arbor stage 3 and stage 4 DLBCL. Moreover, normalization procedures especially for circulating miRNAs need to be further optimized. Ohyashiki normalized all miRNA levels to the most abundant miRNA in their samples, although they observed great variation in the absolute levels of this miRNA. Additional studies are required to clearly establish these putative differences between tissue and plasma.

The critical involvement of specific miRNAs in different types of cancer has sparked the interest in miRNAs as a therapeutic option. MiRNAs target multiple genes and offer the possibility to impact tumor phenotype by either repressing individual oncogenes but also allow for the possibility of targeting a specific subset of genes and as such affecting selected pathways. Approaches to utilize miRNAs for therapy broadly fall into two categories: 1); downregulation of oncogenic miRNAs using antagomiRs, antisense oligonucleotides, sponges or locked nucleic acid (LNA) constructs and 2); replacement of repressed tumor suppressor miRNAs. A promising miRNA that has been utilized for therapeutic purposes is miR-122. Suppression of miR-122 in chimpanzees chronically infected with hepatitis C virus (HCV) using locked nucleic acid (LNA) modified oligos complementary to miR-122 caused long-lasting suppression of HCV virema [20]. LNA-modified antisense oligonucleotide miravirsen are currently in phase 2 clinical trials for treatment of hepatitis C virus (HCV) infection.

MiR-10b has been shown to promote metastasis and invasion by targeting HOXD1 and TIAM1 [21, 22] in breast cancer. Molyneaux et al [23] showed that treatment with miR-10b antagomiRs reduced metastasis of breast tumors to the lungs in mice. As many miRNAs are downregulated in tumors, restoring tumor suppressive miRNAs is another potential therapeutic possibility. In our study we identified 6 MYC regulated miRNAs, i.e. miR-150, miR-26a, miR-26b, miR-29a, miR-29b and miR-101 that strongly inhibit the growth of BL cells. A study by Kota et al [24] showed that therapeutic delivery of miR-26a suppresses tumorigenesis, reduces cancer cell proliferation and induces tumor cell specific apoptosis in a murine liver cancer model. Specific delivery of miR-26a and the five other miRNAs we identified in our study could provide therapeutic options for BL.

In this thesis we focus on the expression and role of miRNA in NHL. MiRNAs belong to the large group of noncoding RNAs (ncRNAs) which are generally divided by their size. Long ncRNAs (lncRNAs) are > 200 bp and consist of long intergenic noncoding (linc) RNAs, pseudogenes, ribosomal genes and antisense RNAs. Non-protein coding RNAs < 200nt are called small ncRNAs and include small nuclear and nucleolar RNAs, ribosomal RNAs, transfer RNAs, Piwi interacting RNAs and miRNAs. As more knowledge has been gained in recent years about these noncoding RNAs their role in lymphogenesis is becoming apparent [25]. For example lncRNA HOTAIR has been shown to be upregulated in primary breast tumours and metastases. Another lncRNA, Malat1 has been shown to be important for colorectal cancer invasiveness and is upregulated in cancers such as neuroblastoma and osteosarcoma.

Future studies on the various species of ncRNA in lymphoma will reveal how widespread their involvement in lymphomagenesis is.

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Nederlandse samenvatting

Micro (mi)RNAs zijn korte, 22 nucleotiden (nt) lange, regulatoire RNA moleculen die onderdeel uitmaken van de familie van niet coderend RNAs. MiRNAs kunnen gericht de expressie van bepaalde genen beïnvloeden, onder andere van tumorsuppressor genen en oncogenen. De laatste 10 jaar heeft onderzoek op het gebied van microRNAs (miRNAs) bijgedragen aan een beter inzicht in de pathogenese en het gedrag van diverse vormen van kanker waaronder B-cel lymfomen en leukemieën.

Dit proefschrift richt zich op de rol van miRNAs in de pathogenese van B-cel lymfomen, specifiek die van het diffuus grootcellig B-cel lymfoom (DLBCL, hoofdstuk 2 en 3) en van het Burkitt lymfoom (BL, hoofdstuk 3, 4 en 5).

