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**Role onkogenní mikroRNA-155 a proto-onkogenu *MYB* u
chronické lymfatické leukémie**

**The Role of oncogenic microRNA-155 and proto-oncogen
MYB in chronic lymphocytic leukemia**

Disertační práce

Ph.D. Thesis

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CONTENT

ABSTRAKT (CZ)	9
ABSTRACT (EN)	10
LIST OF ABBREVIATIONS	11
1. INTRODUCTION	15
1.1 Characterization, diagnosis, progression and therapeutic tools of B-cell chronic lymphocytic leukemia (B-CLL).....	15
1.1.1 What is B-CLL?.....	15
1.1.2 Epidemiology of B-CLL.....	16
1.1.3 Diagnosis of B-CLL.....	16
1.1.4 Clinical staging of B-CLL.....	20
1.1.5 Progression and transformation of B-CLL.....	20
1.1.6 Conventional and novel therapeutic tools of B-CLL.....	21
1.2 Introduction to the microRNAs.....	23
1.2.1 Discovery, nomenclature of microRNAs.....	23
1.2.2 Biological function of microRNAs.....	24
1.2.3 Biogenesis of microRNAs.....	24
1.2.4 Regulation of the microRNA biogenesis.....	27
1.2.5 miRNAs are involved in pathogenesis of B-CLL.....	29
1.3 Characterization of microRNA-155 and its role in B-cells and leukemia.....	32
1.3.1 Overview of microRNA-155/ <i>BIC</i> / <i>MIR155HG</i>	32
1.3.2 Role of miR-155 in normal and leukemic B-cells.....	34
1.3.3 Mouse models of miR-155.....	35
1.3.4 Transcriptional regulation of <i>MIR155HG</i>	36
1.3.5 Relationship between miRNA-155 and its direct target PU.1.....	38
1.4 Characterization of E-box protein MYB and its role in B-cells and leukemia.....	40
1.4.1 Characterization of E-box protein MYB.....	40
1.4.2 Role of MYB in normal and leukemic B-cells.....	41
1.4.3 Transcription factor MYB and its role in leukemia.....	41

1.4.4 Mouse models of Myb.....	42
1.4.5 miR-150 a target of <i>MYB</i> gene in leukemia.....	43
2. INTRODUCTION TO THE THESIS.....	44
2.1 Hypothesis.....	45
2.2 Aims of the Thesis.....	46
2.3 Specific aims of the Thesis.....	47
3. MATERIAL AND METHODS.....	48
3.1 Material.....	48
3.1.1 Biological material.....	48
3.1.1.1 Primary B-CLL patient samples and normal healthy controls.....	48
3.1.1.2 Cell lines.....	51
3.1.2 Chemicals and buffers.....	52
3.1.2.1 Chemicals.....	52
3.1.2.2 Buffers and solutions.....	54
3.1.2.3 Commercial kits.....	55
3.1.2.4 Antibodies.....	56
3.1.2.5 Plasmids.....	56
3.1.3 Instruments.....	56
3.1.4 Programs.....	57
3.2 Methods.....	57
3.2.1 Isolation of primary B-cells and plasma.....	57
3.2.1.1 Isolation of peripheral blood mononuclear cells by Ficoll - Paque.....	57
3.2.1.2 Isolation of CD19+ B-cells by RosetteSep kit.....	58
3.2.1.3 Plasma separation.....	59
3.2.1.4 Lysis of erythrocytes by ammonium chloride (NH ₄ Cl).....	59
3.2.1.5 Magnetic separation of CD19+ B-cells by MACS magnetic beads.....	59
3.2.2 Flow cytometry analysis (FACS).....	60
3.2.2.1 Control measurements for CD19 purity after MACS separation.....	60
3.2.2.2 Propidium iodide (PI) staining.....	60
3.2.2.3 AnnexinV staining.....	60
3.2.3 Transient transfections and transduction.....	61
3.2.3.1 Transfection of primary B-CLL cells by lipofection reagent DMRIE-c.....	61

3.2.3.2	Transfection of HeLa cells by lipofection reagent JetPEI.....	61
3.2.3.3	Transfection of Raji cells by Amaxa nucleofector.....	62
3.2.3.4	Transduction of primary B-CLL cells with lentivirus.....	63
3.2.4	Luciferase reporter assay.....	63
3.2.5	Western blot.....	66
3.2.6	Chromatin immunoprecipitation (ChIP).....	66
3.2.7	Isolation of mRNA/miRNA and preparation of cDNA.....	67
3.2.7.1	Isolation of mRNA/miRNA from PBMCs, B-cells, Raji cells by TRIzol reagent.....	67
3.2.7.2	Preparation of cDNA (complementary).....	67
3.2.7.3	Isolation of miRNAs from B-CLL patient plasma samples by miRNAeasy kit.....	68
3.2.7.4	Preparation of cDNA (complementary).....	68
3.2.8	Performing of RT-qPCR by TaqMan system and with ROCHE probes.....	70
3.2.8.1	RT-qPCR data analysis and statistics.....	74
3.2.9	Microarray mRNA profiling.....	74
3.2.9.1	Microarray data analysis and statistics.....	74
4.	RESULTS.....	76
4.1	Expression and protein profile of B-CLL patients cells.....	76
4.1.1	Expression of miR-155, miR-150 and transcription factors PU.1, FOS, MYB and MYC in B-CLL patients and in normal healthy controls.....	76
4.1.2	Relationship between expressions of miR-155 - miR-150 - PU.1 - MYB in B-CLL samples.....	83
4.1.3	Clinical and prognostic importance of expression of miR-155, miR-150, MYB and PU.1 in B-CLL.....	85
4.2	The occupancy of MYB and epigenetic features at <i>MIR155HG</i> promoter region in B-CLL.....	98
4.2.1	Occupancy of transcription factor MYB on <i>MIR155HG</i> promoter region.....	98
4.2.2	Active chromatin marks associate with elevated levels of miR-155 in B-CLL cells.....	100
4.3	Mutation status of canonical MYB binding motive at the <i>MIR155HG</i> in B-CLL...102	

4.4 Global gene expression profile in B-CLL unrolls deregulation of MYB and miR-155 targets.....	103
4.5 <i>In vitro</i> changes at levels of MYB, miR-155 and PU.1 in B-CLL cells show on the tight relationship between these molecules.....	107
5. DISCUSSION.....	113
5.1 Expression profile of miR-155, PU.1, MYB and miR-150 in relation to the clinical data and epigenetic markers.....	113
5.2 Global gene expression profile: miR-155 and MYB target genes in B-CLL.....	118
5.3 <i>In vitro</i> functional assays confirmed relationship between miR-155, PU.1 and MYB in B-CLL cells.....	122
5.4 Working model of B-CLL and future therapy tools.....	123
6. CONCLUSIONS.....	127
7. SUMMARY.....	129
8. AUTHOR CONTRIBUTION.....	131
9. REFERENCES.....	132
10. SUPPLEMENT.....	154
11. LIST OF PUBLICATIONS.....	170

ABSTRAKT (CZ)

B-buněčná lymfocytární chronická lymfatická leukémie (B-CLL) představuje nádorové onemocnění, pro které je význačná akumulace B-buněk v periferní krvi, kostní dřeni, lymfatických uzlinách a slezině v důsledku poruchy programované smrti. Klinický průběh B-CLL je značně heterogenní, což dokazuje i fakt, že u některých pacientů toto onemocnění rychle progreduje, zatímco jiní pacienti přežívají několik let bez nutnosti léčby. I když se onemocnění charakterizuje stanovováním několika prognostických parametrů, jež jsou typické pro B-CLL, chybí spolehlivá indikace začátku včasné terapie. Z popsaného důvodu je v současnosti velice intenzivně studována nejenom biologická role malých nekódujících RNA (tzv. mikroRNA), ale také spojitost těchto molekul se vznikem a průběhem leukemických onemocnění včetně B-CLL. Proto jsme se zaměřili na studium miR-155, která reguluje diferenciaci buněk krvetvorby, zánětlivých reakcí a produkci protilátek. Zvýšená exprese miR-155 se objevuje v leukemogenezi, kde se předpokládá, že má vliv na zástavu buněčné diferenciace a narušení normální funkce B-buněk. Naše výsledky potvrdily zvýšenou expresi jejího primárního transkriptu miR-155 a také maturované formy u pacientů s B-CLL. Analýza expresních dat kohorty 239 pacientů s B-CLL naznačuje, že vzorky pacientů s nepříznivou prognózou vykazují signifikantně vyšší hladiny obou forem miR-155 s nimiž prokazatelně souvisí nízké hladiny proteinu PU.1 oproti kontrolám. Domníváme se, že jedním z mechanismů vzniku leukémie by mohla být nižší exprese proteinu PU.1 zapříčiněná vysokou hladinou miR-155. Naše výsledky dále ukazují, že transkripční faktor, proto-onkogen MYB z rodiny E-box proteinu se přímo váže do promotorové oblasti genu pro miR-155 - *MIR155HG*, čímž stimuluje jeho transkripci. V oblasti vazebních míst pro MYB jsme detekovali přítomnost chromatinových značek: H3K9Ac a H3K4Me3. Funkční *in vitro* eseje s primárními buňkami pacientů s B-CLL dále prokázaly vzájemný negativní vztah mezi miR-155 a PU.1, a pozitivní vztah mezi MYB a miR-155. Na základě našich výsledků jsme vytvořili pracovní model pro B-CLL, kde MYB stimuluje transkripci miR-155, který následně snižuje produkci PU.1 v B-CLL buňkách. U 20% měřených vzorků B-CLL byl prokázán výše uvedený vztah. Úspěšnost léčby B-CLL spočívá v jejím správném zahájení. Zjistili jsme, že zvýšená exprese miR-155 koreluje s agresivitou B-CLL. Můžeme shrnout, že měření exprese molekul miR-155, MYB a PU.1 u pacientů s B-CLL by tedy mohlo být vhodným nástrojem pro detailnější charakterizaci onemocnění, které by dále umožnilo včasné zahájení terapie.

KLÍČOVÁ SLOVA: B-CLL, terapie, mikroRNA, mRNA, miR-155, MYB, PU.1, genová exprese, leukemie, chromatinové modifikace, DNA, protein.

ABSTRACT (EN)

Chronic lymphocytic leukemia (B-CLL) represents a disease of mature-like B-cells. Due to failed apoptosis but also due to enhanced proliferative signals, the leukemic B-cells accumulate in the peripheral blood, bone marrow, lymph nodes and spleen. The clinical course of B-CLL is very heterogeneous; in some patients B-CLL progresses very rapidly into an aggressive form. Such patients need therapy sooner while in other patients with indolent B-CLL the onset of therapy takes years. Several standard prognostic and disease progression markers are used for disease staging and monitoring, however a reliable marker that will suggest when to start therapy is unknown. Expression of small, non-coding microRNAs is often deregulated and represent important prognostic markers in variety of cancers including leukemia. Hence in our study we concentrated to miR-155, an important molecule regulating differentiation of hematopoietic cells, inflammation process and antibody production. Its aberrant expression was described in Hodgkin`s as well as in non-Hodgkin`s lymphoma, including indolent lymphoproliferations like B-CLL. Our results confirmed elevated levels of both, primary miR-155 transcript and mature form of miR-155 in our B-CLL patient samples (N=239). The aberrant expression of miR-155 in B-CLL samples associated with unfavorable prognosis. Moreover, we also observed elevated miR-155-mediated decrease of its direct target – PU.1 in B-CLL cells that is important regulator of B-cell maturation. Next, we demonstrated that proto-oncogen MYB directly binds at the promoter region of the miR-155 gene - *MIR155HG* and stimulates its transcription. This coincided with enrichment of activated epigenetic marks: hypermethylated histone H3K4 residue and spreaded hyperacetylation of H3K9 at the *MIR155HG* promoter. Our *in vitro* functional assays on the primary B-CLL cells and Raji cell line confirmed the negative relationship between miR-155 and PU.1 and positive relationship between MYB and miR-155. We also have shown that unfavorable prognosis of B-CLL associates with low expression of miR-150 (inhibitor of Myb) and high expression of MYB mRNA. Based on above-mentioned results, we created working model of relationship between miR-155, MYB and PU.1 that can be applied to around 20% of more aggressive B-CLL patients. The success of therapy of B-CLL patients depends on the accurate start of the therapy. Our data also indicate that the levels of miR-155 in B-CLL patients associate with the disease progression and can be used to predict therapy onset. We conclude that measurement of miR-155, MYB and PU.1 expression associates with B-CLL progression and could help in making valuable clinical decisions.

KEY WORDS: B-CLL, therapy, microRNA, mRNA, miR-155, MYB, PU.1, gene expression, oncogene, chromatin modifications, DNA, protein.

LIST OF ABBREVIATIONS

ABC/GC	activated B-cell like/germinal center (type of DLBCL)
ADAR	adenosine deaminase
AGO-1-4	argonaut family proteins 1-4
AID (AICDA)	activation-induced cytidine deaminase
ALL	acute lymphoblastic leukemia
ALV	avian leukosis virus
AML	acute myeloid leukemia
AMV	avian myeloblastosis virus
AP-1	activator protein 1
APL	acute promyelocytic leukemia
ARS2	arsenate resistance protein 2
ATM	ataxia-telangiectasia mutated
AVG	average
BCL2	B-cell lymphoma 2
BCL6	B-cell lymphoma 6
B-CLL	B-cell chronic lymphocytic leukemia
BCR	B-cell receptor
BIC	B-cell Integration Cluster
bp	base pair
BSA	bovine serum albumine
Btk	Bruton`s tyrosine kinase
CD38	cluster of differentiation 38
cDNA	complementary DNA
c-Fos	cellular FBJ murine osteosarcoma viral oncogene homolog
CLP	common lymphoid progenitor
CML	chronic myeloid leukemia
ChIP	chromatin immunoprecipitation
DBD	DNA binding domain
DGCR8	DiGeorge syndrome critical region gene 8
DLBCL	diffuse large B-cell lymphoma
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
ES	enrichment score
ETS family	E twenty-six family proteins
FACS	Fluorescence Activated Cell Sorting
FADD	Fas-associated protein with death domain
FBS	fetal bovine serum
FC	fold change
FCR	Fludarabine-Cyclophosphamide-Rituximab
FISH	Fluorescence <i>in situ</i> hybridization
FITC	Fluorescein isothiocyanate
FosB	FBJ murine osteosarcoma viral oncogene homolog B
FOXP3	forkhead box P3

FUS	fused in sarcoma
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GSEA	gene set enrichment analysis
H2A	histone H2A
H3	histone H3
H3K4Me3	H3K4 methyl3
H3K9Ac	H3K9 acetyl
HIF1A	hypoxia inducible factor 1A
Hsa	<i>Homo sapiens</i>
HSC	hematopoietic stem cell
HSPCs	hematopoietic stem progenitor cells
hrs	hours
IFN α/β	interferon α/β
IFN γ	interferon γ
IgM	immunoglobulin M
IgVH	immunoglobulin heavy chain variable
IL-2	interleukin-2
IMDM	Iscoe's Modified Dulbecco's Medium
IRAK3	interleukin-1 receptor-associated kinase 3
Kb	kilo base
kDa	kilo Dalton
KRAS	Kirsten rat sarcoma viral oncogene homolog
KSHV	Kaposi's sarcoma-associated herpesvirus
LMP-1	latent membrane protein
MAD2, 4	mitotic arrest deficient, yeast, homolog-like 2, 4
MAFB	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B
MAP3K7	mitogen-activated protein kinase kinase kinase 7
MBL	monoclonal B-cell lymphocytosis
MCL	mantle cell lymphoma
MGB	minor groove binder
min	minutes
miRNA	microRNA
miRNA-155	microRNA-155
miR-155	microRNA-155
MIR155HG	miR-155 Host Gene
mmu	<i>Mus musculus</i>
MOI	multiplicity of infection
mRNA	messenger RNA
MYB	v-myb myeloblastosis viral oncogene homolog
MYC	v-myc avian myelocytomatosis viral oncogene homolog
NES	normalized enrichment score
NF κ B	nuclear factor kappa B
NFQ	non-fluorescent quencher
NHL	non-Hodgkin`s lymphoma
nM	nano molar
NRD	negative regulation domain
Nt	nucleotide

ORF	open reading frame
OS	overall survival
P/S	penicillin/streptomycin
PABP	poly(A)-binding protein
PACT	protein activator of PKR
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PI	propidium iodide
PI3K	phosphoinositide 3-kinase
pre-miRNA	precursor microRNA
pri-miRNA	primary transcript of microRNA
RISC	RNA induced silencing complex
RNA	ribonucleic acid
RS	Richter`s syndrome
RT-qPCR	reverse transcription quantitative polymerase chain reaction
SATB1	special AT-rich Binding protein-1
SEM	standard error of the mean
Sfpi1(Pu.1)	spleen focus forming virus proviral integration oncogene (<i>Mus musculus</i>)
SHIP1	inositol polyphosphate-5-phosphatase
SHM	somatic hypermutation
siRNA	small interfering RNA
SLL	small lymphocytic leukemia
SMAD2, 4	Sma- and Mad-related protein 2,4
SPI1 (PU.1)	spleen focus forming virus proviral integration oncogene (<i>Homo sapiens</i>)
STAT3	signal transducer and activator of transcription 3
stRNA	small temporal RNA
TAD	transactivation domain
TF	transcription factor
TGF- β	tumor growth factor β
Th	therapy
TLR	Toll-like receptor
TRBP	ribonucleo protein
tRNAs	transfer RNA
TSS	transcription start site
UTR	untranslated region
WHO	World Health Organisation
wt	wild type
ZAP-70	zeta associated protein about 70kDa

Motto:

The success of therapy of B-CLL patients depends on the accurate start of the therapy.

1. INTRODUCTION

1.1 Characterization, diagnosis, progression and therapeutic tools of B-cell chronic lymphocytic leukemia (B-CLL)

1.1.1 What is B-CLL?

B-CLL represents slow-growing leukemia characterized by an accumulation of leukemic, mature appearing B-cells in the bone marrow, lymph nodes, spleen and in the peripheral blood ^{1,2}. The WHO classification characterizes B-CLL as leukemia of mature B-cells ³. Accumulation of leukemic B-cells is due to imbalance between emerging and apoptosis of B-cells. As a result of failed apoptosis, most B-CLL cells are arrested in G0/G1 ⁴. The high number of lymphocytes in B-CLL patients is caused not only by the prolonged survival of B-cells with failed apoptosis, but also by proliferating of subset B-cells in proliferative centers in lymph nodes and bone marrow ^{5,6}. Messmer *et al.* showed that B-CLL represents a non-static disease where cells displayed dynamic cellular kinetics: some cells proliferate and some die ⁷. The stimulation of endothelial cells and T-cells activates B-CLL cells to divide and reinforces their resistance to apoptosis. The B-CLL cells are then released from proliferative centres into peripheral blood where they start to express markers of activation (CD154, IL-2, CD38). Activated B-CLL cells are further attracted by chemokines back into tissues and proliferative centres ⁸. Normal healthy B-cells originate from bone marrow and mature in the lymph nodes where they are specialized to fight infections by producing antibodies. Leukemic B-cells grow out of control, accumulate in the bone marrow and in peripheral blood and overgrow healthy normally functioning blood cells ⁹. It is under intensive study how the normal B-cell transforms into the leukemic cell. *In vitro* studies on the primary leukemic cells help to understand the pathogenesis of leukemia in more detail.

The etiology of B-CLL is still unclear and actively discussed. The identification of the cellular origin of B-CLL is essential to elucidation of the pathobiology of leukemia development. In most B-CLL cases, the disease manifests initially as a precursor phase, called monoclonal B lymphocytosis (MBL), characterized by asymptomatic monoclonal or oligoclonal proliferation of CD5+ B-cells. The primary leukemogenic event in B-CLL might involve self-renewing hematopoietic stem cells. Thus, the accumulation of leukemic events in B-CLL cells begins at the HSC stage ¹⁰. Moreover, Goldin *et al.*, described that B-CLL

precedes MBL with higher frequency in the hereditary form of B-CLL ¹¹. The MBL shares some similarities with B-CLL, as for example chromosomal aberrations ^{11,12}. Acquisition of chromosomal aberrations as deletion of 11q22-23, 17p13, 13q14 or trisomy of chromosome 12 appears to be the secondary driver of B-CLL development ^{10,13}. Chromosomal aberrations and microenvironment stimuli influence selection and expansion of the B-CLL malignant clone ¹⁴.

The study of small non-coding, regulatory microRNAs in the molecular pathogenesis and development of B-CLL dramatically increased in the last decade ¹⁵. Croce's research group ¹⁶ showed the first evidence that miRNAs are involved in the pathogenesis of B-CLL. Authors found that decreased expression of miR-15-16 cluster associates with frequent deletion of chromosome 13q14 in B-CLL cells ¹⁶. The recently published data showed that miRNAs as: miR-21, miR-150, miR-155, miR-181b, miR-34, miR-29 are differentially expressed in B-CLL ¹⁶⁻¹⁸. The recent evidence suggests that miRNAs could be used as biomarkers for B-CLL prognosis and progression ^{19,20}.

1.1.2 Epidemiology of B-CLL

B-CLL belongs to the most common leukemia in the Western world and affects mainly elderly population with a median age around 70 years. Rarely, B-CLL develops in the individuals under 50 years ²¹. In contrast, in Asian population B-CLL arise with very rare frequency ²². Further, in males B-CLL develops with significantly higher prevalence (the male:female ratio is 2:1) ²³. In the hereditary form of B-CLL the incidence of B-CLL or another lymphoid neoplasm is three times higher as in general population ²⁴. The highest risk of B-CLL development display siblings of the same gender, especially sons where mother was affected with leukemia ²⁵.

