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Zvýšená exprese mikroRNA miR-155 a snížená exprese její cílové mRNA kódující transkripční faktor PU.1 ve vzorcích tumorů z lidských lymfomů.

Up-regulation of microRNA miR-155 is reflected by low levels of its target mRNA encoding transcription factor PU.1 in primary tumors of human lymphomas.

Diplomová práce

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Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

Praha, 29. 4. 2013 Hana Hušková

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CONTENTS

ABSTRACT

Lymphomas are heterogenous class of diseases characterized by proliferation of a malignant lymphocyte clone. MicroRNA miR-155 was found to be a key molecule in immune response, namely in inflammation and germinal reaction of B cells. On the other hand, miR-155 can drive lymphoproliferation in mouse and its levels were found to be elevated in certain lymphoma types in human. MiR-155 down-regulates expression of its target gene PU.1, a hematopoietic transcription factor important for B cell differentiation. Expression of the gene encoding miR-155, known as *MIR155HG*, is controled by several transcription factors, among them MYB, a member of an oncogenic E-box protein family. Levels of MYB itself are controled by microRNA miR-150. In this study, we measured levels of miR-155, PU.1, MYB and miR-150 in lymph nodes of patients with chronic lymphocytic leukemia/small lymphocytic lymphoma (B-CLL/SLL, N=20), diffuse large B-cell lymphoma (DLBCL, N=24), follicular lymphoma (FL, N=29), Hodgkin lymphoma (HL, N=25), marginal zone lymphoma (MZL, N=13), and mantle cell lymphoma (MCL, N=10). We also measured levels of these molecules in lymph nodes with the finding of strong inflammation (N=4). We found that patients of all the diagnoses except of MCL display heterogeneously elevated levels of miR-155 and correspondingly decreased levels of PU.1, possibly in the context of prognosis or other clinical or biological characteristics. This study can add to better understanding of pathogenesis of certain types of lymphoma.

KEYWORDS

microRNA, miR-155, PU.1, MYB, miR-150, lymphoma, gene expression, PCR

ABSTRAKT

Lymfomy jsou pevné nádory lymfocytů, především řady B. V některých lidských lymfomech byla zjištěna zvýšená hladiny mikroRNA miR-155. Tato mikroRNA je důležitá zejména v imunitní reakci, je však take schopna indukovat maligní lymfoproliferaci u myši. Jedním z patogenních mechanismů miR-155 v lymfomech je patrně snížení exprese transkripčního faktoru PU.1, který je v určité hladině nezbytný pro správnou diferenciaci B lymfocytů. Exprese miR-155 je v lymfomech aktivována různými transkripčními faktory, z nichž jedním je MYB z onkogenní rodiny E-box proteinů. Hladina MYB je zase regulována pomocí mikroRNA miR-150. Změřili jsme hladinu mikroRNA miR-155, PU.1, MYB a miR-150 ve vzorcích uzlin pacientů s lymfomy, konkrétně s diagnózami: chronická lymfocytární leukémie/lymfom z malých lymfocytů (B-CLL/SLL, N=20), difuzní velkobuněčný B lymfom (DLBCL, N=24), folikulární lymfom (FL, N=29), Hodgkinův lymfom (HL, N=25), lymfom marginální zóny (MZL, N=13) a lymfom buněk plášťové zóny (MCL, N=10). Expresi zmíněných molekul jsme změřili rovněž v lymfatických uzlinách s nálezem silného zánětu (N=4). Zjistili jsme, že hladina miR-155 je zvýšená u všech diagnóz mimo MCL, a že hladina PU.1 je naopak snížená. Naše výsledky dále naznačují, že hladina miR-155 a PU.1 v individuálních případech souvisí s některými klinickými či biologickými charakteristikami daného nádoru. Tato studie může přispět k lepšímu pochopení patogeneze některých typů lymfomů.

KLÍČOVÁ SLOVA

mikroRNA, miR-155, PU.1, MYB, miR-150, lymfomy, genová exprese, PCR

LIST OF ABBREVIATIONS

ABC	activated B cell-like
B-CLL/SLL	B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma
BCR	B-cell receptor
bic	B-cell integration cluster
BL	Burkitt lymphoma
C	constant
ddH_2O	double-distilled water
cHL	classical Hodgkin lymphoma
DLBCL	diffuse large B-cell lymphoma
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
FL	follicular lymphoma
GCB	germinal center B cell-like
GTFs	general transcription factors
H	heavy
HL	Hodgkin lymphoma
HRS	Hodgkin/Reed-Sternberg
HSC	hematopoietic stem cell
IGH	immunoglobulin heavy-chain gene
IGVH	variable region of immunoglobulin heavy-chain gene
J	joining
L	light
miR	microRNA
MIR155HG	miR-155 host gene
mRNA	messenger RNA
MC	mixed cellularity
MCL	mantle cell lymphoma
MZL	marginal zone lymphoma
NHL	non-Hodgkin lymphoma
no.	number
NS	nodular sclerosis
nt	nucleotide(s)
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PET+	positron emission tomography positive
PET-	positron emission tomography negative
Q-PCR	quantitative real-time PCR
RISC	RNA-induced silencing complex
RLC	RISC-loading complex
RNA	ribonucleic acid
Treg	CD25-positive regulatory T cells
V	variable

1 INTRODUCTION

1.1 Regulation of gene expression by microRNAs

1.1.1 General introduction to regulation of gene expression

Human body consists of about 200 cell types (Junqueira and Mescher, 2009) that have different phenotype and hold various functions. The differences between the cell types are not achieved by differences in genome, since the cells are derived from a single initial cell – the zygote – and therefore share vast majority of genetic information. The differences are achieved by diverse use of this information. Various gene expression programs are engaged in a multistep process of cell differentiation that forms a specific cell type.

A gene is physically a DNA sequence with coding and regulatory regions such as promoter and enhancers/silencers. This sequence is transcribed into RNA which is, in a typical case of a protein-coding gene, translated into a protein. Regulation of gene expression can take place on both DNA and RNA levels.

Regulation on DNA level

Control of gene expression on DNA level is based on regulation of loading of transcriptional machinery. It is largely dependent on proteins called transcription factors. These proteins bind specific sequences on DNA in order to promote or hinder binding of RNA polymerase to DNA. General transcription factors (GTFs) are generally required for transcription initiation. In addition, there are number of transcription factors that act to induce or repress transcription of their target genes. Moreover, these transcription factors can cooperate or block each other; such interactions also add to proper establishment of transcriptional program.

Some transcription factors are involved in modulation of chromatin structure. Chromatin consists of a DNA strand coiled around histone proteins. Histones have high positive charge, whereas DNA has a negative charge, so these molecules hold together. However, N-ends of histones are accessible and they are subjects of covalent modifications, such as methylation and acetylation. These modifications influence charge of histone molecule which leads to loosening or tightening of DNA-histone association and subsequently to facilitation or repression of transcription in specific region of DNA. Transcription factors can bind proteins such as histone-acetyl transferases or histone-methyl transferases that modify N-ends of histones and promote change in chromatin structure and gene expression.

Gene expression is also regulated by methylation of cytosine residues in DNA by DNA methyl transferases. DNA methylation is often located in CpG islands – sequences rich in cytosine-guanine that are parts of gene promoters. Due to methylation, regulatory regions of genes cannot be properly recognized by transcription factors and RNA polymerase. Therefore, expression of methylated genes is silenced.

Regulation on RNA level

A protein-coding gene is first transcribed into pre-mRNA. This molecule is subsequently spliced, modified by adding 5’ cap, poly-adenylated at 3’ end and transported into cytoplasm for translation.

In splicing step, non-coding parts, introns, are removed and exons are reunited to form RNA molecule with correct sequence for translation. Many pre-mRNA molecules – up to 95 % in humans (Pan *et al.*, 2008) – go through alternative splicing. This process is based on modular nature of eukaryote genes, which allows combining exons and thus protein subsections. Proteins arising from this process have different properties (e.g. O'Rourke and Ness, 2008). Therefore, alternative splicing increases coding capacity of genome and regulates gene expression at once.

Gene expression is further regulated by half-life of cytoplasmic mRNAs. One way of regulation of mRNA half-life is binding of microRNAs. These short nucleic acids are part of RNA-induced Silencing Complex (RISC) and mediate degradation of their target mRNAs. MicroRNAs are described in following chapter in greater detail.

1.1.2 MicroRNAs

MicroRNAs are class of small RNA molecules that regulate gene expression at posttranscriptional level. First microRNA, lin-4, was discovered as a regulator of transition from larval stage 3 to stage 4 in *Caenorhabditis elegans* (Lee *et al*., 1993). Today, there are hundreds of microRNAs described in plants and animals - with more than 2000 in human (miRbase, 8.4.2013) -, many of them conserved across taxa. MicroRNAs were found to be regulating number of processes, mainly related to development and differentiation.

Biogenesis of microRNAs

Typical microRNA gene is an independent transcription unit (Lagos-Quintana *et al.*, 2001, Lee *et al.* 2002) that is transcribed by RNA Polymerase II (Lee *et al*., 2004). Long primary transcript (pri-miRNAs) is cleaved by enzyme complex of type III RNAse Drosha and protein DGCR8 (Lee *et al.,* 2003, Landthaler *et. al*, 2004) to generate 60-80 nucleotides (nt) long precursor microRNA (pre-miRNA). Pre-miRNA, a hairpin-like molecule with two-nucleotide overlap on 3' end, is transported from nucleus to cytoplasm by exportin 5 (Yi *et al.,* 2003) (Fig. 1.1). In cytoplasm it serves as a substrate for RISC-loading complex (RLC). This complex consists of protein TRBP and RNAse Dicer. Dicer cleaves pre-miRNA to create miRNA:miRNA*duplex, while TRBP recognizes correct miRNA strand to incorporate into RNA-induced silencing complex (RISC) (Chendrimada *et al*., 2005). Mature microRNA is about 22 nt long.

Mechanisms of microRNA action

MicroRNAs guide RISC to 3' untranslated region (UTR) of target mRNAs. The binding is based on complementarity between the 3' UTR of the mRNA and particular microRNA or its 'seed' sequence (usually 6-8 nt long). Once bound, RISC represses translation of that specific mRNA. This can happen in several ways (Fig. 1.1).

In case of perfect, or almost perfect, complementarity between miRNA and mRNA, RISC cleaves the mRNA, which is subsequently degraded. This 'slicer' property of RISC depends on its core catalytic component from Argonaute family, particularly the protein AGO2 (*rev. in* Tolia and Joshua-Tor, 2006).

In case of imperfect complementarity between microRNA and mRNA, translation is repressed without direct cleavage. It has been found that RISC-mRNA complexes localize into cytoplasmic *foci* called P-bodies (Jakymiw *et al*., 2005, Pillai *et al*., 2005). Main component of P-bodies is protein GW182, a

strong inhibitor of translation (Chekulaeva *et al*., 2009). This protein serves as adaptor for poly-A binding protein and deadenylases. Deadenylated mRNA is directed for degradation (Huntzinger *et al.,* 2013). New levels of complexity have been added to this picture in recent years. First has to do with alternative polyadenylation. This phenomenon has been described already in 1981 *(*Tosi *et al*., 1981): genes can have multiple polyadenylation sites, so the resulting mRNAs can have different 3' UTRs. Majoros and Ohler (2007) have shown that this variability in 3' UTR indeed affects regulation by microRNAs and can be the reason of their context-dependent targeting ability.

Next, it has been shown, that microRNAs can bind not only 3'UTR, but also 5'UTR to promote RISC-dependent translational repression (Lee *et al.,* 2009, Lytle *et al.,* 2007). Furthermore, Ørom and colleagues (2008) described the case of miR-10, which binds 5' UTR of ribosomal proteins and facilitates, not represses their translation. Vasudevan and colleagues (2007) have investigated the relationship of TNF-α mRNA and miR-369-3 in HEK-293 cell line. They found out that miR-369-3 is able to induce or repress translation of TNF-α mRNA, depending on nutrition state.

However, these mechanisms still seem to be marginal mode of action of microRNAs. Therefore we can conclude that microRNAs guide RISC to target mRNA by binding its 3' UTR, which leads to cleavage of such mRNA or its translational repression.

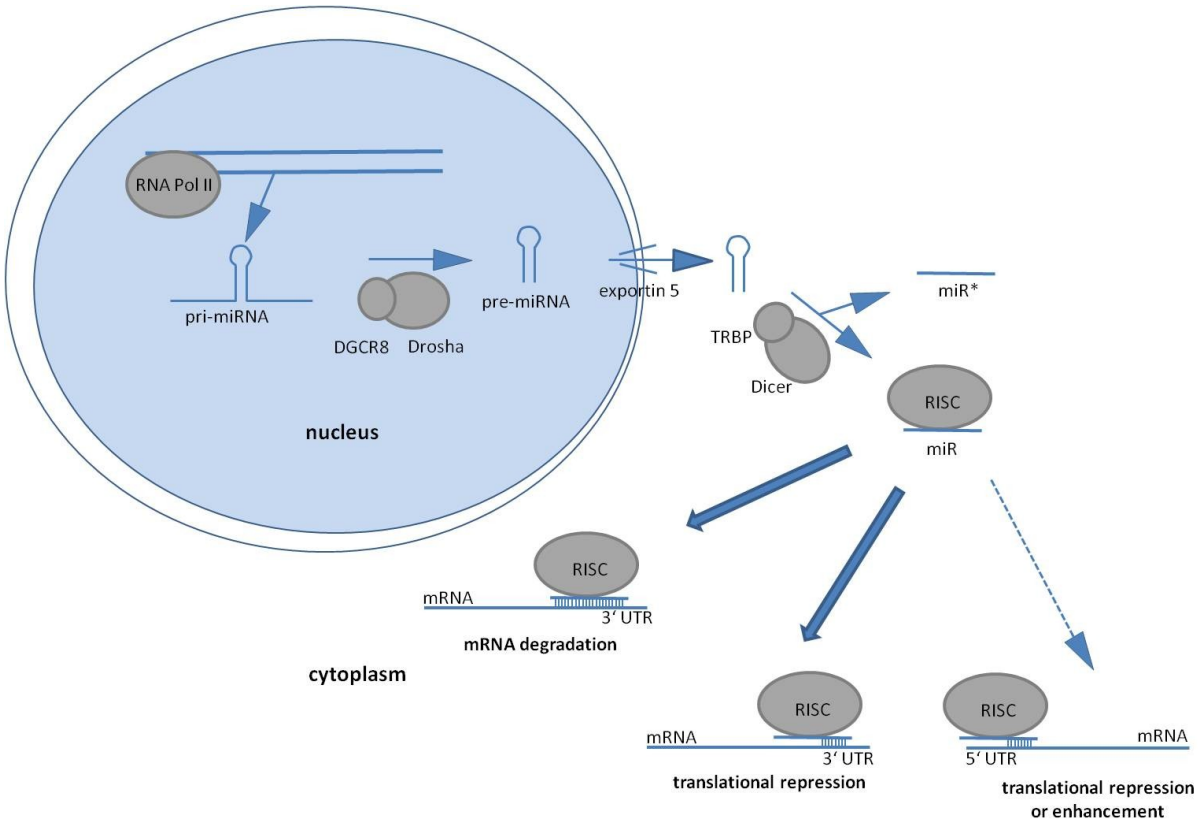

Fig. 1.1. MicroRNA biogenesis and function. MicroRNA genes are transcribed by RNA polymerase II. Primary transcript (pri-miRNA) is cleaved by complex Drosha-DGCR8 to generate precursor transcript (pre-miRNA). Pre-miRNA is transported to cytoplasm by exportin 5 where it is substrate for complex of Dicer-TRBP. Dicer cleaves pre-miRNA to generate mature microRNA strand that is loaded into RISC (RNA-induced silencing complex). As a part of RISC, microRNA participates in posttranscriptional regulation. Binding of RISC to target mRNA usually results in translational repression or degradation of the target, in some cases also to translational enhancement.