Patiënten met DLBCL tonen een grote diversiteit aan klinische symptomen en in de uiteindelijke uitkomst van de ziekte. Het doel van de in hoofdstuk 2 beschreven studie was om te onderzoeken of er in deze heterogene ziekte groepen met een verschillend miRNA expressie patroon zijn te vinden. Hiervoor hebben we ons beperkt tot de expressie van 15 miRNAs. Deze miRNAs waren geselecteerd op basis van hun mogelijke rol in de pathogenese van B-cel lymfomen. De 50 DLBCL waren geselecteerd op basis van duidelijke verschillen in primaire lokalisatie: ze bestonden uit conventionele nodale DLBCL, maar ook uit primaire extranodale DLBCL. Om onduidelijkheid over localisatie te voorkomen betrof het in alle gevallen een DLBCL met zeer beperkte uitzaaiing, dat wil zeggen Ann Arbor stadium I en II. Onze analyse liet zien dat alle DLBCL een zeer hoge expressie hadden van miR-21 en miR-19. DLBCL kunnen op basis van RNA en eiwit expressie onderscheiden worden in van kiemcentrum origine (“germinal centre B-cell” / GCB) of van geactiveerde B cel origine (“activated B-cell” / ABC, ook wel non-GCB genoemd). Voor deze twee subsets zijn in de literatuur verschillende miRNA profielen beschreven, echter een duidelijke consensus is tot op heden niet gevonden. In onze studie vonden wij geen significante verschillen tussen GCB en ABC DLBCL. Wij vonden ook geen verschil toen wij gericht keken binnen alleen de nodale DLBCL subgroep. Het ontbreken van een eenduidig discriminerend miRNA expressiepatroon tussen GCB en ABC DLBCL is mogelijk het gevolg van de heterogene aard van DLBCL en het feit dat de meeste studies verschillende inclusie- en exclusiecriteria hanteren en dat een onderscheid tussen nodaal en extra-nodaal onvoldoende is.

Ongeveer 4% van DLBCL presenteert zich in de testis en het centrale zenuwstelsel (CNS). Dit zijn twee extranodale lokalisaties waar normaal gesproken geen immunologische afweer voorkomt. Deze specifieke subgroep wordt de “immune privileged” of IP-DLBCL genoemd. Deze IP-DLBCL worden gekenmerkt door een specifiek patroon van uitzaaiing, een ABC genexpressie patroon, hoge frequentie van AID-gemedieerde mutaties en frequent

voorkomen van verlies van HLA klasse I en II expressie. Door testis en CNS IP-DLBCL te vergelijken met andere non-IP extranodale en nodale DLBCL vonden wij miRNAs met een significant verschillend expressie niveau. Een van deze miRNAs is miR-17, deze liet een hoge expressie zien in CNS DLBCL in vergelijking tot testis DLBCL. Onze bevindingen komen overeen met eerder gepubliceerde resultaten waarbij miR-17 geïdentificeerd werd als een van de miRNAs die verschillend tot expressie komen wanneer CNS DLBCL wordt vergeleken met nodal DLBCL. In een recente studie werden miR-19, miR-21 en miR-92 geïdentificeerd als kenmerkend voor primair CNS DLBCL. Wij hebben deze drie miRNAs ook geanalyseerd maar vonden geen significante verschillen. De verschillen tussen de studies zijn mogelijk veroorzaakt door verschillen in de methode, het gebruik van andere controle huishoudgenen of het relatief klein aantal lymfomen dat werd bestudeerd. De tweede miRNA die wij in onze studie hebben geïdentificeerd is miR-127. Deze miRNA komt significant hoger tot expressie in testis DLBCL in vergelijking tot CNS DLBCL en nodale DLBCL. Eén van de relevante miR-127 target genen is BCL6. BCL6 speelt een belangrijke rol in de biologie van DLBCL. In overeenstemming hiermee vonden wij een omgekeerde correlatie tussen BCL6 en miR-127 expressie in DLBCL. De verschillen in expressie van zowel miR-17 als miR-127 in testis DLBCL en CNS DLBCL reflecteren dus zeer waarschijnlijk belangrijke stappen in de normale B cel ontwikkeling.