1.1.3 Diagnosis of B-CLL

Typical symptoms of B-CLL include weight loss ($\geq 10\%$) within 6 month; fevers of $>38^{\circ}\text{C}$ for more than 2 weeks without evidence of infection; night sweats; extreme fatigue ²⁶. Among the B-CLL symptoms there are also presented signs of B-CLL as lymphadenopathy (common at the initial phase of B-CLL); painless splenomegaly; hepatomegaly; lesions on skin (macules, papules, ulcers, blisters)²⁷; glomerulonephritis have occasionally been

described. However, in 25% of B-CLL cases, above described symptoms and signs are not present ²⁶.

The diagnosis of B-CLL follows criteria:

A: Peripheral blood analysis

To be diagnosed as B-CLL positive, patient must display more than 5000 of B lymphocytes in 1µl of peripheral blood (lymphocytosis) in duration of 3 months. The clonality of the leukemic B-cells must be confirmed by presence of typical B-CLL surface markers detected by flow cytometry: CD5/CD23/CD19/CD20. The B-CLL cells display reduced levels of IgM, IgD and CD79b as compared to normal B-cells ². Strict immunophenotyping of CD5+ B-cells by flow cytometry confirms the suspected diagnosis of B-CLL and helps to distinguish B-CLL from other lymphoproliferative disorders. The CD5+ B-CLL cells differ in presence of CD23, CD22 surface markers from MCL (mantle cell lymphoma) where these markers are missing ^{21,28}. Elevated level of B2 microglobulin in serum represents another blood abnormality in B-CLL; it is associated with poor prognosis ²⁶.

In the typical blood smear of leukemic B-CLL cells, mature appearing small to medium size B-cells with a compact and visible cytoplasm are clearly detected (Figure 1.1). Nucleus is dense with partially aggregated chromatin and with hard to recognize nucleolus. A small part of B-cells (~10%) may be twice larger with clearly visible nucleolus: these cells are designated as prolymphocyte like cells (Figure 1.2).

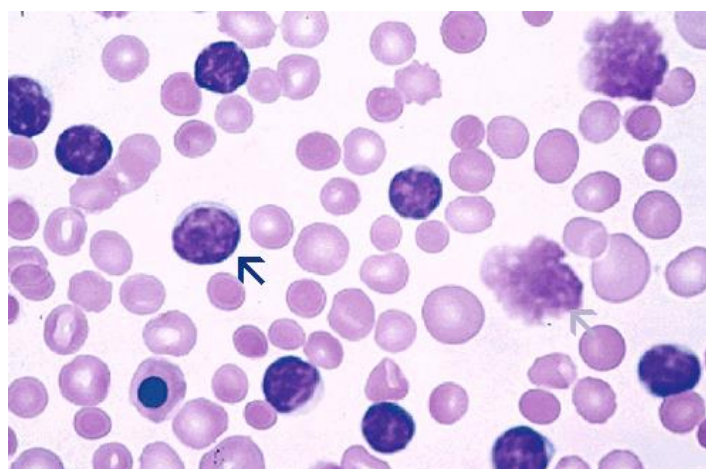


Figure 1.1: Typical blood smear of B-CLL cells (adapted from Brandon Guthery and Nasir Bakshi, 2007 - <http://moon.ouhsc.edu/kfung/jty1/HemeLearn/HemeCase/PB-001-Ans.htm>). Dark blue arrow indicates typical B-CLL cell and gray arrow indicates “smudge cell”, that is very often seen in B-CLL blood smear.

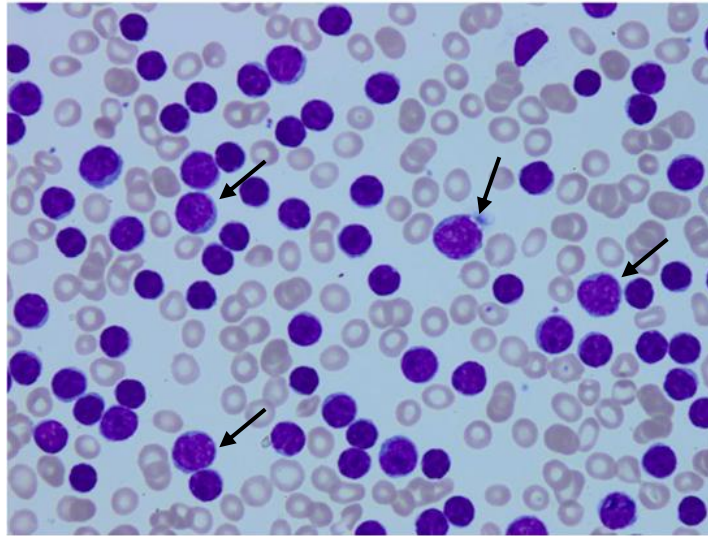


Figure 1.2: Blood smear of B-CLL with prolymphocytes like cells (adapted from ¹⁸). Black arrows indicate prolymphocytes.

B: Bone marrow aspiration, biopsy

The bone marrow examination usually shows more than 30% of mature B-cells. The pattern of marrow infiltration (diffuse, non-diffuse) reflects disease progression, provides prognostic information ². The bone marrow examination is accessory parameter for B-CLL diagnosis.

C: Mutational status of IgVH genes and expression of prognostic markers CD38 and ZAP-70

The process of somatic hypermutation introduces mutations in the variable segments of immunoglobulin variable heavy chain gene (**IgVH**). The proliferating B-CLL cells display mutated IgVH gene as these cells undergo somatic hypermutation in the germinal centers. In contrast, B-CLL cells with unmutated IgVH genes derived from pre-germinal centers ²⁹. The IgVH gene is considered as „mutated“ when shows <98% homology to germ line. The „unmutated“ IgVH is then when the homology to germ line is >98% ^{30,31}.

Determination of presence **ZAP-70** ³² and CD38 on the B-CLL cells ³³ allows to predict the prognosis of asymptomatic patients. ZAP-70 (zeta association protein; 70kDa) is member of family Syk protein tyrosin kinases. The normal B-cells do not express ZAP-70 while the normal T and NK cells express ZAP-70. B-cell receptor enhances expression of ZAP-70 on B-CLL cells ³⁴. ZAP-70 binds on the CD3-zeta and by this engagement activates T-cells ³⁵ Activated T-cells then differentiate, proliferate and secrete a number of cytokines. The estimated cutt-off level of B-cells is usually 20%. Unfortunately this method is not well

standardized ²⁸, moreover by measuring of the expression ratio of ZAP-70 on T-cells/B-CLL cells improves the prognostication and prediction of B-CLL clinical course ³⁶. ZAP-70 represents a rather surrogate prognostic marker in addition to IgVH mutation status and CD38 expression ³⁷.

Measurement of **CD38** surface marker expression on B-CLL cells reflects the situation occurring inside the cell while CD38 represents an indicator of cellular activity (dividing and growing cells). This is a reason why a such CD38+ B-CLL clone is rather associated with the poorer prognosis ³⁸. Generally, the cut-off for CD38 positivity varies from 7-30% ³⁹. The percentage of CD38+ cells together with expression of ZAP-70 on B-CLL cells provides even stronger prognostic value as each marker separately ⁴⁰.

D: Molecular cytogenetic: an optional prognostic test

Evaluation of chromosomal abnormalities by molecular genetics fluorescence *in situ* hybridization (FISH) helps to predict prognosis of B-CLL progression ²⁸. The hierarchical model described by Döhner *et al.*, defines five prognostic categories in B-CLL (1. deletion of 13q14 as a sole aberration; 2. trisomy of 12 chromosome; 3. deletion of 11q22-23; 4. deletion of 17p13; 5. normal karyotype). The most frequent chromosomal abnormality represents loss of **13q14.3** region with prevalence 55% ¹³. This cytogenetic aberration associates with favorable prognosis ¹³.

The second chromosomal abnormality represents trisomy of long arm of 12 chromosome (**t12q**) with prevalence around 12% in B-CLL patients ¹³. This chromosomal aberration represents intermediate risk in B-CLL prognosis ¹³.

The third chromosomal aberration represents deletion of chromosome **11q22-23** with prevalence 18% in B-CLL patients and associates with poor prognosis ¹³. This deleted region includes ataxia-telangiectasia mutated (*ATM*) gene which is mutated in 30% of B-CLL cases and represents sign of rapid disease progression ^{13,41}.

The fourth chromosomal aberration represents deletion of chromosome **17p13** with prevalence 7% ¹³. The deleted region contains gene *TP53*: the guardian of the cell genome. B-CLL patients with deleted 17p13 chromosome have most likely deleted or mutated gene *TP53* and predispose to poor prognosis ⁴².

The last category represents normal karyotype ¹³.

1.1.4 Clinical staging of B-CLL

Standard methods for assessment of B-CLL prognosis represent Rai and Binet staging system. These staging systems are based on the results from physical examinations and blood tests ^{43,44}.

The **Rai staging system** (Kanti R. Rai, USA) defines three categories of risk disease: low, intermediate and high ².

Low risk: lymphocytosis with leukemic cells (>30%) in blood and/or in bone marrow (Rai stage 0)

Intermediate risk: lymphocytosis; enlarged lymph nodes; splenomegaly and/or hepatomegaly (Rai stage I-II)

High risk: anemia (Hb<11g/dl) or thrombocytopenia (Rai stage III-IV)

The **Binet staging system** (Jacques-Louis Binet, France) is based: on A) area of presented enlarged lymph nodes (>1cm) or organomegaly (categories: A-C), B) on presence of anemia or thrombocytopenia (categories: 1-5):

A) Area of presented enlarged lymph nodes:

1. Head and neck.
2. Axillae.
3. Groins.
4. Palpable spleen.
5. Palpable liver.

B) Anemia or thrombocytopenia:

A: Hb 100g/L or more; platelets $100 \times 10^9/L$ or more

B: Hb 100g/L or more; platelets $100 \times 10^9/L$ or more; organomegaly

C: Hb <100g/L; platelets $<100 \times 10^9/L$; irrespective to organomegaly

1.1.5 Progression and transformation of B-CLL

Small lymphocytic leukemia (SLL) shows very similar manifestation as B-CLL. SLL is clinically defined by presence of lymphadenopathy, clonal marrow infiltration, but with absence of peripheral blood lymphocytosis and cytopenia. Number of B-cells in the peripheral blood should not exceed 5000 per μL . The SLL diagnosis needs to be confirmed by histopathological evaluation of lymph nodes ².

B-CLL/SLL could transform into more aggressive non-Hodgkin lymphoma (NHL) called Richter's syndrome (RS) ⁴⁵. RS develops with frequency 5-7% and is mostly represent as diffuse large B-cell lymphoma (DLBCL) ⁴⁶. Patients with RS have a very poor prognosis ⁴⁶. The molecular mechanism leading to RS transformation from B-CLL is unknown.

1.1.6 Conventional and novel therapeutic tools of B-CLL

The manifestation of B-CLL is very heterogeneous. One third of B-CLL patients do not need therapy and survive for years; in another third of B-CLL patients the indolent course of disease progresses; the remaining third of B-CLL patients exhibits aggressive onset at the beginning and needs immediate therapy ²⁹. In many patients, typical symptoms or signs of B-CLL do not manifest; in this case, the B-CLL is revealed simply by routine blood test. Standard treatment of B-CLL is based on "watch and wait" strategy with controls of blood counts and clinical examinations over a period of 3-6 months ²⁶. Therapy to B-CLL patients is applied when the symptoms are visible and the disease progresses rapidly ^{2,26}. We can split the chemotherapy components into several groups ²:

A: Cytostatic agents.

From the purine analogues, the Fludarabine monotherapy is by far the most used and studied as it shows the best results: 7-40% of complete remission rate and overall survival (OS) 66 month ⁴⁷. Nowadays, the standard first line of therapy B-CLL patients in good condition without comorbidities is considered combination of Fludarabine, Cyclophosphamide and Rituximab (FCR). This combined therapy represents very effective treatment as 95% of treated patients responded and from 50-70% showed complete remission ^{48,49}. Among the most common side effects of this therapy belong toxicity, neutropenia, lymphopenia with an increased risk of secondary bacterial infections ⁵⁰. In elder patients and patients with comorbidity is more likely used an alternative therapy protocol FCR-lite, where the toxicity is lower ⁵⁰. For patients where the alkylating agents had failed or patient relapsed after second-line of Fludarabine therapy is applied monotherapy with Alemtuzumab. It is a recombinant, monoclonal antibody against the CD52 antigen. The main use of Alemtuzumab monotherapy was found in "high risk" patients with deleted 17p chromosome or with mutated *TP53* gene ⁵⁰. Another monotherapy alternative for elderly patient with comorbidity is Chlorambucil (alkylation agents), mainly for its low toxicity and low cost. The side effects (cytopenia; myelodysplasia; secondary acute leukemia) and low

complete remission (CR) rate are the main disadvantages of Chlorambucil monotherapy. Chlorambucil in combinations with anti-CD20 antibodies based therapy such as Rituximab shows better results⁵¹.

B: Agents targeting B-cell receptor (BCR) signaling.

Signaling through BCR supports survival of B-CLL cells⁵². BCR signaling needs interaction with different tyrosine kinases: Bruton`s tyrosine kinase (Btk), spleen tyrosine kinases (Syk); Src family of tyrosine kinase and PI3K⁵³. Therefore, novel drugs are based on the inhibition of BCR signaling e.g. inhibitors of Btk⁵⁴. Ibrutinib inhibits Bruton`s tyrosine kinase and induce apoptosis of B-CLL and lymphoma cells⁵⁵. In a recent study, in which Ibrutinib was orally administered to the patients with relapsed or refractory B-cell lymphoma and B-CLL, it was demonstrated that Ibrutinib is well tolerated and induces significant objective responses (16% of patients with complete remission)⁵⁶.

C: BCL-2 inhibitors.

Pro-apoptotic proteins the members of Bcl-2 family represent key regulators of apoptosis⁵⁷. ABT-199 inhibitor selectively inhibits the growth of BCL-2 dependent tumors *in vivo* and spares human platelets. In patients with refractory B-CLL, administration of ABT-199 resulted in lysis of tumor cells⁵⁸.

D: Immunomodulatory drugs.

Lenalidomide with its anti-angiogenic, anti-neoplastic properties was primarily used for treatment of myelodysplastic syndrome and multiple myeloma^{59,60}. Lenalidomide therapy is also applied to the treatment of B-CLL patients. In 4 year study of Lenalidomide as initiating therapy (cohort of 60 B-CLL patients), drug administration resulted in tumor flare reaction with overall survival 82%. Treatment with Lenalidomide was also associated with sustained (36 months or longer) normalization of the absolute number of circulating T-cells (CD3+) and the percentage of CD4+ and CD8+ cells⁶¹.

1.2 Introduction to the microRNAs

1.2.1 Discovery, nomenclature of microRNAs

The microRNAs were discovered in 1993 by Lee *et al.*, in *Caenorhabditis elegans* animal model⁶². This class of small non-protein coding single stranded RNAs (~19-23 nucleotides in length) is now very intensively studied. MiRNAs work as negative post transcriptional regulators of gene expression. MiRNAs, endogenously produced RNAs are evolutionary conserved among all four kingdoms - *Animalia*, *Plants*, *Funghi* and *Viruses*. Due to the role of small RNAs in controlling the proper timing of development, were firstly named as small temporal RNAs (stRNA)⁶³ and later renamed to microRNAs⁶⁴.

One of the first discovered miRNAs were lin-4 (*lineage*) and let-7 (*lethal*) in 1993 by Lee *et al.* in worms *Caenorhabditis elegans*⁶². These miRNAs inhibition mechanism involves sense and anti-sense interactions of RNAs. The lin-4 inhibits translation of *LIN-14* that is responsible for correct timing during the first larval stage and let-7 regulates correct timing and development from the last larval stage to the adult stage⁶⁵.

Nowadays, identification of miRNAs exploits methods as subcloning, *de novo* sequencing studies and bioinformatics approaches. Prediction programs use the conservation of miRNAs within species. Variety miRNA databases were developed: miRBASE⁶⁶, miRNA map⁶⁷, microRNA⁶⁸, coGemIR⁶⁹, miRGEN⁷⁰ and deepBase⁷¹. In publications, computationally predicted miRNA target programs/databases are used: Targetscan⁷², Pictar⁷³, Tarbase⁷⁴, microRNA⁶⁸, Diana-microT⁷⁵, miRecords⁷⁶ and Starbase⁷⁷. Recently, the most used miRNA database – „miRBase“ issued the version 19⁷⁸ which contains 1600 human microRNAs and their number is still rising up.

Terminology of miRNAs distinguishes the precursor miRNA from the mature miRNA. Precursor miRNA is referred to as “hsa-pre-miR-X” while mature as “hsa-miR-X” (where X represents a number in line of discovery). The prefix of three letters refers to the origin of organism (e.g. hsa = *Homo sapiens*, mmu = *Mus musculus*). If there exists a difference in few bases in the sequence of mature miRNA, after numeric value X the letters a, b or c are added (e.g. hsa-miR-106a; hsa-miR-106b). To specify which arm of the hairpin structure is processed from the precursor miRNA, specification 3p (from 3` arm) and 5p (from 5` arm) are used (e.g. hsa-miR-155-3p or hsa-miR-155-5p). By processing of both strands from pre-miRNA, than the less abundant is named as miR-X*. The mature miRNA can result from two

genomic loci, then the numeral suffix after X is added (e.g. hsa-miR-181a-1 or e.g. hsa-miR-181a-2) ^{79,80}. In addition, some miRNAs do not follow such nomenclature (e.g. let-7).

1.2.2 Biological function of microRNAs

The cell fate is under tight control of miRNAs that regulate apoptosis, cell cycle, proliferation or differentiation of cells. Primarily, miRNAs negatively regulate gene expression. MiRNA binds to the 3' untranslated region (3' UTR) of its target mRNA and based on the level of complementarity, the mRNA degradation (*Plants*) or inhibition of translation (*Animalia*) is executed ⁸¹. Besides negative regulation of gene expression, some miRNAs could also promote translation of ribosomal RNA ⁸².

One miRNA can regulate several target mRNAs and on the other hand, several miRNAs can regulate one mRNA target ^{83,84}. The cluster miRNAs as hsa-miR17-92 cluster are processed from one primary transcript that is later spliced into several mature miRNAs. The single miRNAs as miR-155 are transcribed from one primary transcript of host gene. It was estimated that 10–30% of genes are regulated by miRNAs. It is thus not surprising, that miRNAs are involved in cancer development where they are differentially expressed. It is known, that microRNAs are deregulated during leukemia and tumor development ⁸⁵. Important role of microRNAs in tumor genesis is supported by the fact that microRNAs are frequently located at the genomic loci amplified in tumors ⁸⁶ or the loci that frequently undergo genetic mutation: breaks or deletions ⁸⁷. Genes encoding microRNAs and their regulatory regions are often located in vicinity of viral integrations, that leads to deregulation of their expression ⁸⁸. MicroRNAs are important for normal development as documented by the genetic inactivation of several components of microRNA metabolism, resulting in absolute phenotype of early embryonic lethality ⁸⁹.

1.2.3 Biogenesis of microRNAs

Genes that encode miRNAs are localized on the sex and somatic chromosomes with the exception of Y chromosome ⁷⁹. MicroRNA genes are mainly localized in the chromosomal loci that are predisposed to the deletions or amplifications ⁹⁰. The miRNAs are transcribed from RNA transcripts of independent genes or from introns of protein coding genes. MiRNAs can be then divided into three categories depending on their genomic location: 1) exonic

miRNAs in non-coding genes; 2) intronic miRNAs in non-coding genes and 3) intronic miRNAs in protein-coding genes ⁹¹.

Biogenesis of microRNAs (Figure 1.3) starts in the cell nucleus and continues in the cytoplasm. The processing of miRNAs requires RNA polymerase II ⁹² but some miRNAs as miR-515-1, miR-517a, miR-517c and miR-519a-1 could be transcribed also by polymerase III ⁹³. The first product is the primary transcript - **pri-miRNA** that is usually several kilobases long with a typical hairpin structure (Figure 1.3). The endonuclease - RNase III called Drosha digests pri-miRNA. This process is followed by adding three phosphates (3P) at the 5' end on which further binds 7-methyl-guanosine (m⁷G; also called a cap). This cleavage step is important as determines the sequence of the mature miRNA and generates the optimal conditions for further processing of pri-miRNA ⁹⁴.

The second product arising during the biogenesis of miRNA is precursor-miRNA (pre-miRNA) originated from pri-miRNA. The **pre-miRNA** (~70-100nt long) arises by digesting of the one strand by enzyme Drosha and its cofactor DGCR8 (DiGeorge syndrome Critical Region gene 8; dsRNA binding protein). DGCR8 cofactor binds as the molecular anchor to the double-stranded RNA and recognizes the pri-miRNA flanking sequences where undergo subsequent cleavage of stem-loop mediated by the ribonuclease Drosha. Drosha cleaves in approximately 11bp from the junction between the flanking sequences and the stem-loop. Such non-symmetric cleavage leaves two unpaired nucleotides at the 3'OH end ⁹⁵. Drosha complex consists of several factors "accessory components" as EXSR1, FUS, ribonucleoproteins and DEAD-box helicases. These "accessory components" may function as the enhancers of fidelity and activity of miRNA processing by Drosha ^{96,97}. Processing of pri-miRNA to the pre-miRNA is a critical step while defines the mature miRNA sequences (Figure 1.3).