Expression of microRNAs in normal and pathological states

Tissues and cell types can be classified according to mRNA expression profiles; similarly, they also have specific microRNA expression profiles (Lu *et al.,* 2005, Landgraf *et. al.,* 2007). Expression of microRNAs in particular cell varies according to differentiation state (Lu *et al*., 2005, Malumbres *et al.,* 2010) and other physiological processes. Importantly, from both biological and medical points of view, expression of microRNAs changes a lot in pathological states, particularly cancer (Lu *et al*., 2005, Iorio *et al*., 2005, Isken *et al.,* 2008).

Microarray analysis of more than 300 samples of human and mouse tissues and tumors (Lu *et al.*, 2005) found that microRNA expression profiles differentiate between tumors according to tissue of origin. They are also able to classify correctly tumors of uncertain origin, as described by histology (Lu *et al.,* 2005). Moreover, expression of some microRNAs is associated with prognosis (Ambs *et al*., 2008, Li *et al*., 2012, He *et al.,* 2013). These are candidate findings to transfer in clinical practice because initial classification of tumor and estimation of prognosis are crucial for successful therapy. It has been also found that microRNAs are shed within microvesicles in body fluids where they are stable and well accessible for analysis (Weber *et al.,* 2010, Baraniskin *et al*., 2011). All these properties make microRNAs potentially useful cancer biomarkers in clinical medicine.

Aberrant expression of microRNAs in tumor has consequences for tumor pathology. Highly expressed microRNAs are likely to contribute to the neoplasia and serve as oncogenes, whereas down-regulated microRNAs are likely to hold the properties of differentiated cells. There are several microRNAs that are up-regulated in many cancer types, such as miR-17 ~ 92 cluster, miR-21, and miR-155 (Iorio *et al*., 2005 Lee *et al*., 2007, Calin *et al.,* 2004, Meng *et al*., 2007). These microRNAs indeed have antiapoptotic and pro-proliferative effects (Olive *et al*., 2009, Si *et al.,* 2007, O'Connell *et al*., 2009, Pedersen *et al.,* 2009) and therefore they are called oncogenic microRNAs, or oncomiRs. On the other hand, let-7 microRNA has been recognized as a tumor suppressor. This microRNA, often down-regulated in tumors, regulates levels of highly oncogenic protein RAS, which is in turn active and up-regulated in many tumors (Johnson *et al.,* 2005).

Simple classification of microRNAs as oncogenes or tumor suppressors can be misleading because function of microRNAs highly depends on cellular context, as we can see in case of miR-26. This microRNA has a tumor suppressor role in Burkitt lymphoma, but acts as an oncogene in glioma (Sander *et al*., 2008, Huse *et al*., 2009).

Present research focuses on both microRNA profiling, and detailed description of role of individual microRNAs in cancer pathology. Typical example is work of Meng and colleagues (2007) identifying mechanism of miR-21 function in hepatocellular carcinoma. When they transfected anti-miR-21 oligonucleotide, they observed decrease in miR-21 levels and increased levels of PTEN phosphatase, which is a negative regulator of PI3K-PKB pathway. Consequently, carcinoma cells exhibited decreased proliferation, migration and invasion. On the other hand, transfection of miR-21 precursor led to decrease in PTEN level and increase of the malignant characteristics of the cells. Together with additional manipulation of PTEN levels, these results indicate that miR-21 acts as an oncogene in hepatocellular carcinoma at least partly through direct or indirect regulation of PTEN (Meng *et al*., 2007).

Results of *in vitro* microRNA manipulations are promising for clinical medicine; there are attempts to use antimicroRNA oligonucleotides or microRNA precursors as cancer therapeutics (*rev. in* Broderick and Zamore, 2011). The effect of these molecules is clear. The problem is how to deliver them to the

cancer cells, because unprotected oligonucleotides are unstable in blood. An emerging solution is the use of lipidic nanoparticles that were reported to work well in mouse model (Trang *et al.,* 2011, Babar *et al.,* 2012).

Together, current state of knowledge recognizes microRNAs as important regulators of physiological and pathological processes. They are particularly important in pathology of tumors: microRNAs like miR-17~92, miR-21 and miR-155 are potent oncogenes, other microRNAs, such as let-7, work as tumor suppressors. However, microRNAs have various functions in different cellular contexts. There is effort to transfer this knowledge to clinical practice. MicroRNAs are promising tumor biomarkers and even therapeutics. Results of oligonucleotide therapy in mouse models are encouraging for further transfer into human medicine.

1.2B-cell development and disease

1.2.1 B-cell development

B cells are type of lymphocytes whose main role is to produce antibodies, making them key cell type in humoral immunity. They are characterized by a specific receptor called B-cell receptor (BCR). BCR is a membrane-bound immunoglobulin (antibody) and a central molecule in B-cell biology.

BCR consists of two heavy (H) and two light (L) chains that are encoded by distinct genes on human chromosomes 14 (gene *IGH*), 2 and 22 (genes *IGK* and *IGL* for L chains). *IGH* consists of 11 segments coding for different types of H chain constant domains (C, C-end of peptide), 9 segments coding for joining (J) region, cca 50 segments coding for diversity (D) region and cca 100 segments coding for varability (V) region (N-end of peptide). *IGL* and *IGK* code for lambda and kappa light chains; they contain only V, J and C segments (*rev. in* Blackwell and Alt, 1989).

Immunoglobulin genes in the original form are not transcribed. They must be first rearranged. It means that particular V, D, J and C segments (or V, J, C in light chain genes) must be combined in a way that creates an open reading frame leading to stable, functional protein – and therefore to functional BCR and antibodies. Additionally, V segments are mutated and newly synthesized sequences are introduced to the immunoglobulin genes in order to create a specific BCR and antibody (Alt nad Baltimore, 1982). Therefore, each clone of B cells produces different antibody.

Immunoglobulin genes rearrangement and mutation are underlying processes of B-cell development (Fig. 1.2). Shaping of B-cell lineage starts in bone marrow from early B-cell progenitor. (This progenitor itself is derived from hematopoietic stem cell (HSC) in a multistep process.) Early B cell progenitor develops to pro- and subsequently to pre-B cell; VDJ recombination of *IGH* takes place in these steps. Formation of pro- and pre-B cells depends on c-kit and IL-7R signaling (Palacios and Samaridis, 1992, Ryan *et al*., 1997). In case of successful *IGH* recombination, the cell expresses so called pre-BCR and the process continues with recombination of light chain genes (first *IGK*, if it is not functional, then *IGL*) (*rev. in* Blackwell and Alt, 1989).

If rearrangements of both *IGH* and *IGL* are successful, a B cell expresses BCR and migrates from bone marrow to secondary lymphoid organs. There, naïve B cell can recognize an antigen presented by dendritic cells. If it receives second stimulatory signal from a helper T cell, the B cell undergoes cycles of proliferation and affinity maturation, in which process mutations are introduced into immunoglobulin genes to achieve – by chance – an antibody with higher affinity for the initial antigen. This process takes place in germinal centers of lymphoid follicles and therefore is called the germinal

reaction. It is accompanied by high proliferation and apoptosis rate: many cells are generated, many mutations are introduced, but only very few are productive and cells that fail to generate functional highly specific antibody are determined for apoptosis. In an opposite case, a B cell with highly specific BCR goes through so called class switch recombination generating different types of soluble antibodies (*rev. in* Klein and Dalla-Favera, 2008). Terminal differentiation stage of B cell, the plasma cell, resides in blood and produces antibodies during the course of immune reaction.

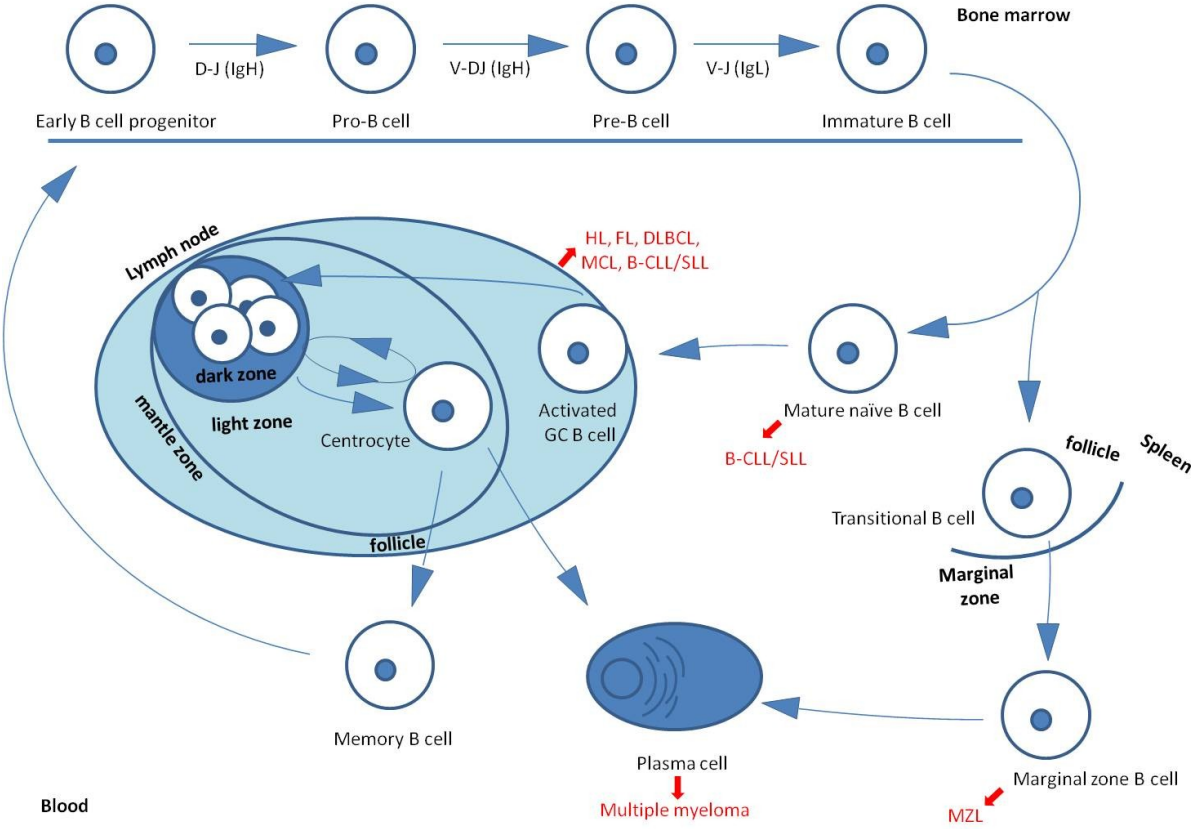

Fig. 1.2. B-cell development and differentiation. Development of B cells starts in the bone marrow. There, mature B cell arises from early B cell progenitor through mutations in immunoglobulin genes. Mature naïve B cells usually migrate into lymph nodes where they are challenged by antigens. When a B cell binds an antigen, it becomes an activated B cell and undergoes proliferation and mutation of variable segment of immunoglobulin genes in order to generate high-specific antibodies (this process is called somatic hypermutation or germinal reaction). Less frequently, B cells can migrate from the bone marrow to lymphoid tissue in spleen and become cells that produce low-specific antibodies often targeted against self-antigens. Nevertheless, cells that successfully pass the whole process differentiate to plasma cells and memory B cells, respectively, and produce antibodies. Cells that fail in any of the steps are determined for apoptosis. Cells that have some defect during the differentiation process but escape apoptosis can generate malignant tumors – leukemias and lymphomas. It is more likely to happen during the germinal reaction because of intense proliferation and mutation in this step. Abbreviations: B-CLL/SLL – B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma, DLBCL – diffuse large B-cell lymphoma, FL – follicular lymphoma, HL – Hodgkin lymphoma, MCL – mantle cell lymphoma, MZL – marginal zone lymphoma.

This developmental process was described for conventional, so called B2 cells. However, there are some smaller B-cell populations, such as B1 cells or marginal zone B cells (Fig. 1.2). These cells produce low-affinity antibodies against some special classes of antigens (*rev. in* Martin and Kearney, 2000).

In summary, B-cell development is a multistep process based on mutations in immunoglobulin genes. Cells that fail in any step are determined for apoptosis; very little portion of cells differentiate successfully into the last stage of specific antibody-producing plasma cells. However, block of differentiation in any step can lead to formation of a tumor. In next chapter, the most frequent solid B-lymphoid malignancies are described.

1.2.2 B-lymphoid malignancies

Lymphoid malignancies can be roughly divided into leukemias and lymphomas. Leukemias are characterized by increase of malignant lymphocytes (T, B, NKT cells) in blood stream, lymphomas are solid tumors of lymphocytes. In this text, we will focus on several frequent B-cell lymphomas.

B-cell lymphomas

B-cell lymphomas are solid tumors of B cells. They often develop in lymph nodes, but also in other sites. Incidence of lymphoma is 6-13 per 100 000 people in a year, higher in developed countries (Boyle *et al*., 2008). Risk factors are immune disorders, both inherited and acquired, and Epstein-Barr virus (EBV) infection in certain lymphoma types. However, generally there is no clear association with any environmental risk factor (Boyle *et al*., 2008). It seems likely that the tendency for malignant transformation is an innate property of B-cell development. This process, as described above, is based on mutation of immunoglobulin genes. Particularly dangerous in terms of transformation is the stage of germinal reaction (Fig. 1.2).