In hoofdstuk 3 hebben we de expressie van het primaire miR-17~92 transcript en de mature miRNAs in B-cel lymfomen onderzocht. Dit oncogene miRNA cluster bevat zogenaamde "stem-loop" structuren (de DNA basis structuur waar een miRNA van kan worden afgelezen) voor 6 miRNAs, miR-17, miR-18a, miR-19a, miR-20a, miR-19b en miR-92. Het primaire transcript heeft twee iso-vormen. Bij de lange isovorm bevinden de stem-loop structuren zich in het exon en bij de andere iso-vorm in een intron. Opvallend is dat de expressie van de individuele mature miRNAs sterk kan verschillen terwijl ze alle zes afkomstig zijn van hetzelfde primaire transcript. Om dit nader te onderzoeken hebben wij de hoeveelheid van de zes miRNAs bepaald in drie B-cel maturatie stadia, 117 B-cel non-Hodgkin lymfomen en in 21 B-cel lymfoom cellijnen. De 4 maturatie stadia waren naïeve B-cellen, kiemcentrum B-cellen, geheugen B-cellen en plasmacellen, allemaal selectief geïsoleerd uit hyperplastische tonsillen. De expressie van miR-92 was in normale B-cel subsets en drie B-cel lymfoom subtypen 10 tot 100x hoger dan de vijf andere miRNAs. Alleen in DLBCL was de expressie van miR-19 hoger dan de expressie van miR-92. In vergelijking met de normale voorloper cellen zagen we een sterk verhoogde expressie van met name miR-19b in alle lymfoom subtypen. Voor miR-92 en de andere vier miRNAs werden echter geen verhoogde expressieniveaus gevonden ten opzichte van de normale B-cellen. De sterke inductie van miR-19b komt overeen met de oncogene effecten in muismodellen. Onze data suggereren

dat er op individueel miRNA niveau een sterke regulatie is van de hoeveelheid mature miRNAs. Mogelijk wordt dit bereikt door verschillen in de processing efficiency van de zes precursor miRNAs en/of door verschillen in de stabiliteit van de mature miRNAs.

Om een completer inzicht in de rol van miRNAs in B-cel lymfomen te krijgen hebben wij in hoofdstuk 4 de miRNA expressieprofielen onderzocht van de vier normale B-cel maturatie stadia (zie hoofdstiuk 3) en vier maligne lymfomen die ook een duidelijk maturatie stadium vertegenwoordigen: mantelcel lymfoom (MCL; reflecteert naïeve B cellen), folliculair lymfoom (FL; reflecteert kiemcentrum B-cellen), Burkitt lymfoom (BL; reflecteert ook kiemcentrum B-cellen) en chronische lymfatische leukemie (CLL; reflecteert geheugen B cellen). Ongesuperviseerd clusteren van de miRNA expressie data toonde twee hoofdgroepen aan, namelijk één voor de normale B-cel maturatie stadia en één voor de vier lymfoom subtypen. In de normale B-cel maturatie stadia vonden wij 23 differentieel tot expressie komende miRNAs waarbij de GC B-cellen en plasmacellen de meest uitgesproken patronen toonden. 73 miRNAs kwamen differentieel tot expressie tussen de vier lymfoom subtypes. Opvallend was het feit dat de meeste van deze miRNAs specifiek gedereguleerd zijn in BL. Binnen het kader van deze studie was BL het enige NHL subtype dat gekarakteriseerd wordt door hoge MYC expressie. In overeenstemming hiermee vonden wij dat 39 van de 49 bekende MYC gereguleerde miRNAs differentieel tot expressie komen tussen BL en de andere lymfoom subtypes. De sterke invloed die wordt uitgeoefend door MYC op het miRNA expressieprofiel in BL suggereert dat MYC de genexpressie op verschillende manieren reguleert, namelijk direct op het niveau van transcriptie van eiwit coderende genen en indirect door middel van regulatoire effecten van miRNAs. Andere studies hebben aangetoond dat de promotor van miR-17~92 niet alleen gebonden wordt door MYC maar ook door inhibitoire leden van de MYC familie. Dit suggereert dat er een complexe relatie bestaat tussen MYC en miR-17~92. Meerdere lymfoom subtypen bevatten een amplificatie van het 13q31 gebied dat ook de genomisch sequenties van het *mir-17~92* gen bevat. Wij hebben de invloed van deze amplificatie op expressie van het primaire miRNA transcript en de mature miRNA clusterleden bestudeerd. Hoge expressie van het miR-17~92 cluster en de zes mature miRNAs was sterk geassocieerd met de aanwezigheid van een 13q31 amplificatie. Onze data komen overeen met de resultaten van andere studies waarin miR-17 expressie gecorreleerd is met 13q31 amplificatie in pediatrische BL en waarin een hogere expressie van miR-17~92 in BL en DLBCL geassocieerd is met amplificatie van 13q31. Daarnaast is een hogere miR-17~92 expressie ook aangetoond in MCL met een 13q31 amplificatie.