Besides the canonical miRNA biogenesis there exists also an alternative biogenesis of miRNA called mirtron pathway (Figure 1.3). The **mirtron** is a short intron with hairpin structure. This pathway uses mRNA splicing mechanisms. After lariat (structure that originates from intron of mRNA during splicing and processing of mRNA/miRNA) releasing the intron by self generates pre-miRNA, however with shorter length as in canonical pre-miRNA pathway, since lack of lower stem of 1 helical turn that typically recruits and mediates cleavage by Drosha/DGCR8 complex ⁹⁸. Cleavage step by Drosha does not occur in the mirtrone pathway ⁹⁹. The miRNAs originated by mirtron pathway are miRNAs with

regulatory function as well as miRNAs that are made up by the canonical biogenesis pathway (Figure 1.3).

The nuclear protein, **Exportin-5** (Ran-GTP receptor) guides transport of pre-miRNA from the nucleus into the cytoplasm. This boundary of pre-miRNA with Exportin-5 ensures two flanking nucleotides on the 3' end of pre-miRNA. RNaseIII (Dicer) cleaves the terminal hair loop of pre-miRNA and give rise to the temporarily present double strand duplex of **miRNA:miRNA*** (~21-23nt). Dicer together with its co-factor TAR RNA binding protein (TRBP) regulates processing and liberation of miRNA:miRNA* duplex. Strand with strongest boundary to the 5' end of miRNA:miRNA* duplex is then incorporated into so called miRNP (ribonucleoprotein) or **RISC** complex (RNA InducedSilencing Complex). The miR* strand plays role in the pathogenesis of some diseases and acts in regulating signaling events ¹⁰⁰. Only the active RISC* complex could bind the mature miRNA and transport it to its target mRNA. The mechanism of miRNA acting lies in guiding the active RISC* complex to the 3'UTR of the target mRNA through "seed sequence". The "seed sequence", usually 6-8nt long is responsible for the correct boundary of miRNA on the 3' end of the target mRNA ¹⁰¹. The key components of RISC are Argonaut proteins 1-4 (**AGO1-4**). All four proteins stabilize the RISC complex, but only AGO-2 has "slicer" activity (slicer activity=cleavage) ^{102,103}. AGO proteins interact also with proteins of RISC complex: GW182 (glycine-tryptophan protein of 182KDa) ¹⁰³. GW182 than further interacts with poly (A) binding protein (PABP) and together recruit deadenylases – to proceed the final step of mRNA deadenylation ¹⁰⁴.

Inhibited targeted mRNA together with miRNA and catalytic components of RISC (AGO1-4) are then collected into the **P bodies** ⁸¹. The P bodies (processing) are subcellular compartments that serve in mRNA turnover. In P bodies' proceeds decapping, degradation of unwanted mRNAs and storage of mRNA until needed for translation. P bodies also act on aiding in translational repression by miRNAs ^{105,106}. Some miRNAs after entering P bodies are not degraded but rather utilized for re-initiation of translation ¹⁰⁷.

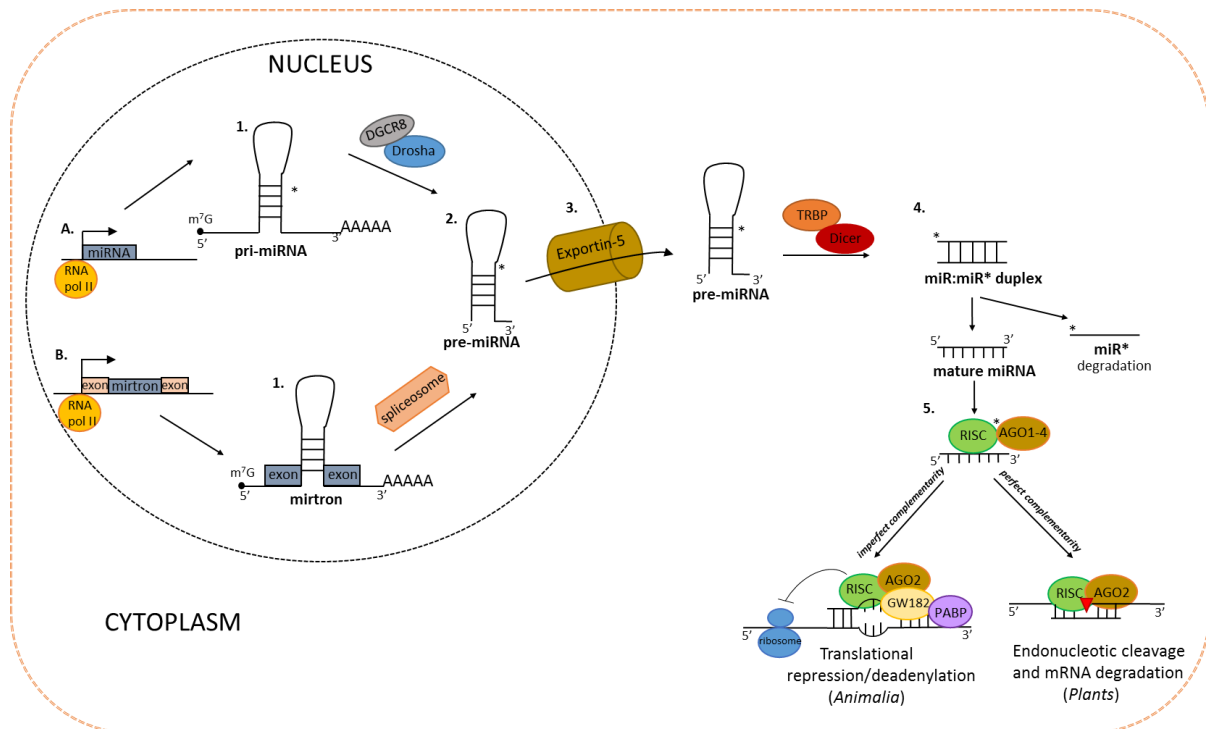


Figure 1.3: Biogenesis of miRNA (adapted and modified from Breving K and Esquela-Kercher A, 2009¹⁰²; Iorio MV and Croce CM, 2011¹⁰⁸; Okamura K *et al.*, 2007¹⁰⁹; Krol J *et al.*, 2010¹⁰³). Biogenesis of miRNAs starts in the cell nucleus and proceeds in the cytoplasm. There are two pathways of biogenesis miRNAs: A) Canonical and B) Alternative mirtron pathway. A) In the canonical pathway, the first step is generation of long primary transcript pri-miRNA by polymerase II/III. The pri-miRNA transcript contains cap at 5' end and polyA tail at the 3' end. In the second step, digestion of pri-miRNA by RNase III-Drosha together with DGCR8 gives rise the precursor - pre-miRNA. B) In the alternative mirtron pathway, the first step is generation of mirtron-encoded by introns. Mirtron hairpins are spliced and debranched in spliceosome to gives rise to pre-miRNA. From this step, both pathways share further steps of generation the mature miRNA. Pre-miRNA is in the third step exported from the nucleus to the cytoplasm by Exportin-5. In the cytoplasm the second RNase III – Dicer together with TRBP further process pre-miRNA. In the next step short existing miR:miR* duplex arise. The mature miRNA originates from the guided strand - with the strongest boundary to the RISC complex and more stable; the miR* strand is usually degraded (but could also play some biological role). Mature miRNA is further loaded into RISC complex mediated by AGO1-4 proteins. In that step, the mature miRNA becomes ready for action: translational repression/deadenylation (in Animals) or mRNA degradation (in Plants).

1.2.4 Regulation of the microRNA biogenesis

Proper timing and precise regulation of miRNAs expression are required for maintaining cell and tissue homeostasis ¹¹⁰.

Both miRNAs and mRNAs have complex promoters regulated by transcription factors, enhancers, silencing elements and by chromatin modifications ^{107,111}. Transcription of miRNAs genes is regulated at the promoter level by: A) Binding of transcription factors, B) Chromatin modifications (Figure 1.4). Direct binding of transcription factors (TF) positively

or negatively regulates miRNA expression. TF c-MYC stimulates expression of miR-17-92 cluster by direct binding at conserved sequence upstream of miR-17-92 host gene ¹¹². Expression of miRNAs is regulated also by chromatin modifications, 5-10% of mammalian miRNAs are regulated epigenetically. The tumor suppressor miR-124a is unmethylated in normal cells but undergoes methylation in cancer cells ¹¹³.

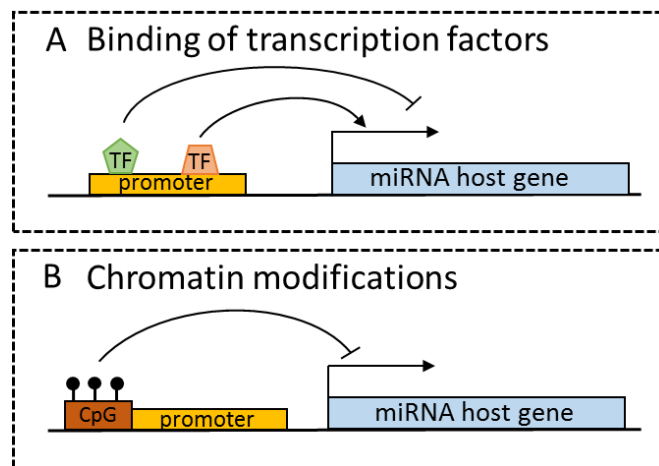


Figure 1.4: Regulation of miRNAs transcription: Transcription of miRNA genes could be positively or negatively regulated by: A) Binding of transcription factors or B) Chromatin modifications. A) Direct binding of TF (transcription factor) at miRNA host gene promoter could positively or negatively regulate the miRNA host genes expression. B) Methylation of CpG at the miRNA host gene promoter region negatively regulates expression of miRNA.

It was shown that processing of miRNAs is regulated by: 1) protein cofactors, 2) protein-protein interactions or 3) through direct interactions with pri-miRNA ¹¹⁴. Each step of the biogenesis of miRNA is tightly regulated alike the regulation of mRNA maturation. The first regulatory step occurs at the processing of **pri-miRNA**. For example, SMAD proteins and p53 protein enhances the maturation of pri-miRNAs through interaction with protein p68 – a component of the Drosha microprocessor complex ^{115,116}. An autoregulation of miRNAs was recently described - e.g. the let-7 promotes processing of its primary transcript ¹¹⁷. Such a regulatory feedback loops are very common in miRNAs and it was postulated that they are critical for maintenance of balance between the levels of the Drosha-DGCR8 complex and their substrate ¹¹⁸. It was described that processing of pri-miR-155, pri-miR-21 and let-7 by Drosha is enhanced by arsenite-resistance protein 2 (ARS2), a component of the nuclear cap binding complex. The low expression or depletion of ARS2 inhibits processing of pri-miRNA-155 ¹¹⁹.

The second level of regulation miRNA maturation occurs at pre-miRNA stage. The processing of **pre-miRNA** is mediated not only by Dicer but also by other cofactors as TRBP and PACT (protein activator of PKR). These two cofactors enhance the specificity of cleavage and promote stability of the Dicer-pre-miRNA complex ¹²⁰. Translocation of processed pre-miRNA from the cell nucleus into the cytoplasm mediates the nuclear pore complex called Exportin-5 ⁹⁴. Exportin-5 together with pre-miRNAs may shuttle also tRNAs (transfer RNAs). Therefore, pre-miRNAs and tRNAs compete for transport by Exportin-5 ⁹⁴. Another regulation of pre-miRNA maturation proceeds during interaction of Dicer with Exportin-5, thus overproduction of Dicer mRNA or pre-miRNA could work as negative or positive feedback loop ¹²¹. The processing of Dicer is negatively regulated by viral miRNA – e.g. miR-BART6-5p encoded by Epstein-Barr virus ¹²². Another negative regulation of pre-miRNA processing respectively export of pre-miRNA represents post-transcriptional process RNA editing mediated by adenosine deaminases (ADAR) ¹⁰³. ADAR proteins catalyze conversion of adenosine to inosine what affects base pairing and structural properties of RNA transcripts ¹²³. ADARs regulate both Drosha-mediated and Dicer-mediated cleavage and prevent the export of pre-miRNAs ¹⁰³.

Mutations in the sequences of pre-miRNAs or mature miRNAs influence further miRNA processing, stability and target selection. Mutations in the pre-miRNA and miRNA sequences originate from changes at the DNA coding sequences and from RNA posttranscriptional modifications ^{124,125}. The single substitutions of base pairs (C to T) in the sequence of pre-miR-15a and pre-miR-16-1 abolish processing of these two miRNAs in B-CLL patient samples ¹²⁶.

Briefly, regulation at the pri-miRNA level by interactions with proteins/cofactors results in different expression of miRNAs. Changes at the base pairs in pre-miRNA and/or mature microRNA sequences alter processing and/or selection of target mRNAs.

1.2.5 miRNAs are involved in pathogenesis of B-CLL

For the first time Calin *et al.*, described the link between B-CLL and miRNAs. Authors described significant down regulation of the microRNA **cluster miR-15-16** (miR-15a and miR-16-1) in B-CLL patient samples. MiR-15-16 cluster is localized in the 13q14 chromosomal region that is often deleted in B-CLL ¹⁶. Klein *et al.*, described on the mouse model, that deletion of 13q14 region with *DLEU2*/miR-15a/miR-16-1 leads to clonal B-cell

lymphoproliferations similarly as in B-CLL patient cells. Moreover, they proved that deletion of cluster miR-15-16 accelerates proliferation of B-cells by enhanced transition of cells from G0/G1 to S phase¹²⁷. Thus, miR-15-16 cluster plays important role in pathogenesis of B-CLL^{128,129}. Another consequence of the deletion 13q14 chromosomal region is elevated production of an anti-apoptotic protein – BCL2, thus absence of miR-15-16 cluster increases resistance of B-CLL cells to the apoptosis. On the other hand, in the normal B-cells, presence of miR-15-16 cluster acts as tumor suppressor gene while it inhibits expression of *BCL2* gene and drives cells to apoptosis¹³⁰. While the BCL2 is in majority of B-CLL patient samples overexpressed, it makes BCL2 molecule a potential target in B-CLL therapy. Recent work by Masood A *et al.*, showed that an inhibitor of BCL2 (AT-101) induces apoptosis in B-CLL cells¹³¹.

It is described that several miRNAs are deregulated in B-CLL cells^{90,130,132}. The **miR-34a/b/c cluster** is the one among them. To the frequent chromosomal aberrations in B-CLL belongs loss of the long arm of chromosome 11 and deletion of the short arm of chromosome 17. The miR-34a/b/c cluster is localized on these two chromosomes. The miR-34 cluster downstream regulates the expression of *TP53* gene that suggests on link between chromosomal deletions (11q, 17p chromosomes) and expression of miR-34a/p53 during B-CLL progression¹³³. Fabbri *et al.*, shown that p53 binds at miR-34b/c cluster and thereby stimulates expression of miR-34b/c cluster in B-CLL¹³⁴. Interestingly, in B-CLL patient cells with deletion or mutation of *TP53* gene the levels of miR-34 cluster decreased. Based on this knowledge, measurement of levels miR-34a could distinguish between the high and low risk B-CLL patients. The B-CLL patient samples with high risk expressed lower levels of miR-34a, displayed shorter time to treatment and shorter doubling time of lymphocytes in comparison to the low risk B-CLL patient samples. Moreover, it was described that low expression of miR-34a due to deletion or mutation of *TP53* gene positively correlates with Fludarabine resistance of B-CLL cells¹³⁵. MiR-34a induces cell cycle arrest and apoptosis in the primary B-CLL cells. The cell cycle and apoptosis are in cooperation with p53 – this suppose an existence of positive feedback loop between miR-34a and p53 protein.

Patients with deletion of 17p chromosome (encoding *TP53* gene) are clinically described as patients with high risk B-CLL. It was shown that B-CLL cells of patients with high risk expressed elevated levels of **miR-21**. Moreover, the B-CLL patients with elevated expression of miR-21 displayed shorter overall survival (12 months) in comparison to the

patients with low miR-21 expression (24 months). Therefore, measurement of miR-21 levels could be in the future used as the marker for overall survival of B-CLL. The statistical analysis based on the cytogenetic data has shown that expression of miR-21 is a marker of an early stage of B-CLL ¹³⁶.

Fulci *et al.*, described three-fold increased levels of **miR-150** in the peripheral blood and lymph nodes of B-CLL patients ¹⁷. The RNA *in situ* staining of leukemic cells showed very clear staining of miR-150 in the outer site of the proliferation centers of leukemic cells in the lymph nodes ¹³⁷. Moreover, expression of miR-150 correlates with poor prognosis as the B-CLL patient cells with unmutated IgVH expressed less miR-150 as IgVH mutated B-CLL cells ^{17,137}.

Decreased expression of **miR-181b** in B-CLL cells overall correlates with worse prognosis of B-CLL. Patients with low levels of miR-181b had higher probability of requiring treatment and display shorter overall survival (5 months) as patients with higher miR-181b expression (14 months). Moreover, the B-CLL cells with deletion of short arm of chromosome 17 expressed less miR-181b as cells without this deletion. Based on this data the low miR-181b expression has become an unfavorable prognostic marker of B-CLL ^{19,136}. The B-CLL patient cells with unmutated IgVH expressed lower levels of miR-181b as mutated IgVH cells ¹³⁸.

Different expression pattern of members **miR-17-92 cluster** appears to be another sign of B-CLL. This cluster encodes six different microRNAs (miR-17p, 18a, 19a, 19b-1, 20a, 92a). Moreover, the miRNA-17-92 cluster regulates proliferation of B-cells and Ig rearrangement as it is highly expressed in progenitor B-cells ^{139,140}. Expression of miR-92, a member of miR-17-92 cluster was increased in the lymph nodes of B-CLL patient samples. Other members as miR-20a and miR-19b-1 were highly expressed in the peripheral blood of B-CLL patients ¹³⁷. The ChIP analysis has shown that the transcription factor MYC stimulates expression of miR-17-92 cluster in B-CLL ¹¹².

The most studied microRNA in B-CLL represents a multifunctional oncogenic microRNA - **miR-155**. The elevated levels of miR-155 were detected in Hodgkin ^{141,142} and non-Hodgkin lymphomas including B-CLL ^{17,130,143,144}. The aberrant expression of miR-155 represents sign of more aggressive course of the disease ^{143,145}. The recent data shows on the potential usage of miR-155 as a prognostic marker in distinguishing between two types of DLBCL forms (ABC/GC) ¹⁴⁶. Based on this information, the measurement of miR-155 levels during

disease progression could be potentially used as a marker of B-CLL progression. Very recently, a prognostic value of miR-155 as marker of B-CLL progression was confirmed²⁰. Authors shown an association between elevated levels of miR-155 in plasma of B-CLL patients and progression of B-CLL from MBL²⁰.

1.3 Characterization of microRNA-155 and its role in B-cells and leukemia

1.3.1 Overview of microRNA-155/*BIC*/*MIR155HG*

MiR-155 is encoded by non-protein coding gene *BIC* identified by Tam as the B cell Integration Cluster while *BIC* gene represents frequent site for integration of the avian leukosis virus (ALV)¹⁴⁷. *BIC* gene lacks an extensive open reading frame (ORF) and contains three exons that form 13kb region within the human chromosome 21q21.3 (Figure 1.5). *BIC* gene does not code any protein-coding gene. The secondary structure of *BIC* gene conserved sequences formed hairpins with imperfectly base paired stem loops. Taken together, *BIC* functions as non-protein coding RNA¹⁴⁷. The human *BIC* cDNA is in 83% identical with the mouse *BIC* cDNA, moreover mature human (hsa) miR-155 differs from mouse (mmu) miR-155 only in a single nucleotide in non seed sequence¹⁴⁸. The *BIC* gene is now designed as *MIR155 Host Gene* (*MIR155HG*).

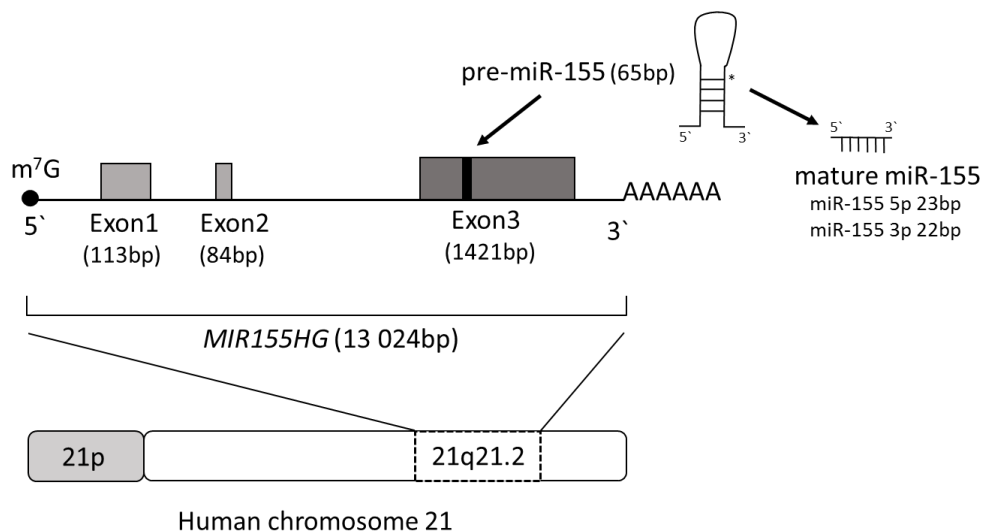


Figure 1.5: Structure of the *MIR155HG/BIC* gene (NC_000021.8). *MIR155HG* (*BIC*) is localized on the 21q21.2 chromosomal locus. The full length of *MIR155HG* spans 13024 bp that contains three exons. The exon 3 encodes precursor pre-miR-155 (1421 bp). After two endonuclease cleavage steps (by endonuclease Drosha and Dicer) arise the mature miR-155 (adapted and modified from Tam W, 2001¹⁴⁸ and <http://www.ncbi.nlm.nih.gov/gene/114614>).