Well defined group of lymphomas are Hodgkin lymphomas. All other lymphomas are classified in a collection group called non-Hodgkin lymphomas.

Hodgkin lymphoma

Hodgkin lymphoma (HL) represents about 30 % of all lymphomas. HL usually arises in lymph nodes of cervical region, primary extranodal involvement is rare. Classical Hodgkin lymphoma (cHL) is vastly most frequent type of HL, accounting for 95 % of cases. cHL is developed either in young age (15-35 years), or in very late age. Chemotherapy in combination with localized radiotherapy cures the disease in 85 % of cHL cases (Swerdlow *et al.,* 2008).

cHL (as well as the rest of HL types) is characterized by specific cells – Hodgkin Reed-Sternberg (HRS) cells. These giant multinucleated cells are the true neoplastic cells. HRS cells probably origin from a germinal-center B cell that failed affinity maturation but evaded apoptosis: they do not produce functional BCR, but they have rearranged immunoglobulin genes with high somatic hypermutation in variable region (Kanzler *et al*., 1996). Pro-proliferation and survival pathways, such as NF-kB and JAK/STAT are activated in HRS and they are highly positive for proliferation marker Ki-67 (*rev. in* Küppers, 2009).

An interesting fact is that HRS cells account only for 0.1 to 10 % of the tumor mass; the rest are other cell types mostly of hematopoietic origin. These cells have supportive role as documented by the observation that it is quite difficult to establish pure HRS cell lines *in vitro* (Drexler, 1993). There is emerging evidence that HRS cells actively manipulate the accompanying cells to create a microenvironment that favors HRS cells' growth (*rev. in* Küppers, 2009).

There are four subtypes of cHL based on histological features. The most frequent is **nodular sclerosis (NS) cHL**, it accounts for approximately 70 % of all cHL cases. Incidence of nodular sclerosis cHL

peaks between 15-34 years of age, the mediastinal involvement is found in 80 % of patients. Up to 40 % of tumors are EBV positive (Swerdlow *et al*., 2008).

Second frequent subtype is **mixed cellularity (MC) cHL** accounting for 20-25 % of cases. This subtype has bimodal age distribution and is found predominantly in men. Ca 75 % of tumors are EBV positive indicating possible role of EBV in pathogenesis of this lymphoma type. Prognosis of mixed cellularity cHL used to be worse than nodular sclerosis cHL but, with the use of modern therapy, the difference almost vanished (Swerdlow *et al.,* 2008).

The last subtypes of cHL, **lymphocyte rich** and **lymphocyte depleted cHL**, are very rare. They account only for 5 and 1 % of the cHL, respectively. They are predominantly diagnosed in men and – in contrary to the first two subtypes – they are more frequently found in developing countries (Swerdlow *et al.,* 2008).

Non-Hodgkin lymphomas

Non-Hodgkin lymphomas (NHL) are very heterogeneous collection of malignancies of diverse biological and clinical features. From the clinical point of view, they are divided to aggressive and indolent lymphomas. Aggressive lymphomas, if untreated, lead to the death of a patient within weeks or months. Once diagnosed, they must be treated without delay. Indolent malignancies can grow for years without clinical symptoms. Even when diagnosed, they often remain untreated (approach called 'watch and wait') because hurried therapy may have negative effect in terms of prognosis.

Diffuse large B-cell lymphoma (DLBCL) is the most frequent NHL accounting for about 30 % of cases (Swerdlow *et al*., 2008). Patients with immunodeficiency are in higher risk of DLBCL than other people. DLBCL – as many other NHL – probably arises from a germinal-center B cell. It arises *de novo* or by transformation from less aggressive malignancies. Gene expression profiling revealed two DLBCL subtypes: germinal center B cell-like (GCB), with expression profile similar to germinal-center B cells, and activated B cell-like (ABC), with expression profile resembling activated peripheral B cells (Alizadeh *et al*., 2000). ABC-DLBCL type is more aggressive and has worse prognosis than GCB-DLBCL type.

Tumor suppressor genes *TP53* and *PTEN* are frequently mutated in DLBCL. *TP53* codes for transcription factor that regulates cell cycle and apoptosis. *PTEN* codes for phosphatase that regulates activity of pro-proliferative PI3K-PKB pathway. This pathway is then constitutively active, which – together with activation of NF-kB pathway and chronic BCR signaling – leads to proliferation and consequently to further mutations in genome (*rev. in* Lenz and Staudt, 2010).

Burkitt lymphoma (BL) is an aggressive B-cell malignancy characterized by translocation t(8;14)(q24;q32) resulting in fusion of *MYC* gene with *IGH* promoter. MYC is a transcription factor and a potent oncogene. Its over-expression due to strong *IGH* promoter is the main cause of BL development.

BL is the most common childhood malignancy in equatorial Africa (so called endemic BL variant). In other regions, BL accounts for only few percent of all lymphomas (so called sporadic BL variant). EBV is detected in all endemic cases and approximately 30 % of sporadic cases, indicating possible involvement of the virus in pathogenesis of BL. There is also immunodeficiency-associated BL which develops mainly in HIV-positive patients (Swerdlow *et al.,* 2008).

Follicular lymphoma (FL) is the most frequent indolent lymphoma, accounting for about 20 % of all lymphomas. The tumor consists of high number of small, closely packed, but poorly defined lymphoid follicles. Majority of FL cases contain translocation t(14;18)(q32;q21) which fuses *BCL2* gene with *IGH* promoter. This translocation leads to high levels of antiapoptotic protein BCL2 resulting in improved cell survival. Tumors are classified in histological grades according to proportion of centroblasts and centrocytes, or proliferating and resting cells. Grade of follicular lymphoma is associated with prognosis. About 30 % of FL cases undergo transformation in lymphoma with higher malignancy, mostly DLBCL. Such tumors are often refractory to therapy and have rapid clinical course (Swerdlow *et al.,* 2008).

Small lymphocytic lymphoma is the solid form of B-cell chronic lymphocytic leukemia. Therefore, it is more proper to use the term **B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma (B-CLL/SLL)**. B-CLL/SLL is the most common lymphoproliferative disorder of the elderly; mean age of diagnosis is 65 years. The disease is characterized by high count of malignant lymphocyte clone in peripheral blood (at least 5 x 10^9 / liter). Other involvements apart from blood and lymph nodes are bone marrow, spleen and liver. Up to 8 % of patients suffer from Richter syndrome – transformation of B-CLL/SLL to DLBCL (Swerdlow *et al.*, 2008).

In B-CLL/SLL, diagnosis is often made thanks to preventive blood tests because the disease remains asymptomatic for a long time. Paradoxically, application of therapy is an adverse prognostic feature, so the strategy 'watch and wait' is often used in order to achieve the right timing of treatment. Once therapy is applied, the patient is determined to be undergoing shortening cycles of remissions, relapses and therapies until death. Therefore, determination of risk is highly important, namely in early B-CLL/SLL. Nowadays, combination of mutation status of variable region of immunoglobulin heavy chain gene (*IGVH*) with ZAP70 and CD38 expression is used. Mutation states of *TP53, ATM*, 13q14 and other loci have been associated with prognosis as well. However, B-CLL/SLL still remains an incurable disease with complex biological and clinical features.

Marginal zone lymphoma (MZL) is an indolent lymphoma accounting for less than 2 % of all B-cell malignancies. The prognosis is overall good, even though transformation in DLBCL may occur. Nodal, extranodal and splenic MZL types are recognized according to the site of involvement.

Mantle cell lymphoma (MCL) is a B-cell malignancy with some special features. It accounts for up to 10 % of NHL; median age of diagnosis is 60 years. Median overall survival is 3-5 years and transformation does not occur. However, vast majority of patients cannot be cured (Swerdlow *et al*., 2008). MCL is cytogenetically characterized by translocation t(11,14)(q13,q32) which leads to over-expression of *CCND1* (coding for cyclin D1) under the *IGH* promoter and to the cell cycle progression. The postulated normal counterpart is peripheral B cell of mantle zone of lymphoid follicle.

1.3MicroRNA-155

Gene for microRNA-155 (miR-155), or miR-155 host gene (*MIR155HG*), is located at short arm of chromosome 21. *MIR155HG* was originally named *bic*, standing for B-cell integration cluster because it was identified as an integration site of avian leucosis virus that causes B-cell lymphoproliferation in birds (Clurman and Hayaward, 1989). Since then, miR-155 has been identified as an important player in both physiological and pathological development of B cells.

MiR-155 is encoded within the exon 3 of human and mouse *MIR155HG* (exon 2 in chicken) (Tam 2001). It is transcribed by RNA Polymerase II and processed in common way as described above. Mature miR-155 is a 23-nt long microRNA with sequence 5'-UUAAUGCUAAUCGUGAUAGGGGU-3' in human; the sequence is highly conserved among vertebrates.

1.3.1 MicroRNA-155 in physiological processes

Under physiological conditions, miR-155 is expressed only in hematopoietic tissue (Ramkissoon *et al.,* 2006), indicating its role in hematopoiesis. In revealing this role, miR-155-deficient mice have been of great importance.

Rodriguez and colleagues (2007) investigated overall phenotype of miR-155-deficient mice. They found out that these mice are viable and fertile, yet tend to develop lung pathology and enteric inflammation, suggesting that miR-155 serves as regulator of immune system. Examination of immune cells revealed that there are no apparent changes in number of B-cells, but in miR-155-deficient mice, there is very little IgM and switched antibody response to challenging by antigen resulting in little antibody protection against pathogen *Salmonella typhimurium*. Similar results were obtained in other studies (Thai *et al.,* 2007, Vigorito *et al*., 2007). They found that miR-155 deficiency results in defect in generation of class-switched plasma cell that can produce high-affinity antibodies. More specifically, the impaired process is not the somatic hypermutation or class-switch recombination, but more likely selection of the right clones of plasmablasts. Together these data show that miR-155 is a molecule crucial for regulation of germinal reaction and antibody immune response.

Vigorito and colleagues (2007) attribute this phenotype to deregulation of PU.1. PU.1 is a transcription factor critical for lymphoid and myeloid development (Arinobu *et al.,* 2007). It is best known as a master regulator of myeloid differentiation, meaning it triggers formation of myeloid lineage and promotes its progression. Concentration of PU.1 in myeloid cells is relatively high (Dahl *et al.,* 2003). However, certain smaller concentration is also important for lymphoid differentiation, mainly for development of B cells (Dahl *et al*., 2003, Medina *et al*., 2004). Therefore, precise regulation of PU.1 expression is needed for accurate formation of both lymphoid and myeloid cells. 3' UTR of PU.1 mRNA harbors site precisely complementary to seed sequence of miR-155. Luciferase reporter assays, Q-PCR and Western blot analyses confirmed that PU.1 is a true target of miR-155 and that miR-155 regulates both mRNA and protein levels of PU.1 (Vigorito *et al*., 2007, Vargova *et al*., 2011*)*. Next, over-expression of PU.1 recapitulates miR-155-deficient phenotype in B cells (Vigorito *et al.,* 2007). These results clearly show that miR-155 regulates levels of PU.1 in B cells in order to secure the right progress of antibody-dependent immune reaction.

MiR-155 is also important for T-cell development. Several studies report that T cells in miR-155-deficient mice are biased towards TH2 population (Rodriguez *et al.,* 2007, Thai *et al.,* 2007). Two likely mechanisms of this alteration have been described. First is based on the observation that lack of miR-155 leads to release of TH2-specific genes, among them c-Maf. C-Maf is a transcription factor that drives expression of TH2 cytokines such as IL-4, IL-5 and IL-10 and therefore modulates the development towards TH2 phenotype (Rodriguez *et al.,* 2007). Second mechanism is based on miR-155 target SOCS1. In equilibrium, miR-155 represses SOCS1, which leads to STAT5 signaling and IL-2 expression. Violation of this cascade leads to low IL-2 expression, lowTH1 phenotype and higher proportion of TH2 population. Low IL-2 production in miR-155-deficient conditions also leads to impaired generation of CD25+ regulatory T cells (Treg) (Lu *et al.,* 2009). Furthermore, FoxP3 – Treg-specific transcription factor – binds *MIR155HG* and activates its expression, suggesting importance of

miR-155 for Treg development (Lu *et al.,* 2009). On the other hand, miR-155 target SOCS1 also reduces signaling by STAT3, which process in T cells induces expression of IL-17 – a hallmark of TH17 cell population (Yao *et al.,* 2012). Treg and TH17 cells have opposite roles in immune system: the first suppress immune reactions namely to self-antigens, the latter are highly pro-inflammatory with a role in autoimmune diseases. The findings summarized in this paragraph illustrate the importance of miR-155 in balancing the immune system by equilibration of T-cell populations.

Finally, miR-155 has been found to be up-regulated during macrophage inflammatory response (O'Connell *et al*., 2007). MiR-155 expression, induced by Toll-like receptor signaling, leads to suppression of C/EBPβ transcription factor and, in turn, to activation of G-CSF expression (O'Connell *et al.,* 2007, Worm *et al.*, 2009). G-CSF is a cytokine that stimulates granulopoiesis in bone marrow. Therefore we can say that miR-155 promotes inflammatory immune response by stimulation of granulocyte development.

1.3.2 MicroRNA-155 in pathological states

MiR-155 in human malignancies

Over-expression of miR-155 is well documented in many human lymphoproliferative disorders. Measurements in cell lines and primary tumor tissues have detected high miR-155 expression in B-CLL/SLL, DLBCL, HL, and FL (van den Berg *et al*., 2003, Kluiver *et al*., 2005, Eis *et al*., 2005, Lawrie *et al.,* 2007, Calin *et al.,* 2005, Vargova *et al*., 2011). Up-regulation of miR-155 has been also documented in 3 cases of MZL *(*Eis *et al.,* 2005, Kluiver *et al.,* 2005). Slight up-regulation was observed in MCL (Zhao *et al*., 2010). In B-CLL/SLL, miR-155 is one of microRNAs that make a specific signature distinguishing between cases with favorable and unfavorable prognosis (Calin *et al.,* 2005). Similarly in DLBCL, miR-155 expression is higher in more aggressive ABC subtype *(*Lawrie *et al.,* 2007).

In BL, miR-155 is up-regulated only if the tumor exhibits type III EBV latency (Kluiver *et al.*, 2007). Latency is a state when a cell is infected but the virus does not multiply. In the case of EBV, there are three types of latency that differ in expression of viral proteins and RNAs. Type I is the most frequent latency type in BL, but type III latency also occurs. It is characterized by expression of latent membrane protein I (LMP1). This protein integrates in cell membranes and activates signal cascades such as NF-kB that induce miR-155 expression (Gatto *et al*., 2008, Yin *et al.,* 2008).