In hoofdstuk 5 hebben wij de rol van 11 MYC gereguleerde miRNAs onderzocht die differentieel tot expressie komen tussen BL en CLL, gebaseerd op de expressiestudies van hoofdstuk 4. De expressieniveaus van tien MYC onderdrukte miRNAs waren verlaagd

in BL (allen met een hoge MYC expressie) en het MYC geïnduceerde miRNA toonde hoge expressie in BL in vergelijking met de CLL (allen met een lage MYC expressie). Verschillen in expressieniveaus werden gevalideerd op dezelfde set van lymfomen als gebruikt in de oorspronkelijke miRNA expressiestudie in hoofdstuk 4 en in een onafhankelijke set van CLL en BL. Om de functie van deze miRNAs te bestuderen hebben we de 10 miRNAs die verlaagd waren in BL cases tot overexpressie gebracht in een BL cellijn. Overexpressie van de MYC onderdrukte miRNAs in BL toonde voor zes van de miRNAs een afname in groei van tenminste 50% gedurende een periode van 18 dagen. Dit geeft aan dat deze zes MYC onderdrukte miRNAs een sterke invloed uitoefenen op de groei van BL cellen. Een van de MYC onderdrukte miRNAs, miR-155, toonde een meer dan 50% toename in groei bij overexpressie, hetgeen indicatief is voor een toegenomen proliferatie. Het is opmerkelijk dat overexpressie van slechts één enkel MYC gereguleerd miRNA al zo'n sterke invloed kan hebben op de proliferatie van BL cellen, omdat MYC een zeer groot aantal eiwit coderende en miRNAs reguleert. Recente studies hebben aangetoond dat MYC waarschijnlijk niet zozeer geassocieerd is met het onderdrukken van gentranscriptie maar meer een algemene versterker is van de transcriptie van die genen die in een bepaalde cel op dat moment al aan staan. Genen die voorheen als MYC onderdrukte genen werden beschreven zijn dan hoogstwaarschijnlijk genen die niet of in mindere mate door MYC worden geïnduceerd in vergelijking tot andere actief getranscribeerde genen. Mogelijk is dit ook het geval voor MYC onderdrukte miRNAs en dus ook de miRNAs beschreven in deze studie. Ondanks dat deze miRNAs mogelijkerwijs niet actief door MYC worden onderdrukt is lage expressie van deze miRNAs klaarblijkelijk van belang voor de proliferatie van BL cellen.

Samenvattend hebben wij in dit proefschrift twee miRNAs geïdentificeerd die CNS en testis DLBCL karakteriseren. Wij laten ook zien dat er duidelijke verschillen zijn in de expressiepatronen van de zes mature miRNAs afkomstig van het oncogene miRNA cluster, miR-17~92, met miR-92 als meest voorkomend miRNA in het merendeel van de gevallen en miR-19b als het meest significant geïnduceerde miRNA in vergelijking met normale B-cellen. Tevens hebben wij aangetoond dat er specifieke miRNA expressieprofielen in verschillende lymfoom subtypes zijn en dat het expressieprofiel van BL sterk beïnvloed wordt door MYC. Als laatste hebben wij zeven MYC gereguleerde miRNAs geïdentificeerd die een zeer sterke invloed uitoefenen op de proliferatie van BL cellen.

Acknowledgments

At last, the light at the end of the tunnel. It's been an interesting and sometimes arduous road taken but none of what has been written in this thesis could have been achieved without the help and input of others. Teamwork is the name of the game.

First and foremost I'd like to thank my promotores Prof. dr. Anke van den Berg and Prof. dr. Philip Kluin. Dear Anke and Philip, your knowledge, dedication and patience have always amazed me and will prove to be an example for me in the future. Your support and kindness in particular last year, is something I will not forget.

Dear Joost, as my copromotor you were unsurpassable when it came to keeping me going in the last fase of my thesis. The retsina will flow!

Members of the reading committee Prof. dr. Ed Schuuring, Prof. dr. Steven T. Pals and Prof. dr. Edo Vellenga, thank you for time and effort. A special thanks to Ed with whom I started my thesis.