The **biogenesis of miR-155** follows canonical miRNA pathway, this process begins in the cell nucleus and continues in the cytoplasm (for details see also Figure 1.3). The primary transcript of miR-155 – pri-miR-155 (13 024nt) is transcribed from *MIR155HG* or *BIC* gene by polymerase II (Figure 1.3 and 1.5). The precursor miR-155 - pre-miR-155 (65nt) originates from the pri-miR-155 by enzymatic cleavage mediated by endonuclease Drosha. This pre-miR-155 is further enzymatically cleaved by Dicer endonuclease to give rise two mature miR-155 - miR-155 5p (23nt) and miR-155 3p (also called miR-155*) (22nt) (Figure 1.5) ¹⁴⁹. The major product represents miR-155 5p that is also the most studied in different cells and tissues including hematopoietic and immune system. However, the minor product, miR-155 3p is less known and it seems that these two products tightly cooperate ¹⁰⁰. Authors described opposite cooperation between these two miRNAs in regulation of IFN α/β (interferon α/β) production in human dendritic cells. Here the miR-155 5p acts as negative regulator and miR-155 3p as positive regulator of production IFN α/β . Their opposing functions are explained by their different mRNA targets: miR-155 3p targets IRAK3 (Interleukin-1 Receptor-associated kinase 3) and miR-155 5p targets TAB2 (TGF- β activated kinas 1/MAP3K7 binding protein 2) ¹⁰⁰.

MiR-155 (5p) represents a **multifunctional microRNA**, while it involves in the various physiological as well as in the pathological processes (reviewed in ^{150,151}). In the normal human hematopoietic stem-progenitor cells (HSPCs) miR-155 maintains early **hematopoietic stem cells** (HSCs) at an early stem-progenitor stage by blocking their further maturation into myeloid, lymphoid and erythroid lineage. The transducing of miR-155 into erythroid K562 cell line as well as into human CD34+ bone marrow cells resulted in decrease of megakaryocytic differentiation and less formation of myeloid and erythroid colonies ¹⁵². The northern analysis revealed that miR-155 expresses all hematopoietic tissues ¹⁵³. Maturation of **B-cells** is under control of miR-155 ^{154,155}. O`Connell has found that permanent expression of miR-155 can increase number of immature granulocytes. Moreover, the increased levels of miR-155 led to the **granulocytes and monocytes** expansion, similarly to the myeloid proliferations as AML ¹⁵⁶. Vigorito and Xiaochun studied the role of miR-155 in **immunity** ^{157,158} and **inflammation** ¹⁵⁹. Trotta *et al.*, described that miR-155 acts as positive regulator of IFN γ production by natural killer cells ¹⁶⁰. The positive functioning of miR-155 on the **inflammation process** underlines also fact that the atherosclerotic plaques and pro-inflammatory **macrophages** displayed high expression of

miR-155 that results in inhibition of inflammatory responses and lipid uptake ¹⁶¹. It has been described that miR-155 involves in pathogenesis of **autoimmune diseases** as rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus ¹⁶². MiR-155 attenuates **apoptosis** via targeting FADD (Fas-Associated protein with Death Domain) ¹⁶³. Haasch *et al.*, demonstrated a strong up regulation of miR-155 expression in CD3/CD28 activated T-cells, suggesting a possible role for miR-155 in **T-cell** activation ¹⁶⁴. Role of miR-155 in the **cardiovascular disorder** support evidence that during the acute inflammatory phase of viral myocarditis the increased expression of miR-155 was specifically localized in the infiltrating macrophages and T-cells ¹⁶⁵. Presence of miR-155 is required for CD8+ T-cell responses to both virus and cancer ¹⁶⁶. MiR-155 is involved also in the development of different types of solid tumors as **breast cancer** ^{167,168}, **lung cancer** ⁸⁵, **colon cancer** ⁸⁵ and **cervical cancer**¹⁶⁹. It was described that miR-155 is over-expressed in **acute myeloid leukemia** (AML) ¹⁷⁰ and in **acute lymphocytic leukemia** (ALL) ¹⁷¹. In practically all **B-cell lymphoproliferations** (Hodgkin's and non-Hodgkin's lymphoma, primary mediastinal B-cell lymphoma, diffuse large B-cell lymphoma (DLBCL)) including **B-CLL** microRNA-155 is aberrantly expressed ^{17,141,144,149,172-174}.

Above-mentioned physiological and pathological processes including leukemia/cancer underlines the wide range of miR-155 functioning.

1.3.2 Role of miR-155 in normal and leukemic B-cells

The B-cell development is regulated by transcriptional factors that are modulated by microRNAs ¹⁵⁴. Deregulated expression of miRNAs and transcription factors are key events in pathogenesis of B-cell malignancies ¹⁷⁵.

During the normal lymphopoiesis the highest level of miR-155 express germinal center cells, intermediate level HSCs and the lowest mature B-cells ¹⁵². In the hematopoietic stem cells, miR-155 functions as blocker of hematopoietic differentiation along all lineages including lymphoid lineage ¹⁵². The normal healthy, germinal center B-cells express aberrant levels of miR-155. In the germinal centers occurs maturation of antibodies and generation of memory B-cells in T-cell dependent antibody responses. As the Hodgkin's lymphoma originate from the germinal center cells, the sustained up regulation of miR-155 is due to block of lymphocyte maturation ¹⁷⁵.

MiR-155 is required for production of antibodies that documents reduced secretion of IgG in B-cells of miR-155 deficient mice. This impaired antibody production is due to defect in differentiation of plasmablast cells and reduced proportion of germinal center B-cells. Another reason of reduced antibody production in miR-155 deficient mice is failure of BCR signaling ¹⁵⁷. Thai *et al.*, described that reduced expression of miR-155 leads to reduced cytokine production ¹⁷⁶.

It was described that miR-155 does not implicate in the somatic hypermutation (SHM) ¹⁵⁷. In contrast, Teng *et al.*, showed that aberrant expression of miR-155 negatively regulates AID (activation-induced cytidine deaminase) expression in B-cells. AID is involved in somatic hypermutation, gene conversion and class-switch recombination of immunoglobulin genes. Authors showed that absence of AID mediated by miR-155 inhibition resulted in imperfect maturation of B-cells ¹⁷⁷.

It has been reported that direct target of miR-155 is *SHIP1* (Src homology-2 domain-containing inositol 5-phosphatase 1) gene that regulates differentiation of B, T-cells and macrophages ¹⁷⁸. The *Ship1* knockout mice displayed spontaneous germinal center formation and antibody class-switching ¹⁷⁹.

During B-cell maturation the presence of miR-155 is necessary for activation of B-cell receptor (BCR): important for antibody production ^{141,180,181}. The B-CLL cells with high expression of miR-155 displayed higher capacity for BCR signaling ³⁴.

The last stage of B-cell development occurs in the peripheral lymphoid tissues where miR-155 is expressed by centroblasts and activated B-cells, necessary for normal B-cell development ¹⁵⁴.

1.3.3 Mouse models of miR-155

Until now it was generated four main miR-155 transgenic mouse models with either enhanced ¹⁸² or deficient miR-155 expression ^{157,176,183}. More information about mechanism of miR-155 acting in immune system may help to understand its role in leukemic process.

In the miR-155 over-expressing transgenic mice ("Eμ-mmu-miR-155" mouse model), pre-leukemic stage with polyclonal B-cell expansion and splenomegaly developed initially (after 3 weeks) ¹⁸². Later, after seven month, pre-leukemic stage progressed into high-grade B-cell lymphoma that is direct evidence of miR-155 functioning as activator of leukemia development ¹⁸².

Vigorito *et al.*, confirmed importance of miR-155 in antibody production in germinal centers ¹⁵⁷. MiR-155 deficient mice had reduced proportion (50%) of germinal center B-cells that led to reduced production of high affinity antibodies. Interestingly, authors firstly thought that impaired antibody production is result of dysfunctioning of somatic hypermutation affected by miR-155. The further experiments showed that miR-155 does not affect somatic hypermutation that occurs in germinal center B-cells (SHM) ¹⁵⁷. Failure of antibody production is due to block in differentiation of plasmablast B-cells (stage of B-cells between plasma and mature B-cells) and in reduced production of cytokines. This was confirmed also by other groups of researchers as Thai and Rodriguez ^{176,183}. Moreover, the miR-155 deficient mice produced less amount of IgM and switched antigen specific antibodies after immunization with pathogen – *Salmonella typhinurium*. The evidence that miR-155 regulates also T-cells represents its strong up regulation in activated T-cells ¹⁷⁶. Rodriguez *et al.*, also proved that dendritic cells failed efficiently activate T-cells ¹⁸³.

As the miR-155 bears the potential to be therapy-targeted molecule, Babar *et al.*, generated miR-155 Cre-loxP tetracycline controlled knock-in mouse model (miR-155^{L^SL^TA}). In these mice, the transcription of miR-155 can be deactivated by doxycycline, thus it provides “TET OFF” system with temporal controlling of miR-155 transcription. MiR-155^{L^SL^TA} mice developed after five month disseminated lymphoma with enlarged lymph nodes and splenomegaly. Surprisingly, mice with such miR-155 induced lymphoma after doxycycline treated food displayed within two days reduced lymph nodes and in 2 weeks the lymphoid organs were as in normal, healthy mice ¹⁸⁴.

To summarize, over expression of miR-155 leads to high grade B -cell lymphoma and miR-155 knockout mice model showed on a marked impairment of miR-155 in T- and B-cell function. The knock-in mouse model gave strong evidence that miR-155 is inductor of B-cell leukemia. Moreover, miR-155 regulates homeostasis of immune system, thus it makes potentially very powerful therapy tool.

1.3.4 Transcriptional regulation of *MIR155HG*

Similarly as mRNA genes, also miRNA host genes are regulated by transcription factors and chromatin modifications ^{102,111}. Aberrant expression of miR-155 in the immune cells and hematopoietic cells usually results in cancer, leukemia and autoimmune disorder ¹⁶².

As miR-155 represents a common target of a broad range of inflammatory mediators and regulators of immune homeostasis, it is not surprising that inflammatory molecules and members of inflammatory pathways can activate the transcription of *MIR155HG*.

It is described that **IFN γ** (Interferon γ), **TNF α** (tumor necrosis factor α), Toll-like receptor (**TLR**) ligands positively regulate *MIR155HG* expression^{185,186}. Van den Berg *et al.*, find a putative NF κ B binding site in -371 to -362bp upstream of the transcription start site of *MIR155HG*¹⁴¹.

TGF- β induces *MIR155HG* transcription through binding of **SMAD4** to the promoter region. Thus, miR-155 is a direct transcriptional target of the TGF- β /Smad4 pathway which activation promotes invasion and metastasis of breast cancer¹⁶⁷.

Another molecule that mediates inflammatory responses in the immune system and induces cytokines represents AP-1. Mathematical and bioinformatical analysis of the promoter *MIR155HG* gene resulted in the identification of **AP-1** (activator protein-1) binding sites -40nt upstream of the first exon *MIR155HG*¹⁴¹. It was confirmed later also by reporter assay¹⁸⁷. B-cells carry out its function through B-cell receptor (BCR). Activated BCR signaling induces expression of transcription factors c-Fos, FosB, and JunB, members of AP-1 family. Transcription factor AP-1 enhances the miR-155 expression through activated BCR and complex of transcription factors (FOS/Jun). The mutation in AP-1 binding site decreased activity of *MIR155HG* promoter and impaired BCR signaling¹⁸⁷.

Several transcription factors regulate expression of *MIR155HG*. One of them is **FOXP3** (Fork-head box P3) a regulator of Tregs (T regulatory cells). In the breast cancer cells, *FOXP3* functions as tumor suppressor gene¹⁸⁸. Over expression of *FOXP3* stimulates *MIR155HG* transcription. MiR-155 by this feed-forward regulatory loop inhibits expression of its direct target: chromatin-remodeling transcription factor **SATB1** (Special AT-rich Binding Protein homeobox 1) that implicates in metastasis of breast cancer cells¹⁸⁹.

Very recently, it was described, that regulator of cell proliferation and survival, **STAT3** (Signal transducer and activator 3) activates *MIR155HG* expression in B-CLL cells. *STAT3* binds to GAS-like elements at the position -709 and -700nt of *MIR155HG* promoter region results in stimulation of *MIR155HG* transcription¹⁹⁰.

The viral proteins and viral microRNAs could also activate transcription of miR-155 in the leukemic cells. Infection by Epstein-Barr virus (**EBV**) stimulates proliferation of B-cells. EBV infection induces expression of viral microRNAs (miR-BART and miR-BHRF) which enhances

the expression of cellular microRNAs. The EBV infected B-cells express high levels of miR-155^{191,192}. EBV encodes latent membrane protein-1 (LMP-1), a functional homologue of the tumor necrosis factor receptor family, contributes to EBV's oncogenic potential through activation of nuclear factor κ B (NF κ B). In the EBV infected B-cells LMP-1 activates transcription of *MIR155HG* through NF κ B and AP-1¹⁹¹. Authors identified the second NF κ B binding site at position -1697nt from TSS of *MIR155HG*. By recruiting both NF κ B binding sites (-1150 and 1697nt from TSS) mediate LMP-1 elevated levels of miR-155^{187,191}. Aberrant expression of miR-155 in the EBV infected B-cells reprograms these cells into rapidly dividing cells and evoke the EBV latency¹⁹². The *MIR155HG* transcription is stimulated also by the oncogenic herpes virus - Kaposi's sarcoma-associated herpes virus (KSHV)^{193,194}.

MiR-155 expression is regulated also epigenetically. Chang S *et al.*, described the BRCA1-mediated silencing of *MIR155HG* promoter in breast cancer cells. Authors found that BRCA1 recruits HDAC2 complex at the *MIR155HG* promoter¹⁶⁸. Therefore is the *MIR155HG* promoter epigenetically silenced through deacetylation of H2A and H3 histones. Silencing of BRCA1 results in increased miR-155 expression¹⁶⁸.

As the miR-155 is in leukemic or cancer cells aberrantly expressed, the transcription factors and inflammatory molecules positively regulate its host gene transcription. The *MIR155HG* is negatively regulated by HDAC2. Viral infection (EBV) contributes to miR-155 expression likewise. Anti-miRNA therapy could be then orientated on inhibition of miR-155 either itself or its activators.

1.3.5 Relationship between miRNA-155 and its direct target PU.1

In silico prediction showed that PU.1 transcription factor belongs among miR-155 target genes. Vigorito *et al.*, validated that miR-155 directly targets PU.1 mRNA in the miR-155 deficient mouse model in which the mRNA and protein levels of PU.1 were increased. The 3'UTR of PU.1 mRNA contains phylogenetically conserved 9nt complementary to miR-155 seed region (9nt) (Figure 1.6). PU.1 rescue experiment *in vitro* on the primary B-cells resulted in impaired IgG production and increased proportion of IgM¹⁵⁷. The luciferase reporter assay confirmed this targeting of PU.1 by miR-155. After transfection of miR-155 mimic into PU.1 luciferase reporter, the luciferase expression of PU.1 reporter was inhibited

¹⁵⁷. Together these data confirm that PU.1 decrease is mediated by elevated level of miR-155.

It was described that over expression of miR-155 leads to decrease of both mRNA and protein level of PU.1 in primary B-CLL cells ¹⁴⁴.

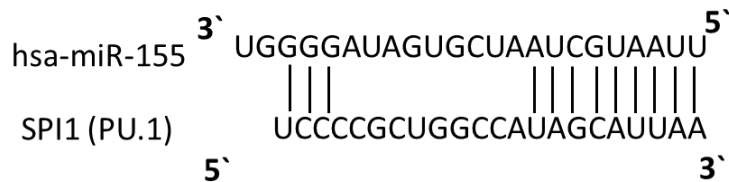


Figure 1.6: The complementary base-pairing between miR-155-5p and the human *SPI1* (spleen focus forming virus proviral integration oncogene) (also known as *PU.1*) mRNA. The miR-155-5p binding site is located 46-53 base pairs downstream from the SPI1 mRNA stop codon. The "seed sequence" base-pairing is represented by 9nt (adapted from miRBASE⁷⁸).

It was described that LMP-1 mediated miR-155 over expression resulted in 2-fold decrease of protein level of PU.1 in EBV negative cell line DeFew ¹⁹¹.

PU.1 also known as *SPI1* (spleen focus forming virus proviral integration oncogene) is a member of ETS family ^{195,196}. PU.1 controls differentiation of common myeloid and lymphoid progenitor cells through regulation of transcription factors ¹⁹⁷. Importance of PU.1 in hematopoiesis underlined PU.1 mouse model (*Sfpi1*^{-/-}) where the null mutation of *Sfpi1* resulted in perinatal lethality and failure in generation of B-cells, macrophages and neutrophils ¹⁹⁸. PU.1 acts as oncogene and as tumor suppressor gene ^{195,196}. Interestingly, different level of PU.1 defines the cell stage during the differentiation. The low level of PU.1 favors in the development of granulocytes and high in the development of monocytes ¹⁹⁹. In the B-cell, the high levels of PU.1 are observed in germinal center B-cells, while PU.1 level decreases with maturation ²⁰⁰. Reduced levels of PU.1 in precursor cells leads to leukemia in mice and humans and also predict poor prognosis ²⁰¹⁻²⁰⁴. Additional data from our lab suggest that PU.1 low mice (PU.1^{ure/ure}) develop after three months acute myeloid leukemia (AML) with aberrant expression of miR-155 and Myb ²⁰⁵.

Hematopoietic transcription factor PU.1 regulates differentiation of both lymphoid and myeloid cell lineages. The highest levels of PU.1 in B-cell line are expressed in germinal center B-cells and with maturation PU.1 expression decreases. Reduced levels of PU.1 in leukemia are mediated by aberrant expression of miR-155.

1.4 Characterization of E-box protein MYB and its role in B-cells and leukemia

1.4.1 Characterization of E-box protein MYB

MYB gene is a cellular homolog of viral *v-MYB* (v-myeloblastosis viral oncogene). It is a founding member of E-box (enhancer box) family transcription factors. *MYB* is a part of genome of two avian myeloblastosis viruses: avian myeloblastosis virus (AMV) and E26 virus²⁰⁶. These viruses induce acute avian leukemia²⁰⁶. The E-box family includes also *MYBL1* (also called *A-MYB*), *MYBL2* (also called *B-MYB*), *MYC*, *N-MYC* and the viral *v-MYB* genes. Each member plays different role and is expressed by different tissues and cells²⁰⁷. The *MYB* gene transcribes from chromosomal locus 6q22-23. Schematic map of *MYB* gene structure shows figure 1.7. *MYB* contains DNA binding domain (DBD), transactivation domain (TAD) and terminal negatively regulated domain (NRD)²⁰⁸. Product, MYB protein (75kDa) functions as DNA binding protein through its binding sequence AACG/TG²⁰⁹. The *MYB* gene contains 15 exons, of which some undergo alternative splicing²¹⁰ (Figure 1.7). The alternative splicing forms (9A, 10A) showed higher transcriptional and transforming potential²¹⁰.

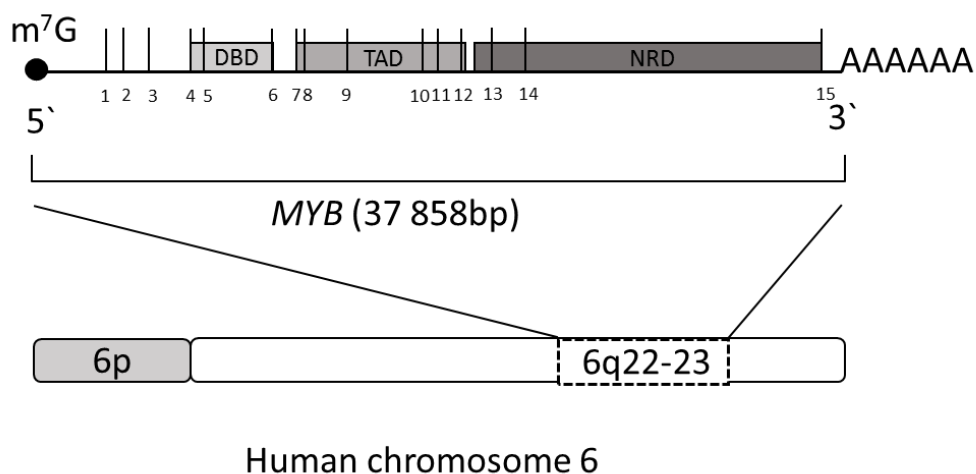


Figure 1.7: Structure of *MYB* gene (NC_000006.11). The *MYB* gene contains three domains: DNA binding domain (DBD) (blue box), central transactivation domain (TAD) (orange box) and terminal negatively regulated domain (NRD) (green box). Human *MYB* spans 37 858bb long mRNA transcript that contains 15 exons (black lines) (adapted and modified from Pattabiram DR and Gonda TJ, 2013 ; O'Rourke JP *et al.*, 2008^{208,210} <http://www.ebi.ac.uk/s4/summary/molecular?term=MYB&classification=9606&tid=synENSMUSG0000019982>).

The key biological function of MYB transcription factor (TF) lies in the maintaining of proliferative potential in progenitor cells. Downregulation of MYB leads to cell differentiation. MYB participates on G2/M cell cycle transition in hematopoietic cells by

direct stimulation of cyclin B1 expression ²¹¹. Moreover, the MYB expression is associated with leukemogenesis. Expression of MYB can be activated by itself and by structural alterations and/or genomic rearrangements ²¹².