Interestingly, high levels of miR-155 have been found also in non-hematopoietic malignancies, such as lung and breast carcinomas (*rev. in* Mattiske *et al*., 2012, Yang *et al.,* 2013) with consequences for biology and prognosis of these tumors (Zang *et al*., 2012).

Finally, there is evidence that miR-155 also play some role in allergy and autoimmune disorders. Studies in atherosclerosis and atopic dermatitis found that miR-155 contributes to pathogenesis of these diseases by modulating macrophage and T-cell responses, respectively (Nazari-Jahantigh *et al.*, 2012, Sonkoly *et al.,* 2010).

Mouse models of miR-155 over-expression

Two mouse models over-expressing miR-155 have been created in order to draw function of miR-155 in establishment of disease. In the first one, mice express miR-155 under *IGH* promoter and develop aggressive polyclonal pre-B cell malignancies (Costinean *et al.,* 2006). The latter model is more

sophisticated: the expression cassette consists of TetO-miniCMV promoter, stop cassette surrounded by loxP sites and miR-155 coding sequence. This construct is targeted to ubiquitously expressed *ROSA26* locus, and therefore crossing a mouse of this genotype to a mouse expressing Cre recombinase under selected promoter leads to excision of stop cassette and expression of miR-155 in cells of interest. Expression of miR-155 can by subsequently repressed by administration of doxycycline, which binds tTA protein – transcriptional activator of TetO-miniCMV promoter (Babar *et al.,* 2012). Authors crossed mice bearing the miR-155 construct to mice expressing Cre recombinase under nestin promoter. Nestin is expressed in central nervous system stem cells and in lymphoid tissue. Removal of doxycycline from feed of those mice led to formation of clonal pre-B-cell lymphoproliferations highly resembling human lymphoproliferations, which are also monoclonal. Furthermore, decrease of miR-155 expression after re-administration of doxycycline led to curing the disease within two weeks (Babar *et al.,* 2012). Interestingly, over-expression of miR-155 in brain did not induce any malignant changes (Babar *et al.,* 2012). All these results indicate that miR-155 is one of a few molecules that can alone drive malignant transformation. At least in some cases, tumors might depend on miR-155 expression with possible consequences for human medicine.

MiR-155 pathways

Up-regulation of miR-155 levels in clinical cases of tumors does not stem from amplification or any other mutation in *MIR155HG*. Therefore, regulation of miR-155 expression must be of great importance.

Regulatory regions of *MIR155HG* contain TATA box, CpG island and other elements.

About 40 nucleotides upstream from the transcription start site, binding site for AP-1 proteins is located (Yin *et al*., 2008). AP-1 proteins JunB and FosB bind this site in response to BCR crosslinking and MAPK signaling and induce miR-155 transcription. This cascade can be also triggered by autocrine TNFα signaling, as described in DLBCL (Pedersen *et al.,* 2009). AP-1 factors cooperate with proteins from Ets family in order to achieve maximal miR-155 expression upon BCR crosslinking (Yin *et al.,* 2008).

Regulatory regions of *MIR155HG* also contain two binding sites for transcription factors NF-kB (Yin *et al.,* 2008, Gatto *et al*., 2008). In B-cell non-Hodgkin lymphoma cells, both of the sites are occupied and activate miR-155 expression (Gatto *et al*., 2008). Study in lymphoblastic cell lines showed that miR-155 is a key molecule for immortalization by EBV (Linnstaedt *et al.,* 2010). In response to EBV infection and NF-kB activation, miR-155 promotes proliferation and inhibits apoptosis (Gatto *et al.,* 2008, Linnstaedt *et al*., 2010).

A mechanistic study, performed by Vargova and colleagues (2011), focused on E-box binding sites in regulatory regions of *MIR155HG*. E-box proteins are known as oncogenic transcription factors. The authors found that primary B-CLL/SLL cells of some patients express high level of E-box transcription factor MYB. In these cases, miR-155 was up-regulated, while levels of PU.1 and miR-150 were decreased compared with cases that express normal levels of MYB. They found that MYB binds 3 E-box sequences within *MIR155HG* regulatory region and activates miR-155 expression.

Other studies (Xiao *et al*., 2007, Wang *et al*., 2008) found out that miR-155 is highly expressed in proliferation centers in solid B-CLL/SLL. In these centers, expression of miR-150 – a microRNA targeting MYB – was low. These findings together indicate that there is relationship between miR-155, miR-150 and MYB in B-CLL/SLL that favors aberrant expression of miR-155.

Of note, MYB has also been identified as target of miR-155 by luciferase reporter assays in HEK293 cells (Imig *et al.,* 2010). However, it seems unlikely that MYB would serve as miR-155 target in B-CLL/SLL.

Next, Vargova and colleagues manipulated levels of MYB, miR-155 and PU.1 and confirmed causal relationships between levels of these molecules. As described above, balanced expression of PU.1 is crucial for B-cell development. Here authors propose following model: in subset of B-CLL/SLL patients, MYB binds *MIR155HG* and stimulates expression of miR-155. High levels of miR-155 lead to decreased levels of PU.1 which result in block of B-cell differentiation and leukemic transformation (Vargova *et al.,* 2011) (Fig. 1.3).

NF-kB, miR-155 and PU.1 have been linked in DLBCL (Thompson *et al.,* 2010). Strong activity of NF-kB cascade in ABC-DLBCL, the more aggressive DLBCL subtype, leads to high level of miR-155 which subsequently represses expression of PU.1. This manifests by low expression of CD10, target of PU.1, in ABC-DLBCL (Thompson *et al.,* 2010).

It should be noticed that NF-kB and MAPK cascades that are aberrantly activated in lymphomas, are also involved in physiological activation of immune response. It has been shown that malignant growth often happens in sites of chronic inflammation (*rev. in* Gonda *et al.,* 2009). Therefore, miR-155 is a possible link between inflammation and cancer (*rev. in* Tili and Croce, 2009).

In summary, miR-155 is a proven oncogene with profound role in B-cell lymphoproliferative disorders. Strong activity of NF-kB, AP-1 and MYB leads to high levels of miR-155 and subsequently diminished levels of its target transcripts, among them PU.1. Imbalance in gene expression leads to block in B-cell differentiation and to tumor formation. In some malignancies, miR-155 levels reflect aggressiveness of the disease. Expression of miR-155 can be a link between inflammation and cancer.

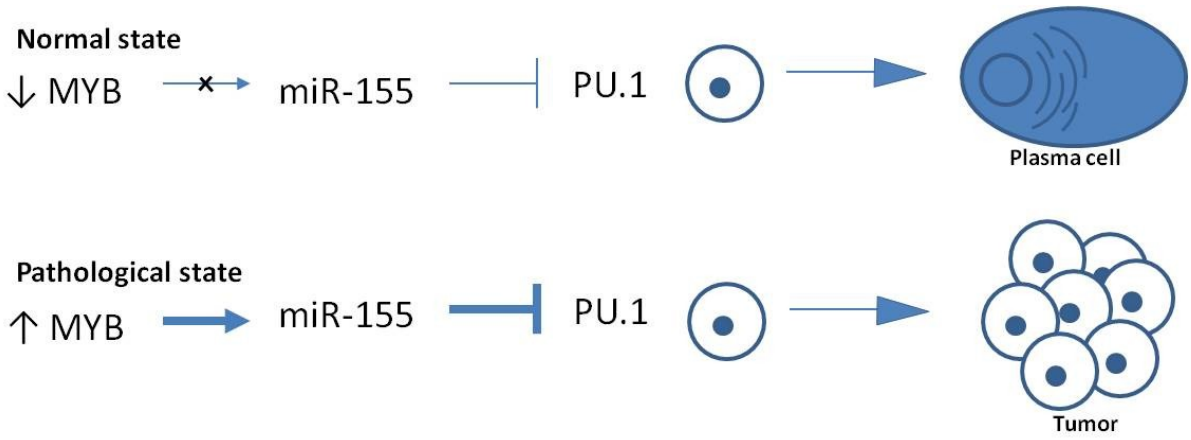

Fig. 1.3. MYB/miR-155/PU.1 model in B-CLL/SLL proposed by Vargova and colleagues (2011). In normal state, MYB levels are low and do not stimulate expression of miR-155. Both miR-155 and PU.1 remain on physiological levels which results in normal B-cell differentiation. High levels of MYB in pathological state lead to high expression of miR-155 and consequently low levels of PU.1 that result in block of B-cell differentiation and malignant lymphoproliferation.

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2 AIMS

A) To measure expression of miR-155 in tumor tissue of patients with various lymphomas and to compare the level of miR-155 in lymphomas with inflammatory lymphoid tissue and normal lymphoid tissue.

B) Based on the findings of Vargova and colleagues (2011), to measure expression of PU.1, MYB and miR-150 in the tissues and correlate them with the levels of miR-155.

C) To determine, whether expression of either of the molecules is associated with some biological or clinical feature.

3 MATERIAL AND METHODS

3.1Material

3.1.1 Samples

The samples, obtained by biopsy of lymph nodes and other tissues, were collected from patients of various diagnoses for the purposes of this research during the years 2009-2012. All biological material was collected on informed consent; the research was approved by ethical committee of 1st Faculty of Medicine.

In cooperation with General Faculty Hospital, data about diagnosis, stage of the disease, risk factors and the course of treatment were collected. Then, expression of selected genes was measured.

Patients were divided in groups according to histological finding. The groups correspond to WHO classification of neoplastic diseases (Swerdlow *et al*., 2008): B-cell neoplasms (B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma, diffuse large B-cell lymphoma, mantle-cell lymphoma, and marginal zone b-cell lymphoma) and Hodgkin lymphoma. We also included group of 4 patients with strong inflammatory picture in the lymph nodes. The control group consists of lymph nodes with histological finding of minor reactive changes.

1. B-cell chronic lymphocytic leukemia/small lymphocyte lymphoma (B-CLL/SLL, Table S1) group consists of 20 patients (12 men and 8 women). Median age of the patients at the time of sample collection was 67.5 years (39-80 years). 4 of the patients were newly diagnosed at the time of the sample collection. For other patients, phase of the disease (progression, stabilization, transformation) was determined at the time of sample collection. 9 of the patients were going through progression and tumors of 7 patients were transforming in diffuse large B-cell lymphoma (process known as Richter syndrome).
2. Diffuse large B-cell lymphoma (DLBCL, Table S2) group consists of 24 patients (14 men and 10 women). Median age of the patients at the time of sample collection was 65 years (23-84 years). 12 of the patients were newly diagnosed at the time of sample collection, 10 were going through relapse and 2 were found to be after transformation from marginal zone lymphoma. In terms of subtype, 15 were ABC-DLBCL, 5 GCB-DLBCL and 4 were of other type of DLBCL.
3. Follicular lymphoma (FL, Table S3) group consists of 29 patients (14 men and 15 women). Median age of the patients at the time of sample collection was 57 years (36-78). 17 of the patients were newly diagnosed at the time of sample collection, 6 were going through relapse and 6 through transformation into more aggressive lymphoma.
4. Hodgkin lymphoma (HL, Table S4) group consists of 25 patients (13 men and 12 women). Median age of the patients at the time of sample collection was 26 years (20-67). 14 of the patients were younger than 30 years. In terms of subtype, 16 were nodular sclerosis (NS), 8 were mixed cellularity (MC) and 1 was another type of HL.
5. Marginal zone lymphoma (MZL, Table S5) group consists of 13 patients (6 men and 7 women). Median age of the patients at the time of sample collection was 64 years (31-78 years). 10 of the patients were newly diagnosed at the time of sample collection, 3 were going through relapse.

6. Mantle cell lymphoma (MCL, Table S6) group consists of 10 patients (5 men, 5 women). Median age of the patients at the time of sample collection was 67 years (50-87 years). 5 of the patients were newly diagnosed at the time of sample collection, 5 were going through relapse.
7. Inflammatory lymph nodes (Table S7) group consists of 4 patients (1 man and 3 women). Median age of the patients at the time of sample collection was 37 years (28-67 years). According to histological finding, the samples were classified as lymphadenitis. The stated possible cause was toxoplasmosis in 3 cases and tularemia in 1 case.
8. Control (table S8) group consists of 6 patients (4 men and 2 women). The samples collected from these patients were enlarged lymph nodes suspected from malignancy; however, the histological analysis revealed only minor reactive changes. Median age of the patients at the time of sample collection was 39 years (18-68 years).

3.1.2 Chemicals

Sodium acetate (Sigma-Aldrich)
Linear acrylamide (Ambion)
Ethanol 96% (Penta)
Phospate buffered saline, PBS (Gibco)
Chlorophorm, $CHCl_3$ (Penta)
Isopropanol (Penta)
Hydrochloric acid (Penta)
Nuclease Eliminator (Amresco)
TaqMan Universal PCR Master Mix II, no UNG (Applied Biosystems)
TRIzol (Life Technologies)
water, double-distilled, ddH_2O
water, nuclease-free (Amresco)

3.1.3 Solutions

Ethanol 80%
To make 80% ethanol, 4 ml of sterile ddH_2O were mixed with 20 ml of 96% etanol.

Sodium acetate 3M, pH 5.5
125.25 g of sodium acetate were diluted in 30 ml of sterile ddH_2O. pH was adjusted to 5.5 by hydrochloric acid.

PBS 1x
50 ml of 10x PBS was mixed with 450 ml of sterile ddH_2O.

3.1.4 Primers and other oligonucleotides

Commercially designed oligonucleotides

PCR
5x primer hsa-miR-155 (Applied Biosystems)
20x primer hsa-miR-155 (Applied Biosystems)

5x primer RNU44 (Applied Biosystems)
20x primer RNU44 (Applied Biosystems)
probes no. 27 , 56 and 60 from Universal ProbeLibrary, 10 μM (Roche)

Custom-designed oligonucleotides
Primers were designed by Juraj Kokavec and synthesized by Sigma-Aldrich. Lyophilized strands were diluted in sterile ddH_2O to final concentration 200 μM and stored in -80°C. Working 20 μM primers were prepared by mixing 10 μl of forward primer with 10 μl of reverse primer and 80 μl of sterile ddH_2O.

Table 1. Primers for Q-PCR.