Also a special thanks to Gert Harms and Tjasso Blokzijl who helped me with getting started in the lab. Debora de Jong, Nancy Halsema, Riks Nynke Schakel and Gertrud Kortman for their help with the various experiments described in this thesis. Of course, everybody in the DNA lab, a big thanks as well for their help along the way! And last but not least, Freke de Jong for logistical support.

To my co-authors, Daphne de Jong, Klaas Kok, Gustaaf van Imhoff, Bart-Jan Kroesen, Arjan Diepstra, Callista Weggemans, Rogier Reijmers, Yvonne Swart, Stefano Rosati, Marije Booman and Berend Tillema. Thanks for the opportunity of working with you and your input throughout this thesis. A special thanks to Klaas for his time spent on the SNP array data analysis.

I'd like to thank Lydia Visser for her support. Also a big thank you to Hans Vos and all the other members of the O and O lab.

Prof. dr. Wim Timens, dear Wim, as head of department, thank you for allowing me the opportunity to finish my thesis in relatively quiet circumstances.

Prof. dr. Harry Hollema, dear Harry, as opleider you were a pillar of support during my seven years as AGIKO. Your door was always open and your calm approach to the problems which arose when combining a PhD with a residency were invaluable! A very heartfelt Thank You.

Dear Wilfred, supervisor and most importantly, friend. Thanks mate! See you and Jane in Rotterdam!

Of course thank you to everybody from the histology lab, cytology lab, cutting room, IT department (thanks Roel), and in particular thank you to Jose Kop, and her colleagues in administration.

My fellow roommates and co-researchers, Wouter Pattje, Michiel Schrijvers, Evert-Jan Boerma, Johan Gibcus, Lu Ping Tan and Wang Miao. Lu Ping, inscrutably cool person and friend. You still ended up being my paranymph! Miao Wang, my enormous admiration for you aside, Paulien and I are waiting for you to come on over to the Netherlands and grace us with a visit.

A thank you to all staff members and (former) residents of the Pathology department, two of whom I'd like to mention by name, Evelien Duiker and Sander de Haas.

Wim en Fransje, Jitte en Gwen, Roos en Cees, José en Jan, Wim en Truus, Paula en Paul, Mariet, Sanne, bedankt voor jullie steun en enthousiasme.

Willem van Wolferen, bedankt!

Jan Jansen, bedankt voor je hulp met de laatste loodjes en alle goede adviezen.

Femke en Willem-Jan, bedankt voor alle support!

Lonneke, ik kan alleen zeggen; "Oh Joy"!

Dear Lorrain, one of the kindest and supportive persons I know, thank you ! And Kevin as well.

Ravi, my best man and buddy, I know you and Suhela couldn't make it this time. But I just want to say thanks for always being there for me. You've been along for the ride from the very beginning. Finally finished …Yeah!

Dirk Jan en Feiko, zonder twijfel mijn meest dierbare vrienden en ook studiegenoten. Beiden inmiddels gepromoveerd en dermatoloog. Natuurlijk promoveer ik als laatste van ons drieën. De uurtjes in de Krommerijn waren toch niet voor niets.

Beste Erwin, wat een geweldig toeval dat wij elkaar weer troffen in Groningen bij de pathologie (de noordoostelijke pathologenclub). En blijkbaar heeft het toeval nog meer in petto! Bedankt voor je vriendschap, kameraadschap en dat je mijn paranymf wilt zijn!

Lieve Sandra, van iedereen uit Utrecht ken jij mij het langste, en als 'geadopteerd broertje' ben ik erg blij en trots dat jij mijn paranymf wilt zijn.

To my parents, dear mummy and daddy, there is so much to thank you for. Thank you both for always being there for me and for the opportunities you gave me, material and immaterial. Dad, thanks for the inquisitiveness and perseverance I seem to have inherited from you. Mummy, I wish you were here.

Joséphine, apple of my eye. Already you seem to be growing up so fast. More time to play!

Mijn mooiste Paulien, eindelijk is het zover, onze nieuwe avontuur in Rotterdam is al begonnen en nu zijn wij als gezin weer met meer tijd samen. Jij hebt mij altijd in alles ondersteund en gefaciliteerd. Nu is het mijn beurt.