1.4.2 Role of MYB in normal and leukemic B-cells

MYB is required for self-renewal in B-cells, T-cells and common lymphoid, myeloid progenitors. The expression of MYB is highest in the long term HSC and its production reduces with maturation of B-cells ²¹³. During the early B-cell development, presence of MYB is crucial for transition from pro-B to pre-B-cells ²¹⁴. Loss of MYB causes a partial block during B-cell development at the pro-B to pre-B cell transition that result in greatly decreased output of new B-cells from the bone marrow. Furthermore, absence of Myb prevents normal B-cell homeostasis due to decreased splenic B-cell survival ²¹⁵. MYB is required for survival and accumulation of CD19+ pro B-cells in the peripheral blood ²¹⁴. Impaired B-cell lymphopoiesis due to loss or mutated MYB is related with defect in IL-7 signaling ²¹³. The cytokine IL-7 regulates pro-B and pre-B cell transition ²¹⁶.

Role of MYB in B-cell malignancies is much less known and studied while MYB was firstly found as inducer of myeloid leukemia in chicken ²⁰⁶. There are some studies where aberrant expression of MYB resulted in acute lymphoid leukemia in humans ²¹⁷ and childhood lymphoid leukemia ^{218,219}. It was described that elevated levels of MYB in subset of B-CLL cells (40%) enhanced the expression of miR-155 ¹⁴⁴. The Sarvaiaya *et al.*, studied knockdown of MYB in the pre-B-ALL leukemic cells. They found that inhibition of MYB decreases the expression of BCL2 – the anti-apoptotic protein and a target of MYB. It resulted in increased apoptosis of pre-B-ALL leukemic cells and sensitivity to chemotherapy ²¹⁹.

1.4.3 Transcription factor MYB and its role in leukemia

MYB is a driver of leukemogenesis that underscores its identification as inducer of avian myeloid leukemia ²⁰⁶.

In the human T-cell acute lymphoid leukemia (T-ALL) Clappier *et al.*, identified genomic alterations as reciprocal translocation t(6;7)(q23;q34) and duplication of MYB loci. In these cases, the expression of MYB was much higher as in cases without genomic

alterations²²⁰. Similar results shown Lahortiga *et al.*, that the aberrant MYB expression in T-ALL associates with duplication of MYB. The knockdown of MYB expression initiates differentiation of T-cells²²¹.

O'Rourke and Ness described that minor change in the MYB structure by alternative splicing gives MYB stronger transforming potential. There is a difference in the expression of alternative transcripts in different hematopoietic cells. Truncated variant of MYB (exon 9A) in human pediatric B-cell ALL, T-ALL and AML samples displayed higher expression and higher oncogenic activity. Thus MYB and its alternative splicing forms strongly contribute to the leukemogenesis²¹⁰.

The increased expression of MYB mRNA²²² mediated by low expression of miR-150 was described in chronic myeloid leukemia (CML)²²³. In subset of primary B-CLL cells we detected elevated expression of MYB mRNA¹⁴⁴.

The aberrant expression of MYB in T-ALL, AML is associated with translocation or duplication of *MYB* gene.

1.4.4 Mouse models of Myb

The null *Myb* mouse model underscores importance of *MYB* gene in hematopoiesis. Homozygous mutant *Myb* mice at 15 day of gestation showed abnormal phenotype characterized by 10-fold decreased hematocrit; low number of multipotent hematopoietic progenitor cells and as well as myeloid and non-nucleated erythrocytes. These mice die at day 15 due to anemia²²⁴.

The *Myb* null mouse model confirmed importance of *MYB* in the B-cell development. These mice displayed 65% decrease of pre-B-cells, 50% decrease of new pre-B-cells in the bone marrow and reduced production of IgM. Loss of *Myb* causes a partial block during the B-cell development at the pro-B to the pre-B cell transition. *Myb* is mainly important for maintenance of pre-B-cells and signals through the pre-BCR and IL-7R. The absence of *Myb* negatively affects also the number of splenic B and follicular B-cells due to decreased bone marrow cells output²¹⁵. Moreover, *Myb* deficiency increase the number of apoptotic B-cells, probably mediated by decreased level of Bcl2 – a target of *Myb*²¹⁵.

Greig *et al.*, described another mouse model with *Myb* deficiency. The bone marrow analysis by flow cytometry showed reduced amount of immature and mature CD19+ B-cells. In absence of *Myb*, mice displayed B lymphopenia with reduced response to IL-7. Authors

demonstrated that conditional deletion of Myb in B-cell progenitors completely abolished B-cell development, thus Myb is required for normal development of common lymphoid progenitor cells (CLPs) as well as for committed B-cell progenitors. Data from microarrays showed that Myb is required for induction of expression several lymphoid genes as *Sfpi1*, *Ikzf1*, *Tcfe2a* within multipotent progenitor cells ²²⁵.

Waldron *et al.*, tested the role of Myb in the pathogenesis of p190^{BCR/ABL} dependent B cell leukemia in Myb deficient transgenic mouse model. Interestingly, loss of one copy of Myb suppresses the leukemogenic potential of B-cells isolated from p190^{BCR/ABL} transgenic mice. Similarly, knockdown of Myb in p190^{BCR/ABL} expressing human B-cell leukemia lines (Z-181, SUP-B15) resulted in reduced proliferation and colony formation B-cells ²²⁶.

These data on mouse models collectively show on the critical role of Myb in the regulation of multiple stages of B-cell development, especially in the pro-B to pre-B cell transition.

1.4.5 miR-150 a target of MYB gene in leukemia

Among the top 10 predicted targets of miR-150 belongs *MYB* gene that is critical for B-cell development as described above (TargetsScan⁷²). MiR-150 is highly B-cell specific microRNA ²²⁷. Xiao *et al.*, validated this computational prediction by luciferase reporter assay in HEK293 cell line where miR-150 targeted MYB. Authors identified three miR-150 putative binding sites at 3'UTR of MYB mRNA. In addition, the enhanced MYB expression in miR-150 deficient B-cells confirmed that miR-150 directly regulates MYB at protein level. This direct relationship was further documented by transfection of progenitor B-cells with mimic-miR-150 that resulted in dose dependent decrease of MYB levels and led to partial block of B cell development with reduced B-cells ²²⁸.

Interestingly, in the B₂20 lymphoma B-cell lines (Daudi, Raji, BJAB, Ramos) the expression of miR-150 is very low. The restoration of miR-150 expression by its stable transfection leads to decreased level of MYB and to terminal differentiation of B-cells. Moreover, elevated level of miR-150 resulted in reduced proliferation of cells that suggest to tumor suppressor role of miR-150 ²²⁹.

MYB represents an evolutionary conserved target of miR-150 that is confirmed by highly evolutionary conserved seed region of miR-150 at MYB ²³⁰.

2. INTRODUCTION TO THE THESIS

To the mostly used prognostic markers of B-CLL belong expression of ZAP-70, CD38, IgVH status and detection of chromosomal abnormalities by FISH³⁹. These markers help to evaluate the status of B-CLL and to predict disease progression³⁹. Used prognostic markers are not sufficient in answering following questions: 1) When to start with the therapy? 2) Will the patient respond to the therapy well?

Since the development of leukemia is a multistep process where any step can be deregulated, more detailed knowledge of regulators of the leukemic process in B-CLL is needed. Transcription factors as PU.1 and MYB are involved in regulation of the normal and pathologic hematopoiesis^{212,231}. While the transcription factors may regulate each other a tight relationship between transcription factors and miRNAs was recently identified²³². The role of miRNAs during the leukemic process and moreover the potential role of miRNAs in prognosis and therapy is at present very intensively studied. Fulci *et al.*, described that B-CLL cells express high levels of miR-155 and miR-150¹⁷. Moreover, elevated levels of miR-155 inhibit the expression of PU.1 in B-CLL in comparison to the normal healthy B-cells^{17,157}.

Recent data show the potential usage of miR-155 as prognostic marker in the breast cancer²³³. High expression of miR-155 correlates with higher tumor grade and advanced disease stage²³³. Application of the antisense miR-155 inhibits cell growth, enhances apoptosis and increases sensitivity of the breast cancer cells to the therapy²³³. Based on this information, the measurement of miR-155 levels during disease progression could be similarly used as a marker of B-CLL progression as well.

During the B-cell development, MYB is required for transition from pro-B to pre-B-cells and for survival of accumulated pro-B-cells²¹⁴. The expression of MYB in the hematopoietic cells differs: the highest levels of MYB are in immature, actively dividing cells and the lowest ones in the mature, differentiated cells^{212,234}. It was described that MYB is involved in acute myeloid²³⁵ and chronic myeloid leukemia²²³, mixed lineage leukemia²³⁶ and in acute lymphoblastic leukemia²¹⁹ but not described in B-CLL yet. *MYB* gene was described among the top 10 target genes of miR-150²²⁸, so we also considered existence of relationship between expression of miR-150, MYB and B-CLL progression.

2.1 Hypothesis

The expression level of oncogenic miR-155 is elevated in hematologic malignancies including B-CLL. The direct target of miR-155 is a key hematopoietic transcription factor PU.1. The miR-155 inhibits PU.1 as a negative post-transcriptional regulator and this leads to progressive blockade of differentiation and leukemogenesis. We hypothesize that MYB proto-oncogene is highly expressed in B-CLL, and that MYB directly promotes the elevation of miR-155 expression level in B-CLL. This might be caused by direct binding of MYB at the promoter region of *MIR155HG* and by this way stimulates the transcription of *MIR155HG* in B-CLL. The detection of high MYB expression levels may be used as the reliable prognostic marker of B-CLL together with miR-155 or miR-150. Moreover, the rescue of PU.1 expression may turn off the leukemic process and may be used as a therapeutic tool in the future.

2.2 Aims of the Thesis

1) To determine whether levels of oncogenic miR-155, miR-150 and transcription factors MYB, PU.1 are related to oncogenesis of B-CLL and to delineate their relationship with the B-CLL disease-prognosis, presence of progression characteristics including ZAP-70, CD38, IgVH and Rai stage and to the therapy response.

2) To determine if proto-oncogene MYB binds to the promoter region of *MIR155HG* and if it stimulates transcription of the *MIR155HG* in B-CLL. To functionally validate direct relationship between MYB, miR-155 and PU.1 in B-CLL cells from patients and in Raji cell line by *in vitro* functional assays.

3) To determine the miR-155 and MYB target genes deregulation in B-CLL in comparison to the normal B-cells using the global microarray approach.

2.3 Specific aims of the Thesis

1) To determine whether levels of oncogenic miR-155, miR-150 and transcription factors MYB, PU.1 are related to oncogenesis of B-CLL and to delineate their relationship with the B-CLL disease-prognosis, presence of progression characteristics including ZAP-70, CD38, IgVH and Rai stage and to the therapy response.

The success of therapy of B-CLL patients is in the proper timing of the therapy. Our aim was to determine the levels of miR-155 in B-CLL patients and test whether there is any correlation between elevated levels of miR-155 and the disease progression (Rai stage, ZAP-70, CD38, IgVH). To answer the question if the expression of miR-155 could be used as reliable prognostic marker, we measured the levels of miR-155 in B-CLL patients in different stages of the disease. MiRNAs negatively regulate transcription of targeted mRNAs. The direct target of miR-155 is a key hematopoietic transcription factor PU.1. Reduced levels of PU.1 were reported to cause leukemia and lymphoma. Here we determined the mRNA and protein levels of PU.1 in B-CLL patient B-cell samples. We also asked if the expression of MYB could be used together with miR-155 as reliable prognostic marker of B-CLL. We measured the mRNA and protein level of MYB in B-CLL patient samples during different

disease stages. Since the miR-150 is a potent inhibitor of MYB, we measured expression level of miR-150 in B-CLL cells in relation with Rai stage and prognostic markers.

2) To determine if proto-oncogene MYB binds to the promoter region of *MIR155HG* and if it stimulates transcription of the *MIR155HG* in B-CLL. To functionally validate direct relationship between MYB, miR-155 and PU.1 in B-CLL cells from patients and in Raji cell line by *in vitro* functional assays.

Transcription factors play crucial role in the development of leukemia. Here we focused on the role of a member of the E-box family - *MYB*. Firstly, we asked whether the mRNA of *MYB* is increased in B-CLL. Next, we set to verify *MYB* binding at the *MIR155HG* promoter region and asked if *MYB* influences transcription of *MIR155HG* in B-CLL. We performed chromatin immunoprecipitation assay of primary B-CLL cells to test if *MYB* binds at the promoter region of *MIR155HG*. We transfected constructs with mutated or unmutated *MYB* binding site into HeLa cells and measured the luciferase activity. We asked whether the studied molecules: *MYB*, miR-155 and PU.1 are in close and mutual relationship within B-CLL. We aimed to verify the relationship between *MYB*, miR-155 and PU.1 by *in vitro* functional assays using the primary B-CLL cells and lymphoblastic cell line Raji. Here we manipulated the levels of *MYB* or miR-155 and determined the outcome as well as rescued the levels of PU.1.

3) To determine the miR-155 and MYB target genes deregulation in B-CLL in comparison to the normal B-cells using the global microarray approach.

We performed the microarray analysis on B-CLL patient samples and normal healthy controls to determine if the target genes of miR-155 and *MYB* are deregulated. Further, by gene set enrichment analysis and by additional tools for prediction of target genes we asked if miR-155 and *MYB* targeted genes are differentially expressed within B-CLL microarray data.

3. MATERIAL AND METHODS

3.1 Material

3.1.1 Biological material

3.1.1.1 Primary B-CLL patient samples and normal healthy controls

The B-CLL patient samples were collected in the period from 2007 until 2012. The patient cohort included overall 239 peripheral blood samples of 210 B-CLL patients from two medical clinics in the Czech Republic: 1) General Faculty Hospital in Prague (under leadership of prof. MUDr. Marek Trněný and in close cooperation with MUDr. Tomáš Stopka, Ph.D and MUDr. Petra Obtrlíková Ph.D) and 2) Medical Faculty and University Hospital in Brno (by kind collaboration with prof. MUDr. Jiří Mayer, CSc. prof. RNDr. Šárka Pospíšilová, Ph.D and MUDr. Marek Mráz, Ph.D). Total number of all patient samples obtained from General Faculty Hospital in Prague was 210 where 23 patient samples were provided two times and three patient samples were provided three times during the study. 71 samples of these 210 patient samples were separated as peripheral blood mononuclear cells (PBMCs) by Ficoll-Paque and 157 samples CD19+ B-CLL cells were separated by Rosette separation kit (Table 3.1). The plasma was collected from all patients in parallel with peripheral blood, but for this Thesis only ten plasma samples were used (Table 3.1). All patient samples (N=41) from Medical Faculty and University Hospital in Brno were separated for CD19+ B-cells by Rosette separation kit (Table 3.1). The patient cohort consists of 142 men and 68 women (Table 3.2). The patient's age ranged from 36 to 95 years with median age 65. All patients signed a written consent in accordance with the ethical guidelines.

Diagnostic and prognostic evaluation of patients was done in both medical institutes based on WHO guidelines (<http://ncicll.com/> and ²⁸). The B-CLL diagnosed in 196 cases out of the 210 patients. B-CLL transformation into Richter's syndrome observed in 14 cases (Table 3.2). Based on the prognostic and cytogenetic data, our patient cohort represents a typical B-CLL population (Table 3.2). The distribution (Nr; %) of patients and patient samples based on the mutation status of IgVH, expression of ZAP-70, CD38 and cytogenetic aberrations are shown in table 3.2. Distribution of patients on Rai staging system as follows: Rai stage 0 (N=42; 20%), Rai stage I (N=47; 23%), Rai stage II (N=46; 22%), Rai stage III (N=19;

9%) and Rai stage IV (N=54; 26%) (Table 3.2). 49% of patients were treated during the study (Table 3.2). For detailed clinical and expression information of patient samples please see section 10. Supplement; Supplemental table: 10.1 and 10.2.

The B-CLL patient samples were used for following experimental methods: isolation of total RNA (including miRNA) (N=239); measurement of protein level by western blot (N=5); chromatin immunoprecipitation (ChIP) (N=15); microarrays (N=12); transient transfections (N=2); transduction (N=1); DNA sequencing (N=2).

The cohort of normal healthy donors included overall 22 peripheral blood samples collected in the period from 2009 until 2010 on the Institute of Hematology and Blood Transfusion in Prague. PBMCs were isolated from 10 samples and CD19+ B-cells were separated from 12 samples. Control cohort consisted of 20 males and 2 women; age of donors ranged from 20 to 43 years with median age 31. From all donors written consents in accordance with the ethical guidelines were obtained. The detailed information about the control samples are in section 10. Supplement; Supplemental table 10.3.

The control patient samples were used for following experimental methods: isolation of total RNA (including miRNA) (N=22); measurement of protein level by western blot (N=1); chromatin immunoprecipitation (ChIP) (N=8); microarrays (N=5); transient transfections (N=5); DNA sequencing (N=1).

Table 3.1: Distribution of patient samples and the sample origin.

Medical Institution	PBMCs	CD19+	Plasma
Prague (N=198)	71	157*	10
Brno (N=41)	0	41	0

*From General Faculty Hospital in Prague were donated in total 198 B-CLL patient samples of the peripheral blood including 10 plasma samples. From 30 patient samples were separated PBMCs and also CD19+ B-CLL cells. From Medical Faculty and University Hospital in Brno were donated in total 41 B-CLL patient samples separated for CD19+ B-cells.

Table 3.2: Clinical and biological characteristics of the B-CLL patient cohort.

Parameter	CLL patients (N=210) No (%)					CLL samples (N=239)* No (%)				
<u>Epidemiological data:</u>										
Age at sample providing (median)	36-95 (65)					36-95 (65)				
Gender (Female/Male)	68 (32%) / 142 (68%)					76 (32%) / 163 (68%)				
<u>Prognostic markers:</u>										
IgVH status	mutated		unmutated			mutated		unmutated		
	48 (26%)		134 (74%)			60 (30%)		141 (70%)		
ZAP-70	positive		negative			positive		negative		
	118 (61%)		76 (39%)			135 (61%)		85 (39%)		
CD38	positive		negative			positive		negative		
	100 (48%)		108 (52%)			113 (48%)		123 (52%)		
<u>Disease progression and transformation:</u>										
Rai stage	0	I	II	III	IV	0	I	II	III	IV
	42 (20%)	47 (23%)	46 (22%)	19 (9%)	54 (26%)	44 (19%)	53 (22%)	55 (23%)	23 (10%)	60 (26%)
Richter's syndrome (DLBCL/MCL)	DLBCL		MCL			DLBCL		MCL		
	11 (5%)		3 (1%)			14 (6%)		3 (1%)		
<u>Cytogenetics:</u>										
Low risk (del13q14)	69 (33%)					78 (33%)				
Intermediate risk (trisomy of 12 chromosome, normal karyotype)	51 (25%)					63 (27%)				
High risk (del11q22-23/del17p13)	14 (7%) / 9 (4%)					20 (9%) / 9 (4%)				
<u>Therapy:</u>										
Therapy prior study	84 (40%)					92 (39%)				
Therapy during study	103 (49%)					124 (52%)				
<u>Overall survival (OS) from dg to last follow up:</u>										
OS <5 years	26 (54%) / 113 (70%)					27 (54%) / 138 (73%)				
OS 5-10 years	15 (31%) / 34 (21%)					15 (30%) / 35 (19%)				
OS >10 years	7 (15%) / 15 (9%)					8 (16%) / 16 (9%)				

*23 patients provided two samples and 3 patients provided three samples during the study (2007-2012).

3.1.1.2 Cell lines

The cell lines were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). Cultivation conditions and media were according DSMZ guidelines.

Raji (#ACC 319) cell line was established from the left maxilla of a 12-year-old African boy with Burkitt's lymphoma in 1963. Raji cell line was classified as risk category 1 according to the German Central Commission for Biological Safety (ZKBS) ²³⁷.

Morphologically, the cells are round, growing in suspension, partly as single cells or in clusters. The cells were cultured in IMDM medium supplemented with 10% FBS, 1% of P/S (Penicillin/Streptomycin) in density of about 0.5×10^6 cells/mL at 37°C with 5% CO₂. Doubling time of Raji cells is 24-36 hours. Saturated Raji cell culture was split at ratio 1:4 twice a week. Raji cells were frozen in medium containing 70% of RPMI-1640 medium, 20% FBS and 10% DMSO at aliquots of approximately 3×10^6 cells per cryotube.

HeLa (#ACC 57) cell line was established from the epithelioid cervix carcinoma of a 31-year-old Afro-American woman (Henrietta Lacks) in 1951. Later the diagnosis changed into adenocarcinoma ²³⁸.

Morphologically, the cells are epithelial-like cells growing in monolayer. HeLa cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 1% of P/S in density of about $1-2 \times 10^6$ cells/mL at 37°C with 5% CO₂. Doubling time of HeLa cells was around 48 hours. Confluent HeLa cell culture was split 1:4 to 1:6 every 3-5 days using trypsin. HeLa cells were frozen in medium containing 70% of RPMI-1640 medium, 20% FBS and 10% DMSO at aliquots of approximately 1×10^6 cells per cryotube.

NB4 (#ACC 207) cell line was established from the bone marrow of a 23-year-old woman with acute promyelocytic leukemia (APL = AML FAB M3) in second relapse in 1989. Cells carry the t(15;17) PML-RARA fusion gene ²³⁹.

Morphologically, the cells are round, growing in suspension. NB4 cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 1% of P/S in density of about 1×10^6 cells/mL at 37°C with 5% CO₂. Doubling time of NB4 cells was around 35-45 hours. Saturated NB4 cell culture was split at ratio 1:2, 1:3 once a week. NB4 cells were frozen in

medium containing 70% of RPMI-1640 medium, 20% FBS and 10% DMSO at aliquots of approximately 7×10^6 cells per cryotube.