Primer Sequence	Code	Length (bp)
AGCCACATCGCTCAGACAC	hGAPDH #60 F	19
GCCCAATACGACCAAATCC	hGAPDH #60 R	19
CCACTGGAGGTGTCTGACG	hSPI1 #27 F	19
CTGGTACAGGCGGATCTTCT	hSPI1 #27 R	20
TGCTCCTAATGTCAACCGAGA	hMYB #56 F	21
AGCTGCATGTGTGGTTCTGT	hMYB #56 R	20

3.1.5 Commercially supplied kits

TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems)

- 10x RT Buffer
- 25x dNTP mix 100 mM
- 10x RT Random Primers
- Multiscribe Reverse Transcriptase 50 U/μl
- RNAse Inhibitor

3.1.6 Laboratory devices

Analytical Balance ALJ 220-4 (KERN)
Eppendorf Centrifuge 5417R (Eppendorf)
Eppendorf Centrifuge 5804R (Eppendorf)
Cycler Mastercycler gradient (Eppendorf)
Cycler for quantitative PCR 7900HT Fast Real-Time PCR System (Applied Biosystems)
Minicentrifuge Rotilabo with ButterFly Rotor (Carl Roth)
Multipipette plus (Eppendorf)
Pipetboy Hirschmann Pipetus (Sigma-Aldrich)
Pipetman (Gilson)
Spectrophotometer ND-1000 (NanoDrop)
Sterilizer Sterilab (BMT Medical Technology)
pH meter PL-600 (M.R.C.)

3.1.7 Software

Microsoft Office 2007
R 2.15.2
RStudio
SDS v2.1
GraphPad Prism 4
Nanodrop ND-1000 v3.0.1
CorelDRAW X5

3.2 Methods

3.2.1 Bioptic specimen treatment

Biopsies were collected surgically, washed in 1x PBS (5 minutes, 300 g) and disintegrated in 0.5-1.0 ml of TRIzol Reagent.

These steps were performed in General Faculty Hospital in Prague and its Central Haematological Laboratories.

The samples were stored in -80°C.

3.2.2 RNA isolation

The bench and all the tools were cleaned with Nuclease Eliminator solution. Sterile tubes and filter tips were used.

The sample was transferred in TRIzol reagent as described above. 400 µl of the sample were mixed with 100 µl of chloroform, vortexed for 20 seconds and centrifuged (20°C, 10 minutes, 13600 g). The aqueous phase was transferred into new tube and 1 volume of chloroform was added; these were mixed and centrifuged as before. The aqueous phase was again transferred into a new tube. To this tube, 1 volume of isopropanol 1/5 volume of 5M sodium acetate (pH 5.5) and 1 µl of linear acryl amide were added. The content was gently mixed; the tube was spun in minicentrifuge and placed in -20°C for precipitation.

Next day, the tube was centrifuged (4°C, 45 minutes, 20200 g) and supernatant was discarded. The pellet was washed with 200 µl of 80% ethanol (4°C, 10 minutes, 20200 g). Supernatant was again discarded; the pellet was dried on air and diluted in 12 µl of RNAse-free water. Concentration of the solution was measured by spectrophotometer.

3.2.3 Measuring gene expression

Reverse transcription
Reverse transcription is a type of polymerase chain reaction (PCR) in which the enzyme reverse transcriptase creates a complementary DNA molecule (cDNA) pursuant to RNA template.
In this study, TaqMan MicroRNA Reverse Transcription Kit was used. This kit contains random primers for reverse transcription of whole RNA and specific stem-loop primers for microRNAs (Fig. 3.1).

Reaction mixture was following:

10x RT Buffer	1.5 µl
10x RT Random Primers	0.5 µl
25x dNTPs	0.4 µl
RNA Inhibitor	0.1 µl
5x Primer hsa-miR-155	1.5 µl
5x Primer has-miR-150	1.5 µl
5x Primer RNU44	1.5 µl
Multiscribe reverse transcriptase	1.0 µl
RNA 100 ng	1.0 µl
Nuclease-free water	7.5 µl

The reaction was performed by Mastercycler gradient device.

Program:

16°C	30 min
42°C	30 min
80°C	5 min
4°C	∞

After the reaction was completed, sterile ddH_2O was added to final volume of 50 µl. The sample was stored in +4°C.

Quantitative real-time PCR (Q-PCR)

System TaqMan (Applied Biosystems) was used for quantification of gene expression (Fig. 3.1). Reactions of 8 µl were mixed in 396well plate.

Reaction mixture was following:

TaqMan Universal PCR Master Mix II, no UNG	4 µl
sterile ddH_2O	3.1 µl
20x primer hsa-miR-155, hsa-miR-150 and RNU44, repsectively	0.4 µl
or	
primer 20 µM + probe	0.3 µl + 0.1 µl
cDNA	0.5 µl

The reaction was performed by 7900HT Fast Real-Time PCR System device.

Program:

50°C	2 min	
95°C	10 min	
95°C	15 s	} 40x
60°C	1 min	
4°C	∞	

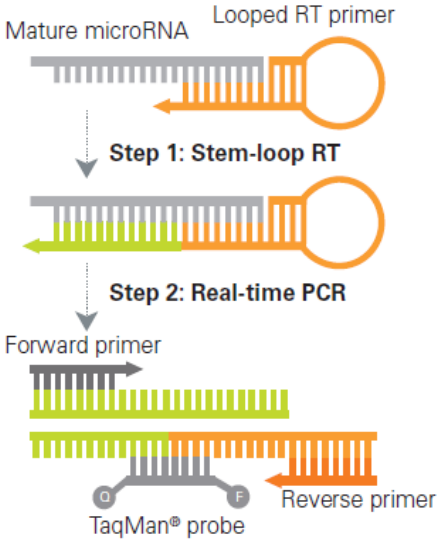

Fig. 3.1: Reverse transcription and Q-PCR of microRNAs in system TaqMan (ABI).
First step involves reverse transcription of a microRNA with the use of stem-loop primer. Product of this step is complementary DNA (cDNA) that is quantified in second step with the use of specific primer and fluorescent probe.

(Source: Life Technologies)

3.2.4 Data mining, processing and statistics

Patient data
Clinical data about patients were collected from Medea database with kind help of dr. Tomáš Stopka and dr. Adela Berkova. The data are listed in Tables S1-S8.

Q-PCR
Cycler for Q-PCR measures the number of cycle in which the fluorescence exceeds certain threshold, which is computed by the device. Resultant value is called CT. These values were used for computing so called fold change (FC), which is the expression level of a target gene relative to the reference gene. In our settings, GAPDH was used as a reference gene for mRNAs and RNU44 for micoRNAs.

The FC values were computed by the ΔΔCT method which is described by following formula:

$$FC = 2^{CT(\text{reference gene})-CT(\text{target gene})}$$

Statistics
Statistical analysis was performed with statistical program R (version 2.15.2) in the interface RStudio. For two-sample testing, we used FC values. All data sets were first tested for normal distribution by Shapiro-Wilk test. If the data came from normal distribution (Shapiro-Wilk p-value > 0.1), two-sample Student t-test was used. In the opposite case, Wilcoxon (Mann-Whitney) two-sample test was used. Corresponding variant of the test was used to determine whether the expression levels are significantly higher or lower.

Simple and multiple regression analyses were ran with ΔCT values. For graphical representation, tools from package *scatterplot3d* were used. Linear models were compared by ANOVA.

Cox proportional-hazards models were calculated using ΔCT values. For these calculations, tools from package *survival* were used.

4 RESULTS

4.1 miR-155 is over-expressed in lymphomas and inflammatory lymph nodes.

Data obtained from TaqMan Q-PCR assay were first used to examine expression of miR-155, PU.1, MYB and miR-150 in each diagnosis. Levels of these molecules in disease states (FC, listed in Tables S1-7) were compared to their levels in lymph nodes with minor reactive changes (i.e. controls) (Table S8).

Data show that levels of miR-155 are significantly elevated in B-CLL/SLL, DLBCL, HL, FL and MZL compared to control lymph nodes (Fig. 4.1A). These results were largely expected. However, expression of miR-155 in MZL was until now documented only in 3 cases. We added other 13 cases; in eleven of them, levels of miR-155 were higher than in controls.

MiR-155 is also up-regulated in our cases of inflammatory lymph nodes to similar level as in lymphomas. High level of miR-155 in inflammatory lymph nodes was expected as well, because miR-155 gets up-regulated during inflammatory immune response.

An exception among studied diseases is MCL: the level of miR-155 in MCL does not differ from that observed in control samples (Fig. 4.1A). This result is consistent with previous report that did not found any signal for pre-miR-155 in MCL lymphoid tissue (Kluiver *et al*., 2005). However, slight up-regulation of miR-155 was observed in leukemic form of MCL in peripheral blood (Zhao *et al*., 2010).

Expression of PU.1, a miR-155 target, shows an opposite pattern. Levels of PU.1 mRNA are reduced in B-CLL/SLL, DLBCL, HL, FL and MZL (Fig. 4.1B). PU.1 is also down-regulated in MCL, but there is no significant difference in PU.1 expression between inflammatory and control lymph nodes (Fig. 4.1B).

Expression of MYB and miR-150 do not give such clear pictures as for miR-155 and PU.1. MYB, which is a target of miR-155 and a regulator of *MIR155HG* at the same time, does not show any change in expression in our set of patients (Fig. 4.1C). Levels of miR-150 are more diverse with decrease in DLBCL and MCL and elevation in HL and inflammatory lymph nodes (Fig. 4.1D).

Together, the data show that miR-155 levels are overall elevated in lymphomas except of MCL, while PU.1 levels are mostly reduced. Yet high variability in the expression of these genes can be observed that might reflect biological and/or clinical features of particular patient within a specific diagnosis.

4.2 MYB/miR-155/PU.1 model in lymphomas

Vargova and colleagues formulated a model of MYB, miR-155 and PU.1 interaction (Vargova *et al.,* 2011). This model says that in a subset of B-CLL/SLL, MYB stimulates the expression of miR-155 that leads to low levels of PU.1 and thus to block of differentiation. We wanted to test, whether the model might generally work in lymphoid malignancies.

To answer this question, we used sequential regression analysis consisting of three linear models. First model was explaining levels of PU.1 by levels of miR-155. In second linear model, we added MYB as another explanatory variable. In case of significant difference between these two models, we can say

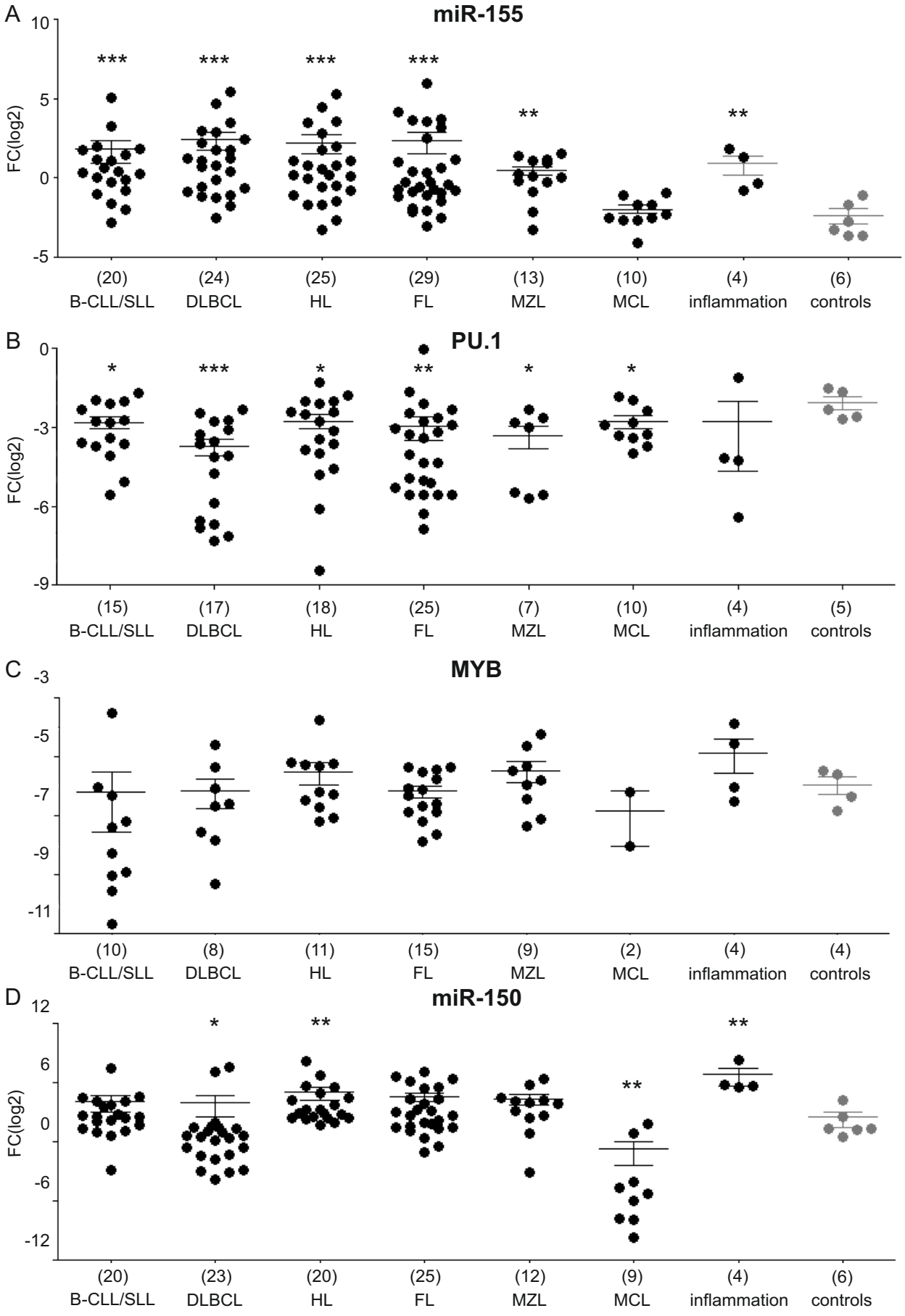

Fig. 4.1. Relative expression of miR-155, PU.1, MYB and miR-150 in lymphomas and inflammatory lymph nodes. The y axis represents the expression of miR-155 (A), PU.1 (B), MYB (C) and miR-150 (D) relative to RNU44 (microRNA) and GAPDH (mRNA), respectively. Data (as fold change, FC) are shown in log2 scale. Mean and SEM are indicated by lines and error bars. Asterisks indicate significance of two-sample t-test or Mann-Whitney-Wilcoxon test for each diagnosis and control group: *** $p<0.001$, ** $p<0.01$, * $p<0.05$. Number of samples used for calculation is written in brackets under each group. B-CLL/SLL - B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma, DLBCL - diffuse large B-cell lymphoma, HL - Hodgkin lymphoma, FL - follicular lymphoma, MZL - marginal zone lymphoma, MCL - mantle cell lymphoma, inflammation - inflammatory lymph nodes, controls - lymph nodes with minor reactive changes.

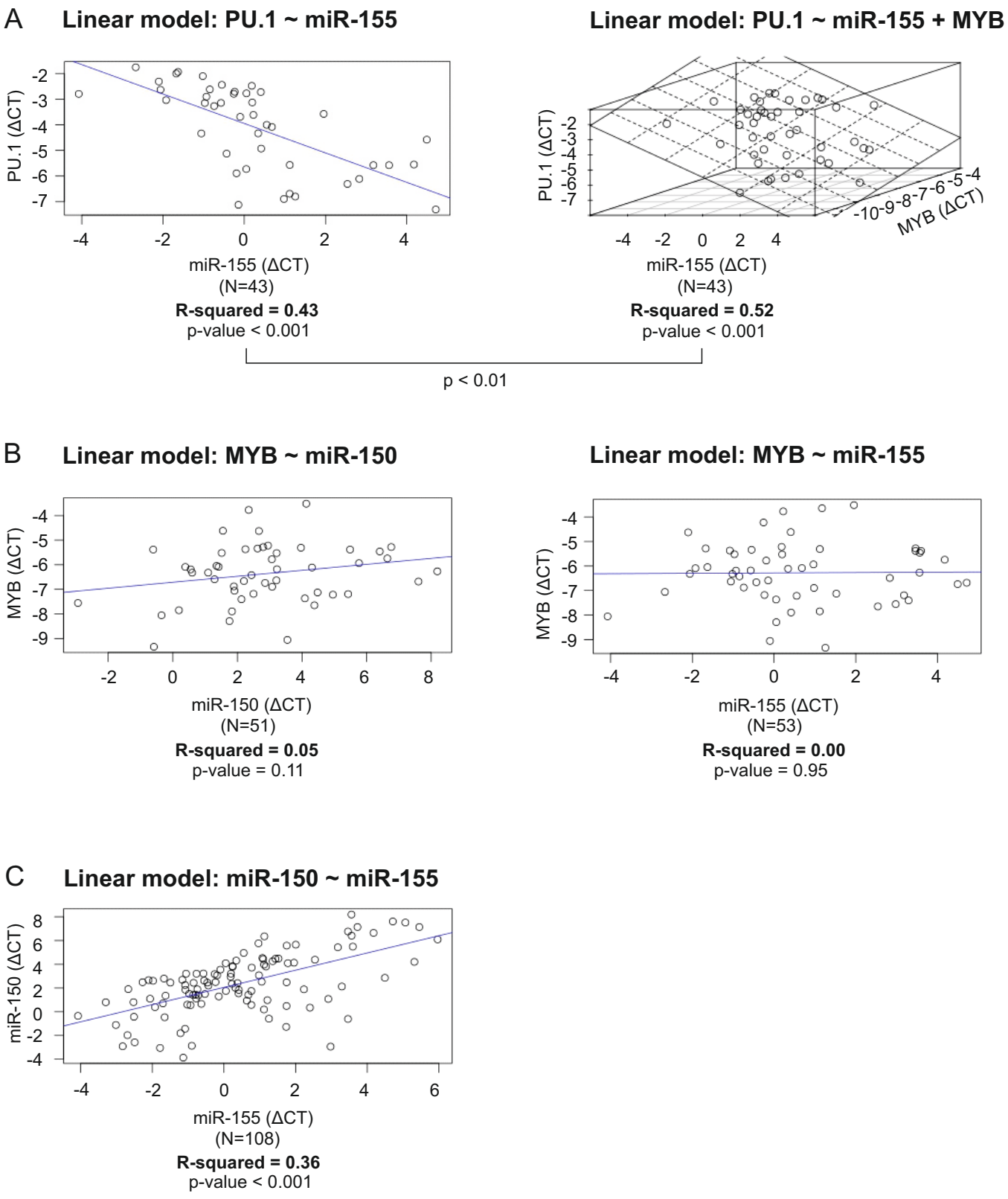

Fig. 4.2. Correlation and linear regression of the expression of microRNAs and their target genes in lymphomas. Correlation and (sequential) regression model for (A) PU.1 depending on miR-155 and MYB, (B) Myb depending on miR-150 or miR-155 and (C) miR-150 depending on miR-155. Data (ΔCT) were counted as follows: $\Delta Ct = CT^{\text{reference gene}} - CT^{\text{target gene}}$; GAPDH and RNU44 were used as reference genes. Lines or planes were added to show the trends. R-squared represents the value of Pearson correlation coefficient for each model. P-values for each model indicate significance of the hypothesis that the correlation is not different from zero. P-value that connects the two models in (A) indicates significance of one-way

that the model proposed by Vargova and colleagues might also work in our set of patients. We also performed simple regression of MYB and PU.1; slope of the regression line should be negative, if the proposed model works. For this analysis, only samples with expression data for all PU.1, miR-155 and MYB were selected.

Note that for the regression analysis we did not use FC values but ΔCT values (listed in Table S9). FC values are basically transformed ΔCT values, but they have a disadvantage: for cases in that the genes are less expressed than a reference gene (GAPDH and RNU44 in our case), the FC value lie between 0 and 1. For cases in that the genes are more expressed than a reference gene, the FC value lie between 1 and ∞. In this setting, the outliers have high impact on linear regression. That is the reason why we have chosen to use ΔCT values instead. They range between -∞ and +∞, so the outliers do not influence the analysis so much. Another advantage of ΔCT values is that they are normally distributed which is an advantage for regression analysis.

We found that miR-155 levels alone can explain 43 % of PU.1 variability in our dataset. When expression of MYB is added to the model, 52 % of PU.1 variability can be explained (Fig. 4.2A). The two models are different at 99% confidence level, as assessed by ANOVA. However, we found positive correlation between the levels of MYB and PU.1. Based on these expression data, we can say that PU.1 might be a target of miR-155 in our collection of malignancies. The results do not support the idea of MYB/miR-155/PU.1 model in this collection.

The malignancies are, however, biologically and clinically very different. We wanted to find out whether they differ in the terms of function of MYB/miR-155/PU.1 model, too. Therefore we performed the same regression analysis as before, but separately for each diagnosis; results are listed in Table S10. We did not get significant results, most likely because of small number of samples in the categories and thus low statistical power. Given these results, we can still assume that PU.1 might be targeted by miR-155 in B-CLL/SLL, HL and FL.

4.3MYB is not a target of either miR-150, or miR-155 in lymphomas.

MYB mRNA contains functional binding sites for both miR-150 and miR-155 (Xiao *et al,.* 2010, Imig *et al.*, 2011). We used simple regression analysis to find out, whether MYB might be a target of miR-150 and/or miR-155 in lymphomas. The first linear model was explaining levels of MYB by the levels of miR-150. In second linear model, miR-155 replaced miR-150 as an explanatory variable. Only samples with expression data for both MYB and miR-150 and MYB and miR-155 were used for the analysis.

We found only very weak correlation between the levels of miR-150 and MYB; miR-155 did not perform better (Fig. 4.2B). This regression analysis was made separately for each diagnosis with similar results (Table S11). We can conclude that MYB is not a target of either miR-150, or miR-155 in lymphomas.

4.4Correlation of miR-155 and miR-150 levels in lymphomas.

Glance at raw data gave us a clue that expression of miR-155 and miR-150 is coordinated among our set of patients. We correlated ΔCT of miR-155 and miR-150 in whole data set and got R-squared of 0.36 (Fig. 4. 2C). This number is reliable because the adjusted R-squared (a correction of R-squared for sample size) has virtually the same value. F-test, analyzing whether slope of specific regression

line is different from zero, was significant at 99.9% level. Altogether these results show fairly strong correlation between the expression levels of miR-155 and miR-150 in lymphomas.

When correlating miR-155 and miR-150 separately for each diagnosis, the R-squared ranged from 0.36 in HL to 0.86 in FL (Table S12). The only exception was MCL, where the correlation was very weak. Interestingly, expression of these microRNAs seems to be correlated also in control samples (R-squared=0.60, adjusted R-squared=0.50), but not in inflammatory lymph nodes.

4.5Detailed analysis of miR-155, PU.1, MYB and miR-150 expression in individual malignancies

As noted in 4.1, general expression patterns of monitored molecules can be observed in individual lymphomas. However, the expression is heterogeneous within each diagnosis. Therefore, we wanted to determine, whether these differences can be attributed to some clinical or biological feature.

We divided the patients into groups according to histological finding. Then, we collected information about diagnosis, treatment, outcome, cytogenetics and other, using the Medea database of General Faculty Hospital. The data are listed in Tables S1-7. We subsequently used the information to detect underlying features of heterogeneity in miR-155, PU.1, MYB and miR-150 expression.

4.5.1 B-CLL/SLL

Different prognostic groups were defined in B-CLL/SLL according to molecular characteristics. Expression of ZAP70 and CD38 in combination with unmutated state of *IGVH* is considered as unfavorable in terms of prognosis. The opposite state (ZAP70- CD38- *IGH* mutated) is considered to have favorable prognosis. Mutations of *TP53*, *ATM*, trisomy of chromosome 12 and complex caryotype have been also linked to unfavorable prognosis, while isolated 13q14 deletion cases have better prognosis (Calin *et al*., 2002).

Small number of samples in our set of nodal B-CLL/SLL did not allow us to determine expression levels of miR-155, MYB, PU.1 and miR-150 according to prognostic groups defined by combination of ZAP70, CD38 and *IGVH*. Therefore, we analyzed individual markers separately. We found difference in PU.1 expression with higher expression in CD38- and ZAP70- cases (95 and 90% level, respectively) (Fig. 4.3). CD38 and ZAP70 are markers of B-cell activation and adverse prognostic markers in B-CLL/SLL, so our results support the idea of PU.1 as a tumor suppressor molecule in B-CLL/SLL.

We next analyzed, whether expression of monitored molecules differs according to clinical characteristics that are associated with disease aggressiveness and prognosis. Such characteristics are Richter syndrome – a process of B-CLL/SLL transformation into lymphoma with higher aggressiveness, usually DLBCL – and number of therapy lines before collection of the sample. Number of therapy lines that patient has received reflects the stage of the disease with respect to its course as described in chapter 1.2.2. The more lines of therapy the patient receives, the worse perspectives he has.

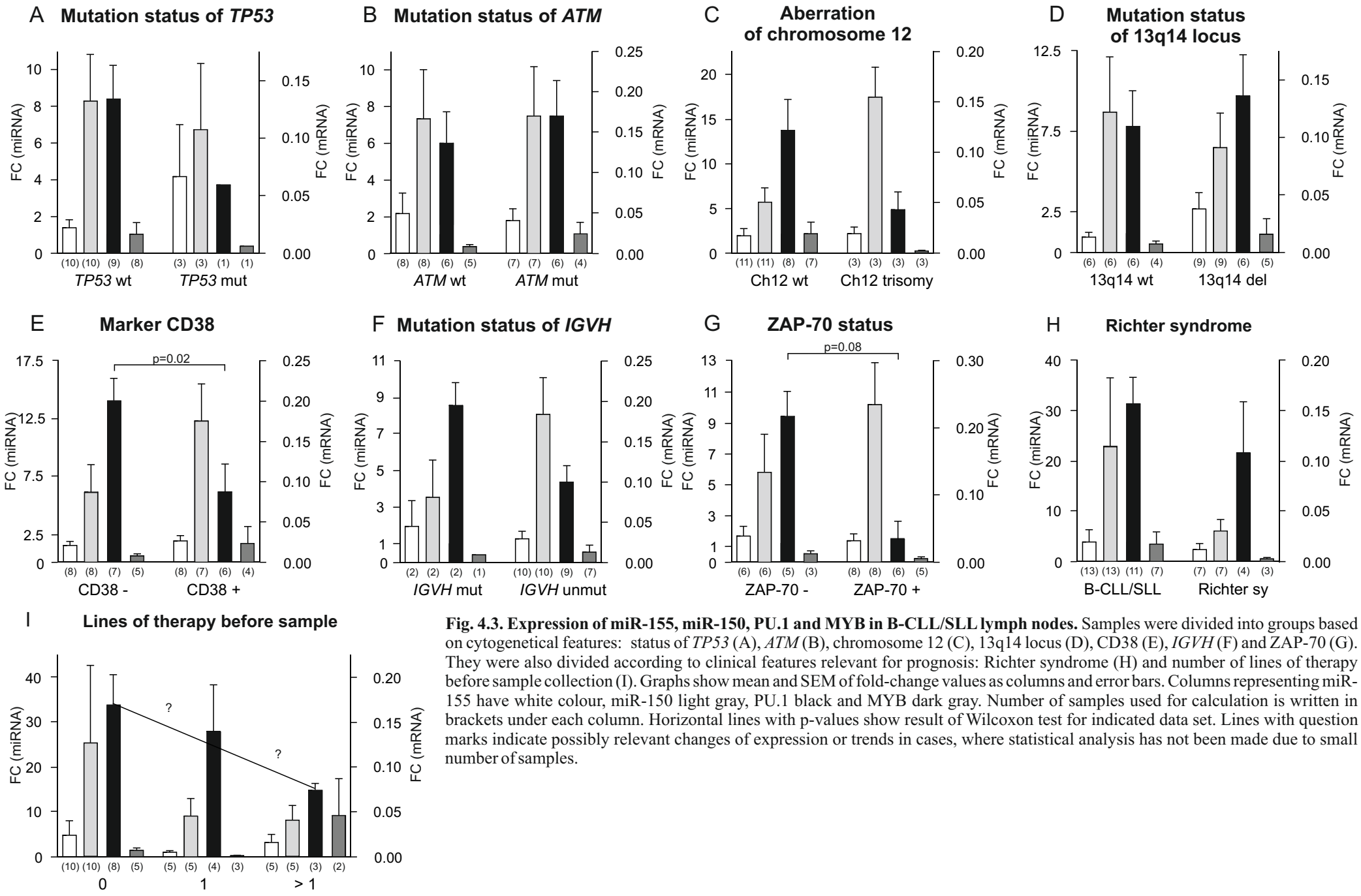

Fig. 4.3. Expression of miR-155, miR-150, PU.1 and MYB in B-CLL/SLL lymph nodes. Samples were divided into groups based on cytogenetical features: status of *TP53* (A), *ATM* (B), chromosome 12 (C), 13q14 locus (D), CD38 (E), *IGVH* (F) and ZAP-70 (G). They were also divided according to clinical features relevant for prognosis: Richter syndrome (H) and number of lines of therapy before sample collection (I). Graphs show mean and SEM of fold-change values as columns and error bars. Columns representing miR-155 have white colour, miR-150 light gray, PU.1 black and MYB dark gray. Number of samples used for calculation is written in brackets under each column. Horizontal lines with p-values show result of Wilcoxon test for indicated data set. Lines with question marks indicate possibly relevant changes of expression or trends in cases, where statistical analysis has not been made due to small number of samples.