3.1.2 Chemicals and buffers

3.1.2.1 Chemicals

Name	Cat #	Company
Acetic acid 99% p.a.	457311	Penta
Albumin, from bovine serum (BSA)	A2153	Sigma-Aldrich
Annexin V FITC	556419	BD Biosciences
Annexin V 10x diluted Binding Buffer	20090907ANXVBB	BD Biosciences
anti-miR miRNA inhibitor negative control#1	AM17010	Ambion
Bradford Reagent	E530	Amresco
Briliant Blau R 250	3862.2	Carl Roth GmbH
CL-XPosure Film (Clear Blue X-ray Film)	34090	Thermo Scientific
Deoxycholic acid (sodium salt)	0613	Amresco
Dimethylsulfoxid (DMSO)	D06502	p_lab
DL-Dithiotherotol (DTT)	0281	Amresco
DMRIE-c	10459-014	Invitrogen
ECL Plus Western Blotting Detection System	RPN2132	GE Healthcare
EDTA	27270	Sigma-Aldrich
EGTA	0732	Amresco
Ethanol 99% p.a.	028621	Penta
Fetal Bovine Serum (FBS)	10270	Gibco, Invitrogen
Ficoll-Paque PREMIUM gradient	17-5442-03	GE Healthcare
Formaldehyde 37% solution	252549	Sigma-Aldrich
Glycine	0167	Amresco
Glycerol	0854	Amresco
Glycogen RNA grade (20mg/mL)	R0551	Fermentas
Chloroform p.a.	256921	Penta
HEPES	0485	Amresco
hsa-miR-155 anti-miRNA inhibitor	AM12601	Ambion

hsa-miR-155 mimic	AM17100	Ambion
jetPEI	101-10-101-10N	Baria
iBlot Gel Transfer Stacks, PVDF, Mini	IB4010-02	LifeTechnologies
Igepal CA-630	I8896	Sigma-Aldrich
IMDM medium	BE12-722F	Lonza
Isopropylalkohol p.a.	593001	Penta
Linear Acrylamide (5mg/ml)	AM9520	Invitrogen
MEM non-essential amino acids (100x)	M714	Sigma-Aldrich
Methanol HPLC	21230-12500	Penta
negative control#1 siRNA	4457289	Ambion
Nuclease eliminator	E891	Amresco
NuPAGE 10% Bis-Tris Gel 1.5mm x 10 well	NP0335BOX	LifeTechnologies
NuPAGE LDS Sample Buffer (4x)	NP0007	LifeTechnologies
NuPAGE MOPS SDS Running Buffer (20x)	NP0001	Life Technologies
OPTI-MEM medium	31985	Gibco, Invitrogen
Penicillin/Streptomycin (P/S) (100x)	15070063	Gibco, Invitrogen
Phosphate Buffered Saline (PBS) (10x)	L1835	Biochrom AG
Polybrane (8mg/ml)	H9268	Sigma-Aldrich
Ponceau S	P3504	Sigma-Aldrich
Precision Protein Dual Color Standards	161-0374	BIO-RAD
Propidium iodid (PI)	P4170	Sigma-Aldric
Protease Inhibitor Cocktail	P8340	Sigma-Aldrich
Protein-A-agarose	P7786	Sigma-Aldrich
Protein-G-Agarose	P4691	Sigma-Aldrich
Ribonuclease A	R4642	Sigma-Aldric
RPMI-1640 medium	R7388	Sigma-Aldrich
siRNA MYB	sc-29855	Santa Cruz
Sodium dodecyl sulfat	M107	Amresco
Sodium chloride	0241	Amresco
Steady-Glo Luciferase Assay System	E1910	Eastport (Promega)
SYBRgreen Master Mix	4368702	LifeTechnologies
TaqMan Universal master Mix II	4427788	LifeTechnologies

Tris-Hcl Ultrapure grade	0234	Amresco
Triton X-100	0694	Amresco
TRIzol reagent	15596-018	Invitrogen
Trypsin (0.25%)	15050	Gibco, Invitrogen
Trypan blue solution (0.4%)	T8154-100mL	Sigma-Aldrich
Tween 20	0777	Amresco
Universal Probe Library (UPL) set	4683633001	ROCHE

3.1.2.2 Buffers and solutions

Buffers and solutions

Content (final concentrations)

RIPA lysis buffer (WB)	50mM Tris-Hcl; pH 8,0; 137 mM NaCl; 1% Igepal CA-630; 0.5% Deoxycholic acid sodium salt; 0.1% SDS; Protease Inhibitor Cocktail (dilution 1:1000)
11% Formaldehyde solution (ChIP)	11% formaldehyd; 0.1M NaCl; EDTA(pH 8.0); 1mM EDTA; 0,5mM EGTA; 50mM HEPES (pH 8.0)
Lysis buffer (ChIP)	50mM HEPES-KOH (pH 7.5); 140mM NaCl; 1mM EDTA; 10% glycerol; 0.5% Igepal CA-630; 0.25% Triton X-100
Lysis buffer II (ChIP)	1mM EDTA (pH 8.0); 0.5mM EGTA (pH 8.0); 10mM Tris-Hcl (pH 8.0); 200mM NaCl
Lysis buffer III (ChIP)	1mM EDTA (pH 8.0); 0.5mM EGTA (pH 8.0); 10 mM Tris-Hcl (pH 8.0)
Paro's IP buffer (ChIP)	0.02% SDS; 2% Triton X-100; 4mM EDTA (pH 8.0); 40mM Tris-Hcl (pH 8.0); 300mM NaCl

Paro's washing buffer I (ChIP)	0.1% SDS; 1% Triton X-100; 2mM EDTA (pH 8.0); 20mM Tris-Hcl (pH 8.0); 50mM NaCl
Paro's washing buffer II (ChIP)	0.1% SDS; 1% Triton X-100; 1% EDTA (pH 8.0); 20mM Tris-Hcl (pH 8.0); 500mM NaCl
Buffer for Proteinase K (ChIP)	0.5% SDS; 25mM EDTA (pH 8.0); 10mM Tris-HCl (pH 8.0); 100mM NaCl
MACS buffer (MACS separation)	0.5% BSA; 2mM EDTA in 1xPBS with Ca ²⁺ , Mg ²⁺ (pH 7.2)
NH ₄ Cl solution (Erythrocytes lysis)	8.34g NH ₄ Cl + 1g NaCl + 0.037g EDTA filled up 1L of ddH ₂ O and sterilized. Stored at 4°C for one month.

3.1.2.3 Commercially kits

Name	Cat #	Company
Anti-FITC MicroBeads	130-048-701	MACS, Miltenyi Biotech
GeneChip HG-U133 Plus 2.0 Array	900467	Affymetrix
Human B cell line nucleofection kit	VPA-1001	Lonza
miRNAeasy kit	217004	Qiagen
hsa-miR-150 (ID:0000473)	4427975	LifeTechnologies
hsa-miR-155 (ID:0002623)	4427975	LifeTechnologies
hsa-pri-miR-155 (ID:Hs03303349_pri)	4427975	LifeTechnologies
RNA Nano chips	5067-1511	Agilent Technologies
RNU44 (ID: 0001094)	4427975	LifeTechnologies
RosetteSep B cell enrichment kit	15064	Stem Cell Technologies
TaqMan microRNA Reverse Transcription Kit	4366596	LifeTechnologies
3'-IVT Express Kit	901228	Affymetrix

3.1.2.4 Antibodies

Name	Cat #	Method	Company
Anti-Actin HRP-conjugated (clone I-19)	sc-1616	WB	Santa Cruz
Anti-CD19 FITC (clone HIB19)	302206	FACS	Biolegend (Luvil)
Anti-H3K4methyl	ab1012	ChIP	Abcam
Anti-H3K9acetyl	07-353	ChIP	Merck Millipore
Anti-v-Myb/MYB (clon EP769Y)	ab45150	ChIP, WB	Abcam
Anti-PU.1 (clone T-21)	sc-352	WB	Santa Cruz
Normal Rabbit IgG	NI01	ChIP	Merck Millipore

3.1.2.5 Plasmids

Name	Cat#	Company
pcDNA	kindly provided by M. Dvořák, Institute of Molecular Genetics in Prague	
pmaxGFP	as part of nukleofection kit	Lonza
pGL4.17	E6721	EastPort Promega
PU.1-IRES-GFP	expressing lentivirus kindly provided by Prof. A.I. Skoultchi, New York	
MYB (wt)	kindly provided by M. Dvořák, Institute of Molecular Genetics in Prague	

(Generic vector maps of plasmids are in the section 10. Supplement; Supplemental material: 10.1).

3.1.3 Instruments

Name	Company
Amxa nucleofector Device II	Lonza
BD FACS Aria IIu cell sorter	BD Biosciences
BD FACS Canto II fluorescence analyser	BD Biosciences
Bioanalyzer 2100	Agilent Technologies
Cellometer Auto T4	Bioscience
Digital sonifier cell disruptor M500	Branson
Genetic Analyzer 3500	Life Technologies
iBlot Gel Transfer System	Life Technologies
Mastercycler Gradient	Eppendorf
Sirius Luminometer	Berthold
Spectrophotometer ND-1000	NanoDrop Technologies
7900HT Fast Real-time PCR System	Life Technologies

3.1.4 Programs

Name	Method	Company
Chromas 2.33	DNA sequencing	Technelysium Pty Ltd
FACSDiva software v.6.3	flow cytometry	BD Biosciences
GeneSpring GX10 software	microarrays	Agilent Technologies
GraphPad Prism (version 5) software	RT-qPCR	GraphPad Software Inc.
Scion Image software Beta 4.0.2	WB	Scion Corporation
ProbeFinder Version 2.45	RT-qPCR	ROCHE
TIGR MeV4 4.0.01	microarrays	TM4 Microarray Software

3.2 Methods

3.2.1 Isolation of primary B-cells and plasma

3.2.1.1 Isolation of peripheral blood mononuclear cells (PBMCs) by Ficoll – Paque

The PBMCs were isolated from 71 peripheral blood samples (from General Faculty Hospital in Prague) from B-CLL patients by Ficoll-Paque PREMIUM gradient.

Briefly, anti-coagulated peripheral blood was diluted with sterile 1xPBS, carefully layered on the Ficoll-Paque PREMIUM solution and centrifuged. Differential migration of cells during the centrifugation results in the formation of layers that contain different cell types (Figure 3.1). The PBMCs are found in the interlayer after centrifugation- seen as “foggy ring”, recovered from this interlayer by short washing step (100g, 6min, 4°C) with a 1xPBS that removes any platelets, rest of Ficoll-Paque PREMIUM and plasma.

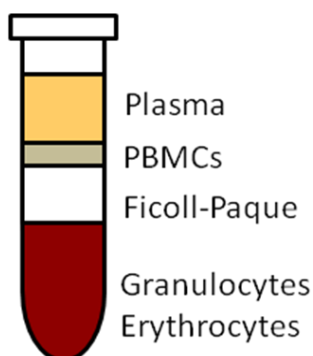


Figure 3.1: Scheme of separated fractions of blood cells by Ficoll-Paque PREMIUM. After centrifugation of peripheral blood on the Ficoll-Paque PREMIUM layer the PBMCs – B, T lymphocytes and ~1% of monocytes, platelets were found in the interlayer between the plasma and the Ficoll-Paque PREMIUM. On the top of the erythrocytes pellet were granulocytes.

With the separated B-CLL/SLL patients PBMC cells was done one or more of the following experimental procedures: 1) cryopreservation: frozen in the freezing medium (90% FBS + 10% DMSO) for 24-48hrs and for the long storage in the liquid nitrogen; 2) transient transfections (N=2) or transduction experiments (N=1); 3) Isolation of mRNA/miRNA for expression profile (N=71) or 4) Microarray profiling (N=12).

The primary PBMCs were cultured one week for maximum (for transient transfections and transduction) *in vitro* in IMDM medium supplemented with 20% FBS, 1% of MEM non-essential amino acids and 1% of P/S at 37°C in humidified atmosphere with 5% of CO₂.

3.2.1.2 Isolation of CD19+ B-cells by RosetteSep kit

The CD19+ B-cells were isolated from 198 peripheral blood samples of B-CLL patients (157 patients samples were from General Faculty Hospital in Prague and 41 from Medical Faculty and University Hospital in Brno) and as well from 12 normal healthy controls from Institute of Hematology and Blood Transfusion in Prague (Table 3.1).

The method of separation the CD19+ B-cells by commercially available Rosette Sep kit is based on the principle involves contacting a sample containing nucleated cells and red blood cells with an antibody composition which allows immunorosettes of the nucleated cells and the red blood cells to form. Briefly, immunorosettes form by crosslinking of B-cell enrichment antibody cocktail with the unwanted cells through multiple erythrocytes. Immunorosettes are targeted for removal with tetramer antibody complexes recognizing CD2, CD3, CD16, CD36, CD56, CD66b and glycophorin A on the erythrocytes. The unwanted cells are then pelleted with the free erythrocytes by centrifugation over a density medium Ficoll-Paque PREMIUM. Desired cells are not labeled with the antibody cocktail and are collected as a highly enriched population at the interlayer between the plasma and the Ficoll-Paque PREMIUM density medium (Figure 3.2).

The purity of separation was confirmed by flow cytometry using anti CD19 FITC antibody. The purity exceeded 93%. The viability of separated cells measured by Trypan blue and Cellometer Auto T4 was more than 90%.

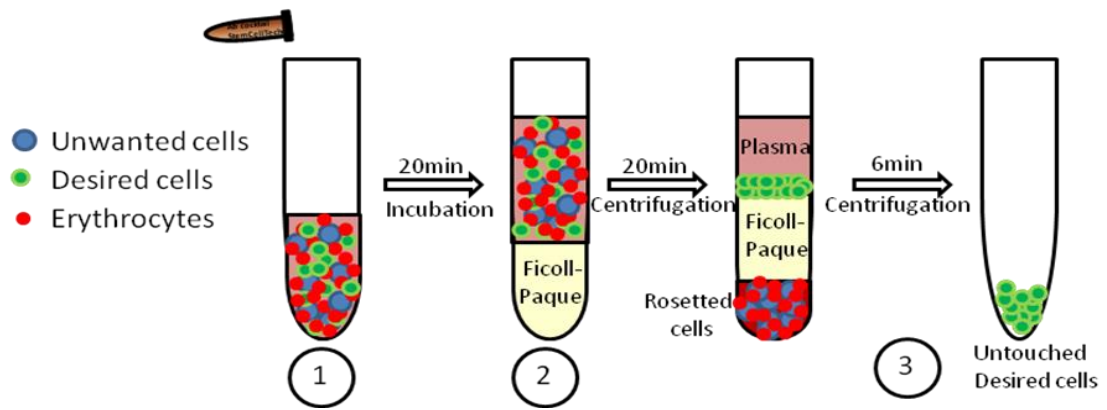


Figure 3.2: Scheme of RosetteSep procedure. During the first step the unwanted cells from whole blood bound to the erythrocytes by using special antibody cocktail. Then the suspension incubated for 20 minutes at room room temperature (RT). During the second step the suspension is loaded on the Ficoll-Paque layer and centrifugated for 20 minutes, RT. The unwanted cells pellet along with the erythrocytes. During the third step enriched CD19+ B-cells are collected. The scheme was adapted from the Stem Cell Technologies: <http://www.stemcell.com/en/Products/All-Products/RosetteSep-Human-B-Cell-Enrichment-Cocktail.aspx>.

3.2.1.3 Plasma separation

The plasma of 10 B-CLL patient peripheral blood samples was obtained by centrifugation at 2000rpm for 10min at 4°C. The miRNAs from plasma samples were isolated by miRNA easy kit following the manufacturer's instructions (see section miRNA isolation). Plasma samples were stored at -80°C.

3.2.1.4 Lysis of erythrocytes by ammonium chloride (NH₄Cl)

The membranes of erythrocytes are effectively permeable to NH₄Cl. The erythrocytes lysis occurs due to unbalanced osmotic pressure. The peripheral blood samples from the normal healthy donors (N=10) were lysed by ammonium chloride (15minutes; 37°C) prior the magnetic separation of CD19+ B-cells.

3.2.1.5 Magnetic separation of CD19+ B-cells by MACS magnetic beads

After erythrocytes lysis, the CD19+ B-cells were isolated by magnetic beads separation. In the first step, the cells were stained with FITC-labeled anti-CD19 antibody. In the second step, anti-FITC magnetic beads were bound to the CD19+ (FITC stained) cells. The third step was magnetic separation of CD19+ cells labeled with magnetic beads on the MACS columns in the magnetic field, where non-labeled cells goes through the column, and labeled cells stay inside the column. Labeled cells were than collected from column.

3.2.2 Flow cytometry analysis (FACS)

3.2.2.1 Control measurements for CD19 purity after MACS separation

The purity of magnetically enriched CD19⁺ B-cells was confirmed by flow cytometry (FACS). The cell viability and apoptosis was measured by propidium iodide (PI) and AnnexinV staining. The purity of MACS isolated cell population exceeds 93% and cell viability exceeds 90%. The separated CD19⁺ B-cells were further proceeded for RNA/miRNA isolation (N=10).

3.2.2.2 Propidium iodide (PI) staining

PI is a membrane non-permissible dye that is generally excluded from viable cells. PI will stain necrotic and late apoptotic cells with compromised membranes. In dead (or apoptotic) cells, PI binds to the double stranded DNA by intercalating between the base pairs. 1 μ L of PI was added per sample directly before FACS analysis.

3.2.2.3 AnnexinV staining

AnnexinV belongs to family of calcium-dependent phospholipid-binding proteins. AnnexinV marks mid to late stage apoptotic cells by labeling phosphatidyl serine (PS). Under normal physiologic conditions, PS is predominantly located in the inner site of the cell membrane. After initiation of apoptosis, PS is translocated on the outer surface of the cell membrane. This phenomenon is used for the detection of apoptotic cells by fluorescently labeled antibodies to AnnexinV.

Briefly, cells were harvested and washed with 1xPBS and binding buffer. Stained with FITC conjugated AnnexinV for 10-15 minutes at room temperature, in dark. After AnnexinV staining, cells were washed with binding buffer and analysed by flow cytometry within one hour.

3.2.3 Transient transfections and transduction

3.2.3.1 Transfection of primary B-CLL cells by lipofection reagent DMRIE-c

Primary PBMC cells isolated from 2 B-CLL patient samples were transfected with following molecules: 1) siRNAs MYB (pool of three target specific siRNAs) and negative control #1 siRNA; 2) hsa-miR-155 anti-miRNA Inhibitor and miRNA inhibitor negative control #1; 30) pmaxGFP plasmid (2µg). The pmaxGFP plasmid was used for optimization of transfections - to find the final and most efficient concentration of desired oligonucleotides.

Transfection reagent DMRIE-c (1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide and cholesterol) represents a liposomal component that forms complexes with nucleic acids (RNA, DNA). DMRIE-c is efficient for transfections of mammalian cells, particularly lymphoid suspension cells.

The confluency of cells was around 80%. Cells were washed with the serum and antibiotics-free medium OPTI-MEM prior to transfection. The siRNA against MYB (30nM) or anti-miR-155 (40nM) as well as negative controls (added in the same final concentrations as the concentrations of specific siRNA MYB/anti-miR-155) were mixed with DMRIE-c and OPTI-MEM medium. Transfection suspension was added to the cell suspension and incubated in humidified atmosphere with 5% CO₂ at 37°C for 5 hours. After 5hrs of incubation with transfection reagents the equal volume of cultivation IMDM medium was added to the cells. The transfection efficiency was controlled by fluorescence activity of pmaxGFP, efficacy of B-CLL cells was around 60%. Transfected cells were harvested after 48 hrs (MYB siRNA) or 96 hrs (anti-miR-155). Total RNA was extracted and RT-qPCR was performed (see section RNA isolation and RT-qPCR).

3.2.3.2 Transfection of HeLa cells by lipofection reagent JetPEI

Into HeLa cells were transfected MYB (wt; mutated MYB binding sites) and control pcDNA plasmids by lipofection reagent JetPEI (linear polyethylenimine). The confluence of HeLa cells prior to transfection was around 60%. Transfection reaction contained plasmid DNA, jetPEI solution and NaCl solution. The transfection reagents were mixed and incubated for 25 minutes at room temperature to form the complexes. The formed complexes were added to the HeLa cells by slow pipetting. HeLa cells were incubated in the humidified

atmosphere with 5% CO₂ at 37°C. After 48 hrs, the HeLa cells were harvested and lysates for luciferase assay and for western blot were prepared.

3.2.3.3 Transfection of Raji cells by Amaxa nucleofactor

Raji cells were transfected with following molecules: 1) MYB siRNAs (pool of the three target specific siRNAs) and siRNA negative control#1; 2) mimic hsa-miR-155 and miRNA negative control#1; 3) hsa-miR-155 anti-miRNA Inhibitor and miRNA inhibitor negative control#1; 4) MYB and control pcDNA plasmids. The pmaxGFP plasmid was used for optimization of transfections - to find the final and most efficient concentration of desired oligonucleotides.

The Raji cells were transfected by nucleofactor Amaxa with Human B-cells nucleofection kit. 24 hrs prior to transfection the cell number was determined, cells were seeded in the ~70% of confluency in 6 well plates and cultured until transfection in IMDM medium with FBS, but without antibiotics. On the next day, the cultured Raji cells were centrifuged and washed once with 1xPBS. Two million of Raji's cells were mixed with the nucleofection solution and transfected with oligonucleotides of hsa-miR-155 (40nM and 100nM final concentration), anti-miR-155 (40nM and 100nM final concentration/well), siRNA MYB (30nM and 100nM final concentration/well), MYB and control pcDNA plasmids (0.2µg and 2µg/well) and co-transfected with pmaxGFP plasmid (2µg/well) provided with the transfection kit. The program M-013 was used for transfection as recommended. After nucleofection the cells were immediately transferred into warmed up culture medium (37°C) and incubated in humidified atmosphere with 5% CO₂ at 37°C. Transfected cells were harvested after 24 hrs for mimic hsa-miR-155 transfection and 48 hrs for transfection of siRNA MYB, anti-miR-155, plasmid MYB. The transfection efficiency was controlled by fluorescence microscopy by fluorescence activity of pmaxGFP. The transfection efficacy was ~70%. The RNA was extracted and RT-qPCR was performed.