We did not observe any differences between B-CLL/SLL and Richter syndrome in terms of miR-155, PU.1, MYB and miR-150 expression. We observed possible trend in decrease of PU.1 levels with higher number of therapy lines (Fig. 4.3). However, this observation cannot be supported by statistical test because of small number of samples.

Together our data provide another small piece of evidence to the idea that miR-155 is an oncogenic molecule in B-CLL/SLL, whereas PU.1 serves as a tumor suppressor.

4.5.2 DLBCL

In DLBCL, we divided the samples in two groups according to gene expression, i. e. immunophenotype. ABC, the more aggressive one, was reported to have higher levels of miR-155 than GCB, the less aggressive type (Eis *et al*., 2005, Lawrie *et al.*, 2007) but in our set, we do not see any changes in levels of observed molecules; the p-value is virtually 1 (Fig. 4.4A).

We next divided the samples according to response to therapy. This characteristic is a powerful predictor of prognosis mainly in Hodgkin lymphoma (according to Jan Koren, head physician of Clinic of Hematology, General Faculty Hospital in Prague). We decided to check also in DLBCL and FL.

After diagnosis of lymphoma, patients are usually appointed for first-choice therapy for each lymphoma type. If this therapy does not work, the patients get other, more aggressive type of therapy. Response to the therapy is basically of three categories: complete remission, partial remission and stable disease. In complete remission, the tumor is not detectable. Partial remission means that the tumor shrinks more than 50 % of its size; in stable disease, the therapy does not affect the tumor very much or at all. Additional examination by positron emission tomography (PET) determines the proliferation activity of tumor cells. Proliferating cells utilize fluorodeoxyglucose and are visible on PET scan. This case is called PET-positive (PET+), opposite case is PET-negative (PET-). Persisting positivity after therapy is an adverse feature, because it means that – despite the therapy – tumor maintained its proliferative activity.

We divided the cases according to response to therapy in two groups. In favorable group we appointed cases that responded by complete remission or PET- partial remission. The unfavorable group consists of cases that responded by PET+ partial remission, or did not respond (i.e. stable disease). We did not find any difference between the groups in terms of miR-155, PU.1, MYB and miR-150 expression (Fig. 4.4B).

Finally, we divided the samples according to the type of diagnosis, meaning if they come from newly diagnosed tumor, or they represent relapse or they have history of transformation from tumor with less aggressiveness (in our case MZL). Again, we did not find any true or possible differences between the groups (Fig. 4.4C).

Together, we did not find any differences in expression among the studied molecules in tested categories in DLBCL – not even in immunophenotypic groups that were previously shown to have different levels of miR-155.

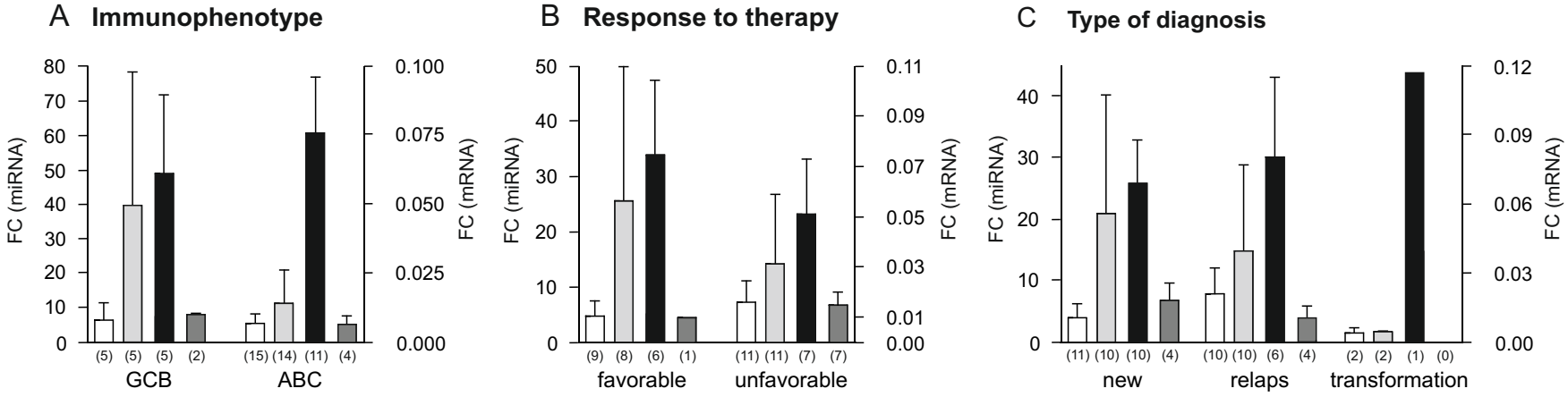

Fig. 4.4. Expression of miR-155, miR-150, PU.1 and MYB in DLBCL. Samples were divided into groups according to immunophenotype (A), response to therapy after the sample (B), and type of diagnosis (C). Graphs show mean and SEM of fold-change values as columns and error bars. Columns representing miR-155 have white colour, miR-150 light gray, PU.1 black and MYB dark gray. Number of samples used for calculation is written in brackets under each column.

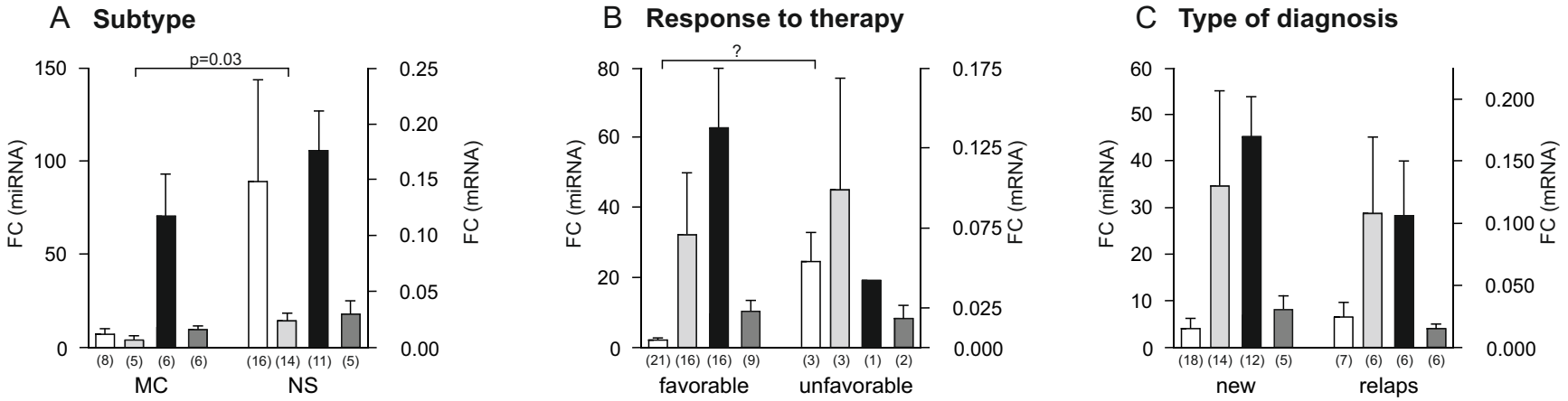

Fig. 4.5. Expression of miR-155, miR-150, PU.1 and MYB in HL. Samples were divided into groups according to subtype (A, response to therapy after the sample (B) and type of diagnosis (C). Graphs show mean and SEM of fold-change values as columns and error bars. Columns representing miR-155 have white colour, miR-150 light gray, PU.1 black and MYB dark gray. Number of samples used for calculation is written in brackets under each column. Horizontal line with p-value shows the result of Wilcoxon test for indicated data set. Line with a question mark indicates possibly relevant change of expression in the case where statistical analysis has not been made due to small number of samples.

4.5.3 HL

In HL, we analyzed expression of miR-155, PU.1, MYB and miR-150 according to cHL subtype, response to therapy and type of diagnosis.

We found higher expression of miR-150 in NS subtype of cHL ($p < 0.05$). In our collection of HL samples, miR-150 is up-regulated compared to controls (Fig. 4.1C) and there seem to be a difference between the two subtypes (Fig. 4.5A). These results are in disagreement with previous finding of Gibcus and colleagues (2009) but their data come from HL cell lines. Our data come from lymph nodes diagnosed with HL, so the tissue is a mixture of HRS and other cells mainly of hematopoietic origin which could account for the difference.

For response to therapy, we did not obtain any significant result. However, we got very interesting picture for expression of miR-155. The 3 samples in unfavorable group have FC 11.0, 22.5 and 39.8. In favorable group, there are 21 samples with median FC 1.1 (range 0.1 - 11.8) (Fig. 4.5B). This result gives us a hint that levels of miR-155 might be associated with response to therapy in HL.

Similarly as in DLBCL, we did not find any difference in expression of studied molecules according to type of diagnosis, i.e. newly diagnosed tumor versus relapse (Fig. 4.5C).

In summary, our results show different levels of miR-150 in HL subtypes and that miR-155 might be a predictor of response to therapy in HL.

4.5.4 FL

In FL, we analyzed expression of miR-155, PU.1, MYB and miR-150 according to 3 clinical features and 2 biological features. First clinical feature analyzed was follicular lymphoma international prognostic index (FLIPI) –an index combining age, stage of the disease, lactate dehydrogenase serum level, number of involved lymph nodes and level of serum hemoglobin. Other analyzed clinical features were response to therapy and type of the diagnosis. The biological features were grade of the tumor and positivity for proliferation markers Ki-67 or MIB1.

From all of the studied differences, one proved to be significant, and that is difference in miR-155 expression between the samples with high and low positivity for proliferation markers (Fig. 4.6E). Expression of miR-155 is significantly higher ($p < 0.05$) in samples with proliferation fraction of 70-100%, compared with samples with proliferation fraction of 0-20%. This result supports the idea of miR-155 as a powerful oncogene in B cells.

4.5.5 MZL and MCL

Our MZL and MCL groups are very small, consisting of 13 and 10 patients, respectively. Therefore, it is tricky to analyze them deeper. We only focused on PU.1 in MCL because of its overall down-regulated expression (Fig. 4.1B). Interestingly, we found out that levels of PU.1 differ between patients with newly diagnosed MCL and those with relapse ($p < 0.1$, Fig. 4.7A). We also found possible trend in decreasing levels of PU.1 transcript according to increasing proliferation rate in the tumor (Fig. 4.7B). Of course, the number of samples is very little, so the results do not allow us to make any specific conclusion. However, these findings suggest that PU.1 can be an important molecule in MCL pathology and its role is worth further investigation.

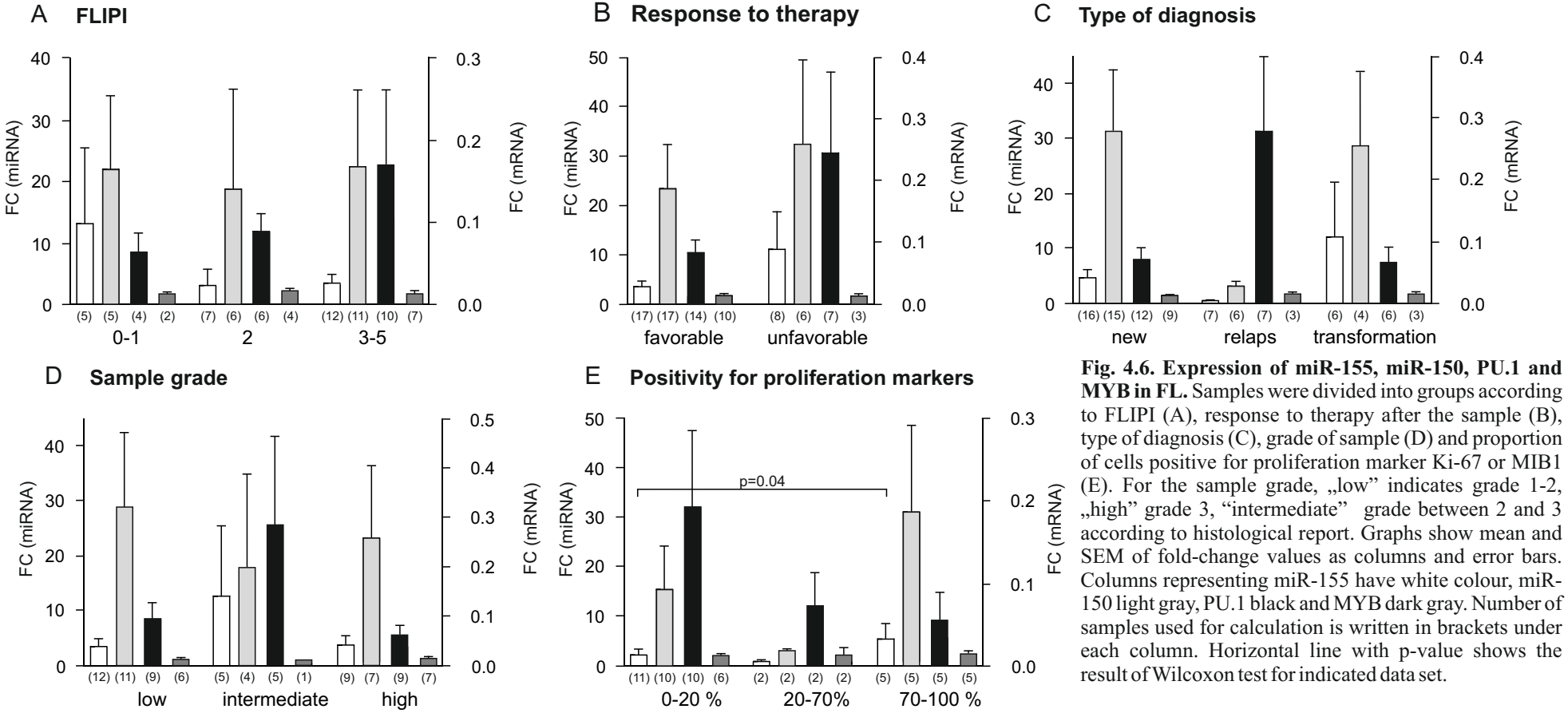

Fig. 4.6. Expression of miR-155, miR-150, PU.1 and MYB in FL. Samples were divided into groups according to FLIPI (A), response to therapy after the sample (B), type of diagnosis (C), grade of sample (D) and proportion of cells positive for proliferation marker Ki-67 or MIB1 (E). For the sample grade, „low" indicates grade 1-2, „high" grade 3, "intermediate" grade between 2 and 3 according to histological report. Graphs show mean and SEM of fold-change values as columns and error bars. Columns representing miR-155 have white colour, miR-150 light gray, PU.1 black and MYB dark gray. Number of samples used for calculation is written in brackets under each column. Horizontal line with p-value shows the result of Wilcoxon test for indicated data set.

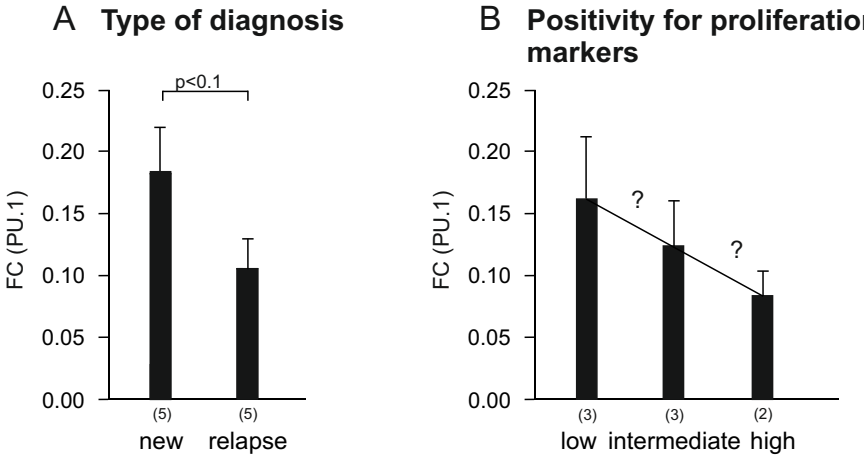

Fig. 4.7. Expression of PU.1 in MCL. Samples were divided into groups according to type of diagnosis (A) and positivity for proliferation markers (B). Number of samples used for calculation is written in brackets under each column. Horizontal line with p-value shows result of Wilcoxon test for indicated data set. Line with question marks indicates possible trend in the case, where statistical analysis has not been made due to small number of samples.

4.6Cox proportional-hazards models for lymphomas

Cox proportional-hazards models are regression models that are used to estimation of impact of a studied factor on the incidence of an event (infection, tumor, death and so on).

Incidence is a central measure in epidemiology. It is a number of cases in which an event occurs in a specific time period. From this, we can calculate incidence rate – probability that an event occurs in someone who is at risk (e.g. that the lung cancer occurs in a smoker). Incidence rate is a proportion: in nominator is incidence (i.e. number of events in a time period), in denominator is number of cases at risk at the same time period. Incidence rate is often called 'hazard rate'. With incidence or hazard rate, so called incidence or hazard ratio can be calculated. This ratio reflects the difference in risk between control ('baseline risk') and study population (e.g. lung cancer in smokers and non-smokers). With this, regression models can be ran in order to estimate the impact of studied factor (e.g. smoking) on incidence of lung cancer. If the hazard ratio is 1.5, we can say that smokers have 50% higher risk of developing lung cancer than non-smokers in our example. The problem is that the data from control population are often missing. Nevertheless, if we assume that the hazard ratio remains the same at time, we won't need the value of incidence rate in control population to calculate the impact of studied condition on the incidence rate. This assumption is used by Cox model (Cox, 1972). This model is written as

$$\ln h_i(t) = \alpha(t) + \beta_1 x_{i1} + \beta_2 x_{i2} + \ldots + \beta_h x_{ih}$$

The baseline risk is unspecified and the hazard ratio is independent of time. Therefore, the model is called Cox proportional-hazards model.

We calculated Cox proportional-hazards models for miR-155, PU.1 and miR-150 for each diagnosis. We found an adverse effect of miR-155 for survival of HL patients but it vanished after adding age as a confounding factor. For PU.1, we found positive effect for survival in B-CLL/SLL patients that also remained after adding age as a confounding factor ($p < 0.05$). Nevertheless, it would probably disappear after adding other confounding factors, such as combination of *IGVH* with ZAP70 and CD38, *TP53* or *ATM*. For miR-150, we did not find any effect in any diagnosis.

5 DISCUSSION

We measured expression of miR-155, PU.1, MYB and miR-150 in lymph nodes and other tissues infiltrated by several types of lymphoproliferative malignancies: 20 cases of B-CLL/SLL, 24 DLBCL, 25 HL, 29 FL, 13 MZL, and 10 MCL. Additionally, we measured expression of these molecules in 4 inflammatory lymph nodes. Lymph nodes with minor reactive changes (N=6) were used as controls.

We found significantly higher levels of miR-155 in all malignancies – except of MCL – compared with control lymph nodes. Levels of miR-155 in inflammatory lymph nodes were also significantly higher than in control lymph nodes and comparable with the levels in lymphoproliferations. Our results support previous observations of elevated levels of either precursor or mature miR-155 in lymph nodes with B-CLL/SLL, DLBCL, HL and FL involvement (Eis *et al*., 2005, Kluiver *et al*., 2005, Lawrie *et al*., 2007, Wang *et al.,* 2008, Xiao *et al.,* 2007). We added 13 cases of MZL to the 3 previously observed (Eis *et al*., 2005, Kluiver *et al*., 2005) and therefore conclude that levels of miR-155 are overall elevated in this disease.

We did not observe any changes of miR-155 expression in MCL; it is consistently low as in control lymph nodes. 6 cases of nodal MCL have been previously shown to be negative for pre-miR-155 by *in situ* hybridization (Kluiver *et al.,* 2005). On the other hand, slight up-regulation of miR-155 has been observed in peripheral blood of 30 MCL cases by microarray technique (Zhao *et al.,* 2010). MCL is located primarily in lymph nodes, but peripheral blood-involvement is found in virtually all cases (Ferrer *et al.,* 2007). We may hypothesize that MCL elements in circulation have different properties than those in lymph nodes and that these properties are reflected by the levels of miR-155. The results reported in circulating MCL can be also attributed to inaccuracy of microarrays; they were not validated by PCR. Nevertheless, from both our and published results we conclude that miR-155 is not over-expressed in nodal MCL.

As for the levels of miR-155 in inflammatory lymph nodes, Tili and Croce (2009) observed very high miR-155 expression in inflammation and intermediate expression in tumors (higher than normal but not so high as in inflammation, unpublished data). Given that miR-155 is able to induce transformation in vivo (Costinean *et al*., 2006), Tili and Croce suggest that 'different levels of miR-155 have different outputs' (Tili and Croce, 2009) and subsequently speculate about possible mechanisms. However, levels of miR-155 in our set of inflammatory lymph nodes are similar to those observed in lymphomas; therefore our results do not support suggestion of Tili and Croce.

For expression of PU.1 we found an opposite pattern than for miR-155; they are significantly lower in all studied malignancies, including MCL. Correlation between miR-155 and PU.1 in pooled set of malignancies is negative with miR-155 explaining 43% of PU.1 variability. In individual diagnoses, the correlation is significantly different from zero in B-CLL/SLL, HL and FL. PU.1 transcript is a validated target of miR-155 in B cells. Certain level of PU.1 is necessary for proper differentiation of B cells. It was shown that altered miR-155 levels lead to deregulation of PU.1; down-regulation of PU.1 has been reported as one of the mechanisms of miR-155 action in lymphomagenesis (Thompson *et al*., 2010). Our results give indirect evidence that PU.1 is a target of miR-155 at least in B-CLL, HL and FL.

We also used the regression analysis to see, whether MYB/miR-155/PU.1 model proposed for B-CLL/SLL (Vargova *et al*., 2011) might generally work in B-cell malignancies. In this model, MYB stimulates miR-155 expression which leads to decreased levels of PU.1, block of B-cell differentiation and malignant proliferation. Linear model with levels of MYB transcript and miR-155 as predictor

variables explains 52 % of PU.1 variability in our data set, which is significantly different from prediction by miR-155 alone. However, the correlation between PU.1 and MYB was positive. (The models were not significantly different from zero when analyzed separately for each malignancy, probably because of small number of samples.) The results do not support the idea of general function of MYB/miR-155/PU.1 model in B-cell malignancies. It seems that the mechanism is restricted to certain subset of patients, as originally described (Vargova *et al.,* 2011).

MYB has been described as a target of both miR-155 and miR-150 (Xiao *et al.,* 2007, Imig *et al*., 2011). We therefore correlated MYB levels with levels of miR-155 and miR-150, respectively. The correlation coefficients were virtually zero. Therefore we can conclude that MYB is not likely to serve as a target transcript of miR-155, nor miR-150 in analyzed lymphoproliferative malignancies. This finding can be explained as follows: I) targeting by microRNAs depends on cellular and molecular context and MYB was described as miR-155 target in HEK293 cell, not in B cells; II) even though MYB has been described as miR-150 target in B cells, it can maybe escape the regulation – possibly also due to pathological changes in overall gene expression – and does not serve as miR-150 target in lymphomas any more. MYB still might be a target of miR-150 in our set of control lymph nodes ($p<0.1$); however, the evidence is based on small data set (N=4) and therefore weak.

We then found positive correlation between levels of miR-150 and miR-155 (R-squared 0.36-0.86 according to data set) except of MCL and inflammatory lymph nodes. This result might account for some general changes in efficiency of microRNA pathway or possibly to casuality – maybe an effort to compensate miR-155 levels by increasing levels of miR-150.The important thing to look at is still the difference between normal and disease state – positive correlation of the microRNAs does not necessarily mean that both are over-expressed or down-regulated, as seen in Fig. 4.1.

We analyzed the expression of miR-155, PU.1, MYB and miR-150 in individual diagnostic groups in relation to clinical and molecular characteristics of the patients and tumors from which we had the samples. In B-CLL/SLL, we found significantly lower expression of PU.1 in cases positive for ZAP70 or CD38 and a possible trend of decreasing PU.1 levels with increasing number of therapeutic lines. All these characteristics – positivity for ZAP70 and CD38 and number of therapeutic lines – are adverse prognostic features in B-CLL/SLL. Therefore we can assume that expression of PU.1 is getting lower in B-CLL/SLL patients with unfavorable prognosis. Together with overall decreased levels of PU.1 in MCL, we also found lower levels of PU.1 in relapse of MCL compared with newly diagnosed MCL and a possible trend of decreasing PU.1 with increasing proportion of proliferating cells. These results indicate that PU.1 might be an important molecule in B-CLL/SLL and MCL pathology with possible clinical consequences.

Next interesting finding suggests that miR-155 can separate HL cases according to response to the therapy that follows the sample collection. Our initial finding must be first validated using larger data set. If true, miR-155 could be used in clinics as a prognostic marker for HL. From biological point of view, it would be interesting to find out whether miR-155 actually contributes to the tumor resistance and what would be the mechanisms. For these consequences, we find the result promising and worth attention.

We further found higher expression of miR-155 in FL samples with high (70-100 %) proliferation fraction. It is well documented that miR-155 is both target and enhancer of survival and proliferation pathways (Gatto *et al*., 2008, O'Connell *et al.,* 2009, Yin *et al*., 2008). It would be interesting to find out if or how miR-155 contributes to proliferation in FL and whether there are some clinical implications.

Last difference we observed in our data set was that levels of miR-150 are higher in NS type of cHL compared with the MC type. At histological level, NS differs from MC type by presence of sclerotic fibers that surround malignant follicles. However, miR-150 was reported to regulate levels of integrin β3 and subsequently level of collagen I (Honda *et al*., 2013). According to this, we would expect reduced levels of miR-150 in NS. Due to absence of more information, it is rather difficult to interpret our finding.

What we expected to find, but did not, were the different levels of miR-155 between ABC and GCB subtypes of DLBCL. Our expectation was based on one publication of miR-155 levels in 18 ABC and 17 GCB cases (Lawrie *et al.,* 2007), supported by one publication of miR-155 levels in 6 DLBCL cell lines (4 GCB, 2 ABC; Thompson *et al*., 2011), and pre-miR-155 measurement in 12 DLBCL cases (10 GCB, 2 ABC; Eis *et al*., 2005). Lawrie and colleagues used the same type of samples and the same method of measurement as we did, so the disagreement is not likely to be attributed to the methodological bias. However, although Lawrie did find significant difference in miR-155 expression between the two DLBCL subtypes, range of miR-155 expression in his GCB-DLBCL set was quite broad (Fig. 2C *in* Lawrie *et al.,* 2007). It is possible that our 5 cases of GCB-DLBCL are those with relatively high miR-155 levels. Therefore, we would have to analyze more GCB-DLBCL cases to be able to challenge Lawrie's finding.

Small number of samples is an overall limitation of our study, affecting particularly the deeper analysis of the role of miR-155, PU.1, MYB and miR-150 in studied lymphoproliferations. Small number of samples also compromises the predicative value of Cox proportional-hazards models. Although we identified PU.1 as a possible predictor of survival in B-CLL/SLL, the model was using only age as a confounding factor. It is likely that PU.1 as a predictor would not remain significant after adding more confounding factors, such as Rai stage, *TP53* mutation status, and combination of ZAP70, CD38 and *IGVH*. Nevertheless, we are quite confident about overall expression results that are shown in Fig. 4.1 and 4.2.

This study is additionally limited by the facts that I) the expression is analyzed in mixed cellular populations, and II) the analysis is restricted to RNA level. These features of the study do not allow us to make clear statements about the underlying biology because the studied cell population is not precisely defined and for PU.1 and MYB, the information about actual level of the functional protein is missing. However, these limitations can turn into advantages seeing from the clinical point of view. The measurement of RNA is simple, relatively cheap and performed on actual biopsies from patients. All of this favors the approach for clinical use in case that the observed differences would be validated.

The next steps after this pilot study would be clearly to extend the number of analyzed cases, particularly in the unfavorable response to therapy group in HL. We also think that expression of PU.1 should be further investigated in B-CLL/SLL and MCL – both in terms of disease prognosis and pathological mechanisms. In case of MCL, the issue of miR-155 expression in nodal versus circulating form should be addressed in greater detail as well.

In summary, we found that miR-155 is up-regulated in almost all examined lymphoproliferative diseases, while its target PU.1 is down-regulated. The pathological levels may reflect prognosis of the patient and state or aggressiveness of the disease, which is promising for potential clinical application. More investigation of both clinical and biological consequences of miR-155 and PU.1 expression in lymphomas is needed.

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