3.2.3.4 Transduction of primary B-CLL cells with lentivirus

Primary PBMC cells from B-CLL patient sample (P40) were infected with either GFP expressing plasmid or *PU.1*-IRES-GFP expressing lentivirus. Infection of primary B-CLL cells was performed with MOI=3 [MOI=multiplicity of infection; means the ratio of infectious agents (e.g. virus) to infection targets (e.g. cell)]. After 48 hrs, the GFP positive infected cells were sorted out (Fluorescence activated cell sorting, BD FACS Arianllu), and cultured for 24 hrs in the IMDM medium supplemented with 10% of FBS and 1% of MEM non-essential amino acids in humidified atmosphere with 5% CO₂ at 37°C. The viability of infected cells was determined by PI and AnnexinV staining (see PI and AnnexinV staining).

3.2.4 Luciferase reporter assay

To test the putative MYB binding site at the promoter region of *MIR155HG* the mutated region in MYB binding site was subcloned into pGL4.17 plasmid. The mutated region originated from the region of MYB binding site (E-box1) localized at -397nt to – 335nt from TSS. It was deletion of 5nt CCACC in forward direction and deletion of 5nt GGTGG in reverse direction (Figure 3.3). The MYB “wt” and MYB “mutated” plasmids were transiently transfected into HeLa cells by transfection reagent jetPEI (see also section transfection). The HeLa cells were harvested 48hrs post transfection. Following the manufacturer`s instruction the luciferase assay was performed.

The luciferase activity was measured using Dual Luciferase Assay on Sirius luminometer with the collection time 10 seconds. The values of luciferase activity were normalized to the protein content relative to the signal of transfected cells with the empty reporter vector pGL4.17.

Scheme of DNA sequence of *MIR155HG* with CpG islands and putative MYB binding sites

- CpG -33 to 349bp (relative to TSS)
- MYB binding motif (AACT/G) -399 to -394bp (E-box 1)
209 to 215bp (E-box 2)
1201 to 1207bp (E-box 3)
- Primers for ChIP and sequencing

2401	CTTTCCATCC	CCCAAAATCA	AATGGCTGCT	CCAGGAAAAA	GTTCCCCTTG	TGGCAGGGTC	CGGGGAAAAA	GAGAGAAGCG	ACAAAACCAA	AAATTA AAC
	GAAAGGTAGG	GGGTTTTAGT	TTACCGACGA	GGTCCCTTTA	CAAGGGGAAC	ACCGTCCCAG	GCCCTCTTTT	CTCTCTTCGC	TGTTTTGGTT	TTAATTTTG
2501	GACCGAAGTC	CCATATGCTC	CAGGAATATG	TCCTGGAGAT	GGGAGTGGAG	GGCAGGGGGA	GAAATGTTGT	GAGGTCAAAA	TTTTTGAAAT	TTAAGTCCT
	CTGGCTTCAG	GGTATACGAG	GTCCTTATAC	AGGACCTCTA	CCCTCACCTC	CCGTCCCCTC	CTTACAACAA	CTCCAGTTTT	AAAAACTTCA	AAATTCAGGA
2601	ATATCTTGAC	ATCCCGAGTA	TAAATGCGGG	TACCAGACAC	AGTACAAAAC	TTCTCAAAGC	CCAGTTACGT	ATTCCAAAAC	AAACGCGGGC	TCTTGAAGGG
	TATAGAACTG	TAGGGGCTCAT	ATTACGCCC	ATGGTCTGTG	TCATGTTTGC	AAGAGTTTCG	SGTCAATGCA	TAAGGTTTGG	TTTGGCCCGG	AGAACTTCCC
2701	TGATGAGGTA	GGGATGAAAT	CCCTCTTCTC	CTGAAGACCA	TTTCTTCTCT	TCTTAGGGAC	CTGCTGGTCT	CCAGCTGATT	CGGTCCAGGA	GGAAAAACCT
	ACTACTCCAT	CCCTACTTTA	GGCTCTCTCT	GACTTCTGGT	AAAGAAGGAG	AGAATCCCTG	GACGACCAGA	GGTCGACTAA	GCCAGGTCTC	CCTTTTTTGG
2801	CCCACTTGCT	CCTCTCGGGC	TCCTGCAAG	GAGAGAGTAG	AGACACTCCT	GCCACTCAGT	TGCAAGAAGT	CGCCACTTCC	CCCTCCAGCC	GACTGAAAAT
	GGTGAAACGA	GGAGAGCCCG	AGGGACGTTT	CTCTCTCATC	TCTGTGAGGA	CGGTGCTCA	ACGTTCTTCA	GCGGTGAAGG	GGGAGTCCGG	CTGACTTTCA
2901	TCGGGCGAGC	TCTGGCCGCT	CATTTGAAGG	CGTTTCCCTT	TCTTTAAGAA	CAAAGTGTGG	AGCCCAAGCC	TTGCGGCGCG	GTGCAGGAAA	GTACACGGCG
	AGCCCGCTGC	AGACCCGGCA	GTAACCTTCC	GCAAAAGAAA	AGAAATTCCT	GTTTCCAACC	TCGGGTTCCG	AACGCCGCGC	CACGTCCTTT	CATGTGCCCG
3001	TGTGTTGAGA	GAAAAAAAT	ACACACACGC	AATGACCCAC	GAGAAAAGGA	AAGGGGAAAA	CACCAACTAC	CCGGGCGCTG	GGCTTTTTTC	ACTTTTCTTT
	ACACAACCTCT	CTTTTTTTTA	TGTGTGTGCG	TTACTGGGTG	CTCTTTCCCT	TTCCCTTTTT	GTGGTTGATG	GGCCCGCGAC	CCGAAAAAGC	TGAAAAGGAA
3101	TAAAAAGAAA	AAAGTTTTTC	AAGCTGTAGG	TTCCAAGAAC	AGGCAGGAGG	GGGAGAAAG	GGGGGGGGGT	TGCAGAAAAG	GCGCCTGGTC	GGTTATGAGT
	ATTTTTCTTT	TTTCAAAAAG	TTTGACATCC	AAGGTTCTTG	TCCGTCCTCC	CCCCTCTTCC	CCCCCCCCCA	AGTCTTTTTC	CGCGGACCAG	CCAATACTCA
3201	CACAAGTGAG	TATATAAAGG	GTGGCACGTT	CGCAGGCGCG	GGCTTCTGT	GCGCGTCA	CGGGGGGG	AGGCGGCTT	GGAGCTTGG	GAAGGAGGCG
	GTGTTCACTC	AATATTTTCC	CAGCGTGCAA	GCGTCCGCGC	CCGAAGGACA	GCGCGTCT	CGGGGGGG	AGGCGGCTT	GGAGCTTGG	GAAGGAGGCG
3301	CTTTCCCTCT	CTTGAACGTC	GCAGGGACGC	CGGGGGACTT	CGGTGCGAGG	GTCACCCCGC	GGTTAACTGG	CGAGGCAAGG	CGGGGGCAGC	GCGCACGTGG
	GGAAAAGGAGA	GAACCTGCAC	CGTCCCTGCG	GCCCCCTGAA	GCCACGCTCC	CAGTGGGGGC	CCAAATTGAC	GCTCCGTTCC	GCCCCGCTGG	GCGGTGCACC
3501	CCGTGGAGCC	CGCCCTGGTC	CGCGCGCGCG	CTGCGGGTGC	CCCCTGGGGA	CTCAGTGGTG	TGCCTCGCC	CGGGACCAGA	GATTGCGCTG	GATGGATTCC
	GGCACCTCGG	GCCGGACCAG	GGCGCGCGCG	GACGCCACG	GGGGACCCCT	GAGTACCAC	AGCGGAGCGG	GCCCTGGTCT	CTAACCGGAC	CTACCTAAGG
3601	CGCGGGCAGA	GGCAGGGGGA	AGGAGGGGTG	TTCGAAAACCT	AATACTTGG	CTTCTTTGCA	AAGTTTCTTT	GGATGGTTGG	GGACGTACCT	GTATAATGCC
	GCGCCCGTCT	CCGTCCCCCT	TCCTCCCCAC	AAGCTTTGGA	TTATGAACCT	GAAGAAAACG	TTCAAAGGAA	CCTACCAACC	CCTGCATGGA	CATATTACCG
3701	CCTGGACCAG	CTTCCCTGTT	GGAGTGGCCA	GAGAAGTGTG	TAAAACACAC	TAGAGGGGCA	GGGTGAAAAA	AGAGACTGCC	TTCAAAAACCT	GTATCTTTTC
	GGACCTGGTC	GAAGGGACAA	CCTCACCCGT	CTCTTACAC	ATTTTGTGTG	ATCTCCCGGT	CCCACCTTTT	TCTCTGACGG	AAGTTTTGAA	CATAGAAAAG
3801	GATTTCAATTT	TGAAAAATAA	CTACAAATCT	ATTTTAATTT	TACAAAAGTTA	GACTCATAGC	ATTTTAGATA	TCAAATGCTT	CATTTAACAG	AAGTGAAGAT
	CTAAAATAAA	ACTTTTTTATT	GATGTTTAGA	TAAAATTTAA	ATGTTTCAAT	CTGAGTATCG	TAAAATCTAT	AGTTACAGAA	GTAATTGTGC	TTCACCTCTA
3901	GGAGCAAACG	CTCAATCAGC	GCTGTATTTT	ATTCGCTCCT	GTTGTGCCAG	GGTGCCTTTT	TGCCGAGCGG	TTGCCTTTCT	TTACTACAAA	AACCCCTTGG

(E-box 3)

Primers F, R (+1.6kb)

4301	GGACATTATG	TGTATATTCC	AAAGCCAAAA	TTACTGCTTG	TAAATCCAT	TCATCTCTCC	GAAGACGTAG	TGCCTCTTTT	ATATGGCTTT	AACCTAATGT
	CCTGTAATAC	ACATAAAGG	TTTCGGTTTT	AATGACGAAC	ATTATAGGTA	AGTAGAGAGG	CTTCTGCATC	ACGGAGAAAA	TATACCGAAA	TTGGATTACA
4401	ATAATCGAAG	TGAATTAGGG	CAGCCAGACT	TTTAATCACA	GGACTGATCT	TTTAAATCCG	GGTATATGTA	AAGTTAATTG	CTTATCTATG	TGAACAGTAA
	TATTAGCTTC	ACTTAATCCC	GTCGGTCTGA	AAATTAGTGT	CCTGACTAGA	AAATTTTTGC	CAATATACAT	TTC AATTAAC	GAATAGATAC	ACTTGTTCATT
4501	TATACACTAT	AAAAAGTTTT	CAAGGCAACT	TCCTGCCTGT	GGTTAATTT	GATAGTCACA	ATGATAGGAA	ATTGGCAGGG	TTAGGTGGTG	GTATCTACAT
	ATATGTGATA	TTTTTCAAAA	GTTCCGTTGA	AGGACGGACA	CCAAATTTAA	CTATCAGTGT	TACTATCCTT	TAACCGTCCC	AATCCACCAC	CATAGATGTA
4601	TTTGCCCATG	AGGACTTGTG	GCTCAGAGAA	TTTGAGTTTT	TTCTAGTAAG	GTCACTAGGT	CCCTAGTGCA	CAGCCTGCTT	TTAGGTACGG	ATGTGGGATG
	AAACGGGTAC	TCCTGAACAC	CGAGTCTCTT	AAACTCAAAA	AAGATCATTC	CAGTGATCCA	GGGATCACGT	GTCGGACGAA	AATCCATGCC	TACACCCTAC
4701	GGGGTGTCCG	CAGACCACAG	GGTTTTGACT	CTCAGTTTGG	GCTCATTTC	ATGACTCCTT	GCTGCCCTCC	TGATTTCTGT	CAAAATGACCA	AAGCACATTT
	CCCCACAGCC	GTCTGGTGTG	CCAAAACCTGA	GAGTCAAACC	CGAGTAAAGG	TACTGAGGAA	CGACGGAGGG	ACTAAAGACA	GTTTACTGGT	TTCGTGTA
4801	AGTAATTACT	TCTGCTGCAA	GGACTACTCC	TTATTAGGCT	GTGATAAGTA	AGTTTCCTCA	CATAGTGGGT	CAGCTCACTC	TGGCCACAGG	ACCCAGCTTC
	TCATTAATGA	AGACGACGTT	CCTGATGAGG	AATAATCCGA	CACTATTCAT	TCAAAGGACT	GTATCACCCA	GTCGAGTGAG	ACCGGTGTCC	TGGGTGGAAG
4901	CTAACCCAC	ACATTAAGAA	AGAGAAAAAT	TAGCACTGTC	TGAGACCTAC	AACCACTTCA	AGGGAGAACA	GTGGTGTITG	CACAAATGTC	TACTTTTGT
	GATTGGTGTG	TGTAATTCTT	TCTCTTTTAA	ATCGTGACAG	ACTCTGGATG	TTGGTGAAGT	TCCCTCTTGT	CACCACAAAC	GTGTTTACAG	ATGAAAACAA
5001	TTAACCTAGT	CATGTGTAAA	AAGTGTAAAT	CACTGGTCTT	TACCAAAAAA	AAAAAAAAAA	AAACAACAAC	AAAAAAAAAA	CAGGCATCAT	AGTCTGCCTG
	AATTGGATCA	GTACACATTT	TTCACATTA	GTGACCAGAA	ATGGTTTTTT	TTTTTTTTTT	TTTGTGTG	TTTTTTTTTT	GTCCGTAGTA	TCAGACGGAC

Figure 3.3: Scheme of DNA sequence of *MIR155HG* with CpG islands and putative MYB binding sites. The scheme shows the DNA sequence of *MIR155HG* with highlighted E-box sequences (blue) and putative MYB binding sites (E-box 1, 2, 3 (pink)), CpG islands (green color), TSS (red), primers for ChIP: -0.5kb, +0.1kb, +1.6kb from TSS (yellow), TATA box (-44bp; dark yellow). The DNA sequence of *MIR155HG* (NT_000021.8) was obtained from GenBank database and further worked out in the program Vector NTI suite 9.

Sequences of primers for subcloning of wt versus mutated MYB binding site into pGL4.17 plasmid:

(Oligonucleotides containing the putative MYB binding site (in bold and italic) and oligonucleotides with mutation/deletion of putative MYB binding site (E-box 1) are highlighted in gray color:

Primer 1F (wt) (in the region of E-box 1 (red box))

TCGAGTC GACTCCTG**CCACCCAGTTG**CAAGAAGTCGCCACTTCCCCCTCCAGCCGA

Primer 1R (wt) (in the region of E-box 1 (red box))

AGCTTCGGCTGGAGGGGGAAGTGGCGACTTCTTGC**AACTGGGTGG**CAGGAGTGTC GAC

Primer 2F (mut) (in the region of E-box 1 (red box)) with deletion of CCACC

TCGAGTC GACTCCTG**CAGTTG**CAAGAAGTCGCCACTTCCCCCTCCAGCCGA

Primer 2R (mut) (in the region of E-box 1 (red box)) with deletion of GGTGG

AGCTTCGGCTGGAGGGGGAAGTGGCGACTTCTTGC**AACTG**CAGGAGTGTC GAC

3.2.5 Western blot

Primary PBMC cells of B-CLL patient samples (N=5) and NB4, HeLa cells were lysed and sonicated. Denatured cell lysates were loaded on the Bis-Tris gel. The gels were dry-blotted by iBlot Gel Transfer System. Prior to overnight incubation with primary antibody the membranes were blocked by non-fat milk in PBS/Tween 20. The membrane was washed with PBS/Tween 20 and incubated with the secondary antibody HRP-conjugated. For visualization of bands the ECLPlus Western Blotting Detection System and X-ray films were used. As a loading control antibody the anti-Actin HRP-conjugated antibody was used. The optical density of protein bands was measured by Scion Image software.

3.2.6 Chromatin immunoprecipitation (ChIP)

Chromatin from 10×10^6 primary PBMCs cells (isolated from B-CLL patients samples, N=15 and normal healthy B-cells (N=8)) was cross-linked with 1% formaldehyde for 10 minutes at room temperature. Subsequently cells were lysed by a set of lysis buffers to isolate the nuclei from cells that were resuspended in 2ml of low-salt buffer and sonicated (45% intensity, 500 cycles of 2 seconds, in ice-ethanol cooling bath) with a Branson Sonic Dismembrator equipped with a microtip to yield 200bp to 400bp DNA fragments^{240,241}. ChIP was performed using the following antibodies: antibodies against H3K9Ac, H3K4Me3, MYB and isotypic normal rabbit IgG as a control nonspecific antibody. DNA extracted from the immunoprecipitates was used as template for SYBRGreen based qPCR reactions. Specific occupancy on DNA ("percentage of input") was defined as a copy number of a specific DNA fragment in each immunoprecipitate compared with the copy number of that DNA fragment within 1/100 input dilution used for immunoprecipitation (1% input DNA). The control antibody values were subtracted from the values obtained using the specific antibodies.

The quantitative PCR was performed on the 384 well plates in the real time PCR machine and samples were runned in duplicates. The single qPCR reaction composed of $8 \mu\text{L} = 1 \mu\text{L}$ of DNA + $0.4 \mu\text{L}$ of $20 \mu\text{M}$ primers (F+R) + $4 \mu\text{L}$ of SYBRgreen Master Mix + $2.6 \mu\text{L}$ of sterile ddH₂O.

The sequence of primers used for qPCR amplification of precipitated DNA were as follows:

putative MYB binding site 1 (E-box 1):

MIR155HG (-0.5kb) F: TCTCTTAGGGACCTGCTGGTCTCCAG

MIR155HG (-0.5kb) R: TGTCTCTACTCTCTCCTTG CAGGGAGC

putative MYB binding site 2(E-box 2):

MIR155HG (+0.1kb) F: ACCAAGGAGACGCTCCTGGCACTG

MIR155HG (+0.1kb) R: CGTCCCTGCCACGTTCAAGAGAGG

putative MYB binding site 3 (E-box 3):

MIR155HG (+1.6kb) F: TCACATAGTGGGTCAGCTCACTCTGG

MIR155HG (+1.6kb) R: GTTCTCCCTTGAAGTGGTTGTAGGTCTC

Program for SYBRgreen based qPCR:

50°C 2min, 95°C 10min, 95°C 15sec, 60°C 1min, 95°C 15sec, 60°C 15sec, 95°C 15sec for 40 cycles.

Presence of chromatin modifications of histones – “percentage of input” is defined as a copy number of specific DNA locus in each specific DNA immunoprecipitate normalized on the copy number of the same locus in the same immunoprecipitate sample in dilution 1:100 (1% of DNA input). From the obtained PCR values of “percentage of input” from the specific antibody, the “percentage of input” the control antibody was subtracted.

3.2.7 Isolation of mRNA/miRNA and preparation of cDNA

3.2.7.1 Isolation of mRNA/miRNA from PBMCs, B-cells, Raji cells by TRIzol reagent

Total RNA was extracted from all patients samples (N=239) and control samples (N=22) by TRIzol reagent followed by manufacturer`s protocol with modifications. The precipitation step with isopropanol at -20°C overnight was enhanced by adding linear acrylamide. On the next day, the RNA precipitate was centrifuged at 14000rpm for 30min at 4°C. RNA precipitate was washed with 75% Ethanol, dried on the air and finally resuspended in RNase free sterile water. The RNA quantity was determined by ND-1000 Spectrophotometer and quality was determined by Agilent 2100 Bioanalyzer. Isolated RNA was stored at -80°C or overwritten into cDNA and run RT-qPCR (see section cDNA and RT-qPCR).

3.2.7.2 Preparation of cDNA (complementary)

For cDNA preparation the TaqMan microRNA Reverse Transcription Kit was used. The manufacturer's instructions were followed. Single cDNA reaction with components of kit and RNA composed of 15 μ L (set up of single cDNA reaction is below) and reverse transcription reaction proceed at thermal cycler with program: 30min 16°C, 30min 42°C, 5min 85°C, after hold at 4°C. The 5x primers of specific microRNAs (RNU44, miR-155, miR-150) were as part of specific miRNA assay kit. After reverse transcription reaction complete, sterile H₂O was added to reach the final volume 50 μ L. The RT-qPCR was performed. The cDNAs were stored at -20°C.

<u>cDNA reaction:</u>	1x
10x RT buffer	1.5 μ L
25xdNTPs (100mM)	0.4 μ L
RNAse inhibitors	0.1 μ L
RT Random primers (10x)	0.5 μ L
5x primer (for specific miRNA)	1.5 μ L
Multiscribe Reverse Transcriptase (RT)	1.0 μ L
RNA (100ng/ μ L)	1.0 μ L
ddH ₂ O	9.0 μ L
	<hr/>
	15.0 μ L

3.2.7.3 Isolation of miRNAs from B-CLL patient plasma samples by miRNAeasy kit

MicroRNAs (miR-155, miR-24 and let-7a) were isolated from 10 B-CLL plasma samples by miRNA easy kit following the manufacturer's protocol. Briefly, plasma was lysed and homogenized in QIAzol lysis reagent (monophasic solution of phenol and guanidine thiocyanate). By adding chloroform and centrifugation the plasma lysate separated into aqueous and organic phase. The upper aqueous phase was extracted where the miRNA occurred. By adding ethanol the RNA bonded on the RNAeasy mini elute spin column. From the column was washed out phenol and other contaminants by washing buffers provided by kit. The separated miRNAs were eluted in RNAse free water.

3.2.7.4 Preparation of cDNA (complementary)

The miRNAs from plasma were reverse transcribed by using TaqMan microRNA cDNA Reverse Transcription kit with specific miRNA primers by following the manufacturer's instructions. Single cDNA reaction composed of 15µL and run at thermal cycler with following program: 30min 16°C, 30min 42°C, 5min 85°C, hold 4°C. After completing, the reverse transcription was proceeded in Real Time qPCR (RT-qPCR). The cDNAs were stored at -20°C.

<u>cDNA reaction:</u>	1x
10x RT buffer	1.5µL
25xdNTPs (100mM)	0.4µL
RNAse inhibitors	0.1µL
5x primer (for specific miRNA)	1.5µL
Multiscribe Reverse Transcriptase (RT)	1.0µL
miRNA	5.0µL
ddH ₂ O	5.5µL
	<hr/>
	15.0µL

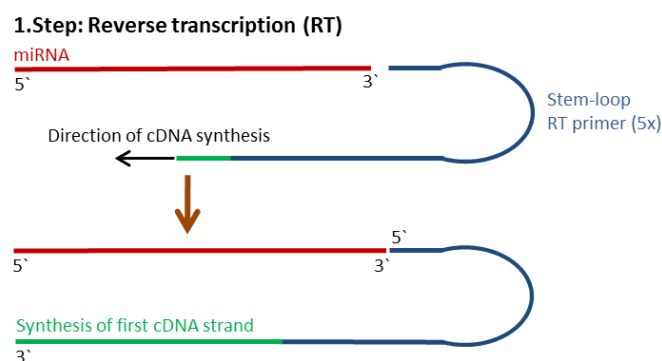
3.2.8 Performing of RT-qPCR by TaqMan system and with ROCHE probes

The expression of mRNAs/miRNAs was determined by TaqMan chemistry together with mRNA primers with specific probe and specific miRNA primers provided by microRNA assay kits.

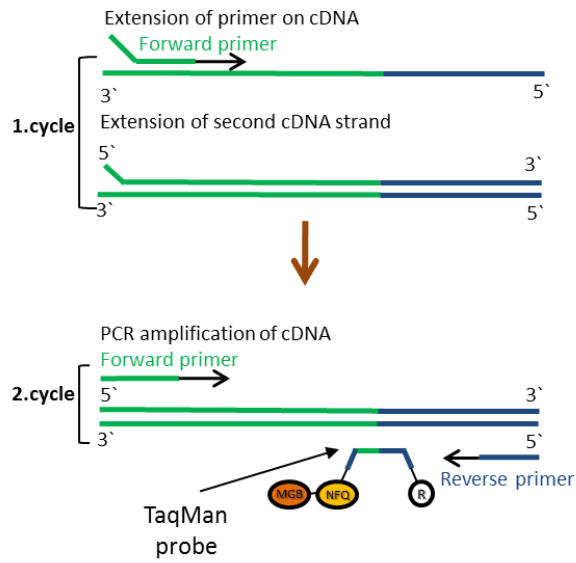
The TaqMan RT-qPCR system is based on fluorogenic-labeled probes that use the 5' nuclease activity of Taq DNA polymerase that detects specific PCR products.

The quantification by the miRNA assays pass over in the two-step RT-qPCR (Figure 4, step 1 and 2). During the reverse transcription (RT) the stem-loop specific miRNA primer (5x) gave rise to the first strand of cDNA (Figure 3.4, step 1). Later, during the qPCR are amplified the PCR products from cDNAs using the TaqMan specific miRNA assay primers (20x) together with the TaqMan Universal PCR Master Mix II that contains the TaqMan probe. TaqMan probes are dual labeled (FAM, NFG) and hydrolysis of probes increases the specificity of RT-qPCR. TaqMan probes contain a FAM dye label on the 5' end and the minor groove binder (MGB) that increases the melting temperature (T_m). The MGB is attached on

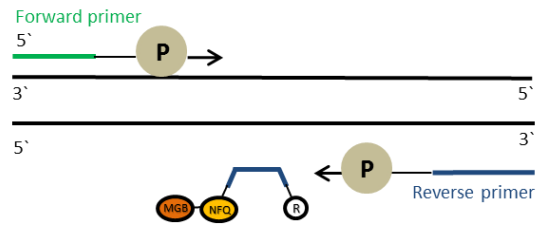
the non-fluorescent quencher (NFQ) localized on the 3' end that inhibits fluorescence when the probe is intact (Figure 3.4, step 3). In the presence of target sequence, the TaqMan probe anneals between the primer sites and the Taq DNA polymerase with 5' nuclease activity cleaves this boundary during the extension of strand. The reporter dye molecules are cleaved from their respective probes with each cycle (Figure 3.4, step 3). The FAM dye separates from NFQ and the reporter signal increases. The removing of the TaqMan probe from target strand allows primer extension and continuing of the polymerization of the template to the end (Figure 3.4, step 4). The increase of fluorescence intensity is equal to the amount of produced amplicons. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. The 5' nuclease assay process (Figure 3.4, steps 4 - 6) takes place during PCR amplification. This process occurs in every cycle and does not interfere with the exponential accumulation of product. During PCR, the TaqMan MGB probe anneals specifically to a complementary sequence between the forward and reverse primer sites (Figure 3.4, step 3). When the probe is intact (Figure 3.4, step 3 and 4), the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence. The Taq polymerase cleaves only probes that are hybridized to the target sequence (Figure 3.4, step 5). Cleavage separates the reporter dye from the quencher dye; the separation of the reporter dye from the quencher dye results in increased fluorescence by the reporter. The increase of fluorescence signal occurs only when the target sequence is complementary to the TaqMan probe and is amplified during qPCR. Because of these requirements, non-specific amplification is not detected. Polymerization of the strand continues, but because the 3' end of the probe is blocked, there is no extension of the probe during PCR (Figure 3.4, step 6).



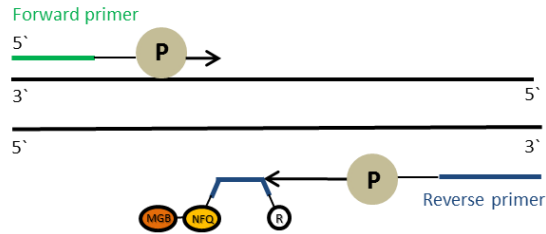
2.Step: qPCR







3.Step: Polymerization



4.Step: Strand displacement



-  Minor groove binder
-  Non-fluorescence quencher
-  Reporter
-  Taq polymerase

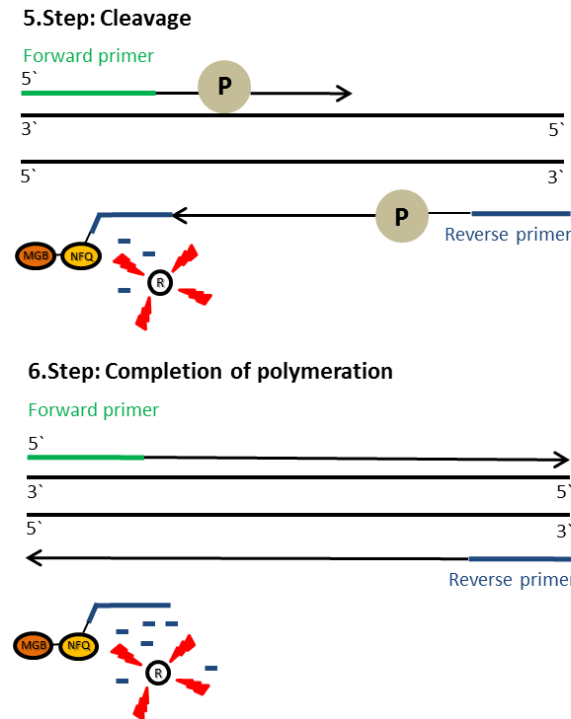


Figure 3.4: Scheme of the TaqMan system RT-qPCR. Scheme describes the preparation of cDNA and process of RT-qPCR. In the first step the miRNA specific primer gave rise to first strand of cDNA, the next steps describes the process of prolongation of strand during qPCR. During the polymerization the FAM and NFQ are attached to the TaqMan probe. During the each extension cycle the reporter dye FAM is cleaved from TaqMan probe. After separation of the FAM from NFQ the reporter dye emits fluorescence that is measured during qPCR. (adapted from http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_041461.pdf).

The single reaction RT-qPCR reaction was composed from cDNA master mix (MM) and primer master mix (miRNA primer MM or mRNA primer MM) with the final volume of 8µL. The detailed formula for the single master mix reactions are below:

<u>Single reaction – cDNA master mix:</u>	1x
TaqMan Universal PCR Master Mix II	2.0µL
ddH ₂ O	1.5µL
cDNA	0.5µL

	4.0µL
+	
<u>Single reaction – mRNA primer master mix:</u>	1x
TaqMan Universal PCR Master Mix II	2.0µL
Primer F+R (20µM)	0.4µL
Probe (10µM)	0.1µL
ddH ₂ O	1.5µL

	4.0µL

or

<u>Single reaction – miRNA primer master mix:</u>	1x
TaqMan Universal PCR Master Mix	2.0µL
miR primer (20x)	0.4µL
ddH ₂ O	1.6 µL

	4.0µL

The RT-qPCR reactions were applied on the 384 well plates and run on the qPCR instrument according to manufacturer's instructions. Program for cycling of the TaqMan based RT-qPCR reactions was as follows: 95 °C 15s and 60 °C 1'; 40 cycles.

The mRNA primers and specific probes were designed by software ProbeFinder Version 2.45 and Vector NTI Suite 9. The sequences of mRNA primers with specific ROCHE probes are shown in Table 3.3.

Table 3.3: Sequences of mRNA primers with specific ROCHE probes.

Primer name	Probe #	Probe sequence	Primer sequence
GAPDH F	60	CTTCCCCA	AGCCACATCGCTCAGACAC
GAPDH R	60	CTTCCCCA	GCCCAATACGACCAAATCC
PU.1 F	27	CAGGCAGC	CCACTGGAGGTGTCTGACG
PU.1 R	27	CAGGCAGC	CTGGTACAGGCGGATCTTCT
FOS F	67	TGCTGGAG	CTACCACTCACCCGCACT
FOS R	67	TGCTGGAG	AGGTCCGTGCAGAAGTCCT
MYB F	56	TGCTGTCC	TGCTCCTAATGTCAACCGAGA
MYB R	56	TGCTGTCC	AGCTGCATGTGTGGTTCTGT
MYC F	34	CTGCCTCT	CACCAGCAGCGACTCTGA
MYC R	34	CTGCCTCT	GATCCAGACTCTGACCTTTTGC

F=Forward; R=Reverse

3.2.8.1 RT-qPCR data analysis and statistics

For the RT-qPCR data analysis the delta-delta Ct method was used, where the Ct (cycle threshold) values of specific (s) were subtracted from Ct values of reference gene (r); calculated by $2^{\Delta\Delta(\text{Ctr}-\text{Cts})}$ equation. MicroRNA expression levels were normalized on the expression levels of reference gene - RNU44 (small nucleolar RNA, C/D box 44) and mRNA expression levels were normalized on the reference gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase). In case of plasma samples, the normalization was done on average (AVG) of miR-24 and let-7a Ct values. Expression data are presented as mean (two biological duplicates) and error bars indicate the standard error of the mean (SEM).

For statistical analysis of expression data from B-CLL patient samples (PBMCs, CD19+) and controls the Student's t test and Mann-Whitney test was used (GraphPad software) was used. Any difference for which the P value was <0.05 was regarded as statistically significant (*).

3.2.9 Microarray mRNA profiling

To perform the genome-wide microarray mRNA profiling by the Affymetrix HG-U133 Plus2 GeneChip we separated B-cells from B-CLL peripheral blood patient samples (N=16) and also from peripheral blood samples of healthy donors (N=5). We filtered out 4 B-CLL patient samples due to GeneSpring Quality Control Principal Component Analysis (PCA) analysis.

Briefly, total RNA was purified by TRIzol reagent (see section RNA isolation) followed by preparation of biotin-labeled cRNA (complementary) target population according to 3'-IVT Express Kit protocol. The cRNA probes were used for subsequent hybridization on the gene expression chip containing nearly 50 000 probes followed by fluorescent staining, fluidics processing and scanning according to the Affymetrix recommendations.

3.2.9.1 Microarray data analysis and statistics

High throughput data analyses were performed using GeneSpring GX10 and TIGR MeV4 softwares²⁴². GeneSpring software was used for probe level data preprocessing and summarization (using RMA), quality control analysis based primarily on Principal Component

Analysis (PCA) and partially for statistical analysis of microarray data. MeV4 software was used for data visualization, for creating the heat maps.

To determine the expression of **miR-155 targets** in B-CLL patients samples we used the 6 following target prediction tools (data are available on: <http://dnasuite.com/gsea/>):

PicTar:<http://pictar.mdc-berlin.de/>

Targetscan:<http://www.targetscan.org/>

Microrna:<http://www.microrna.org/>

Microcosm:<http://www.ebi.ac.uk/enright-srv/microcosm/>

TargetsDiana:<http://diana.cslab.ece.ntua.gr/tarbase/>

GSEAsset:<http://www.broadinstitute.org/gsea/msigdb/cards/AGCATTAMIR-155.html>

To determine pattern of expression of **MYB targets** we used 3 different lists of MYB target prediction tools (data are available on: <http://dnasuite.com/gsea/>):

Set MYB TARGETS GENEGO: <http://www.genego.com/>

Set MYB TARGETS RULAI.CSHL.EDU:<http://rulai.cshl.edu/cgi-bin/TRED/>

Set MYB TARGETS LITERATURE is based on the literature search.

To show that notable part of MYB and miR-155 downstream targets are differentially regulated we used GSEA (Gene Set Enrichment Analysis)²⁴³.

GSEA represents the analytical method for interpreting of gene expression data. This method focuses on the gene sets that share common biological function, chromosomal location or regulation pathway. It is a tool how to show significance of a gene list among whole genome expression data of all genes ordered by some significance measure (i.e. t-test p-value of a gene expression between two phenotypes or some other measure of differential regulation). Significant lists are those in which notable part of genes lies at the beginning or at the end of the whole genome ranked list. GSEA scores provided lists of genes by enrichment score (ES) and normalized enrichment score (NES). Primary measurement of the significance of a list is q-value (lower is better). Default GSEA setting marks as significant those list with q-value ≤ 0.25 . The complete GSEA results from our B-CLL patient samples and controls are available on: <http://www1.lf1.cuni.cz/~vkulv/gsea>.

The detailed protocols are in section 10. Supplement; Supplemental material 10.2.

4. RESULTS

4.1 Expression of candidate oncogenic microRNAs and their targets in B-CLL

4.1.1 Expression of miR-155, miR-150 and transcription factors PU.1, FOS, MYB and MYC in B-CLL patients and in normal healthy controls

By quantitative PCR and by microarray approach we measured the levels of both: mature form of microRNA-155 (miR-155) ($N_{CD19+/PBMC}=113$; $N_{CD19+}=156$) and its primary transcript generated from the *MIR155HG* (pri-miR-155) ($N_{B-cells}=11$; $N_{CD19+}=155$) (Figure 4.1 and 2).

Our data show significant ($P<0.001$, Student's t-test; Mann-Whitney test) increase of mature miR-155 in the circulating leukemic B-cells in comparison to normal B-cells (Figure 4.1A, B). Expression of miR-155 increased from 4-fold up to 32-fold in comparison to the normal CD19+ B-cells in both PBMCs (peripheral blood mononuclear cells) (graph A) and in separated CD19+ B-cells (graph B).

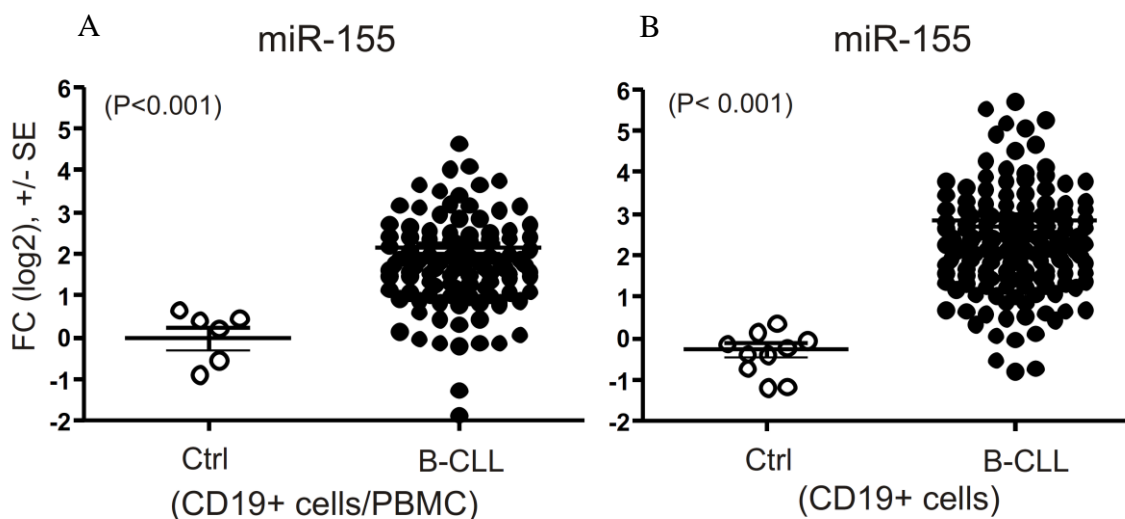


Figure 4.1: Expression of miR-155 in B-CLL patient samples. Results of TaqMan based RT-qPCR showed elevated levels of miR-155 in B-CLL patient samples [(A) $N_{CD19+/PBMC}=113$; (B) $N_{CD19+}=156$] in comparison with normal CD19+ B-cells [(A) $N_{ctrl\ CD19+}=6$; (B) $N_{ctrl\ CD19+}=10$]. The Y-axis represents relative abundance of mature miR-155 relative to reference gene RNU44. Data are shown as fold change (FC) in log2 scale and baseline normalized to the RNA levels of controls (control measurements were set equal to 1). Error bars represent standard error of the mean (SEM). Statistics was done by Student's t-test (A) or Mann-Whitney test (B). P values are shown in parentheses.

We detected 16-32 fold over-production of the pri-miR-155 transcript in B-CLL patient cells by RT-qPCR (Figure 4.2A) as well as using a microarray approach (Figure 4.2B) in comparison to normal CD19+ B-cells.

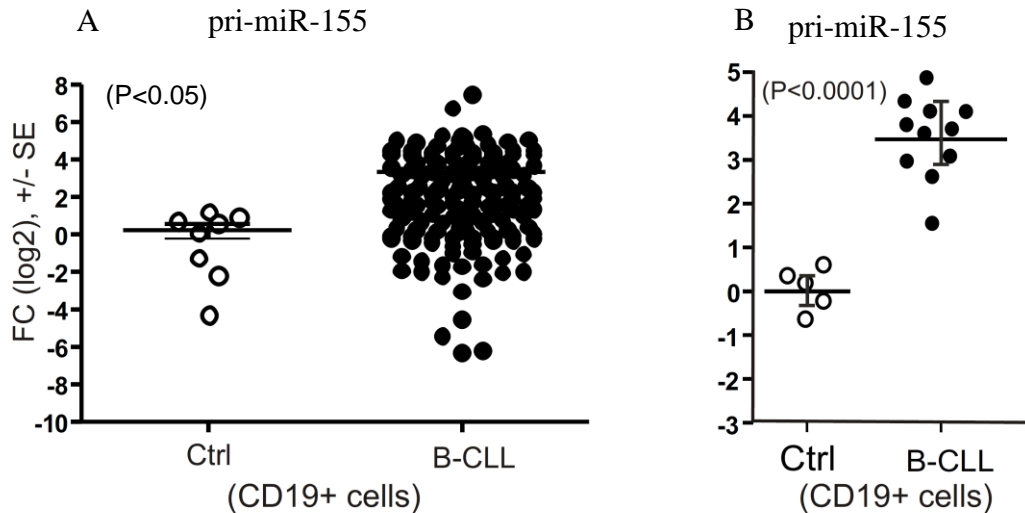


Figure 4.2: Elevated levels of primary transcript (pri-miR-155) a product of *MIR155HG* gene in B-CLL samples. (A) Results of TaqMan based RT-qPCR shown an elevated levels of pri-miR-155 in B-CLL patient samples ($N_{CD19+}=155$) as compared with normal CD19+ B-cells ($N_{CD19+}=8$). The Y-axis represents relative abundance of pri-miR-155 relative to GAPDH. Data are shown as fold change (FC) in log2 scale and normalized to the RNA levels of controls (control measurements were set equal to 1). Error bars represent standard error of the mean (SEM). (B) Results from microarrays shown an elevated levels of pri-miR-155 in B-CLL ($N_{CD19+}=11$) as compared with normal CD19+ B-cells ($N_{CD19+}=5$). Statistics was done by Student's t-test (A) or Mann-Whitney test (B). P values are shown in parentheses.

We measured in parallel the expression of mature miR-155 in the peripheral CD19+ B-cells (Figure 4.1) and also in the plasma samples (N=10) (Figure 4.3A). For the measurement of miR-155 level in plasma we selected the samples with the intermediate (~2-fold) and high (~7-fold) expression of mature miR-155 (obtained from measurements in the CD19+ cell population). Indeed, positive correlation between expression of miR-155 in CD19+ B-cells and in plasma ($r=0.5$) was observed (Figure 4.3B).

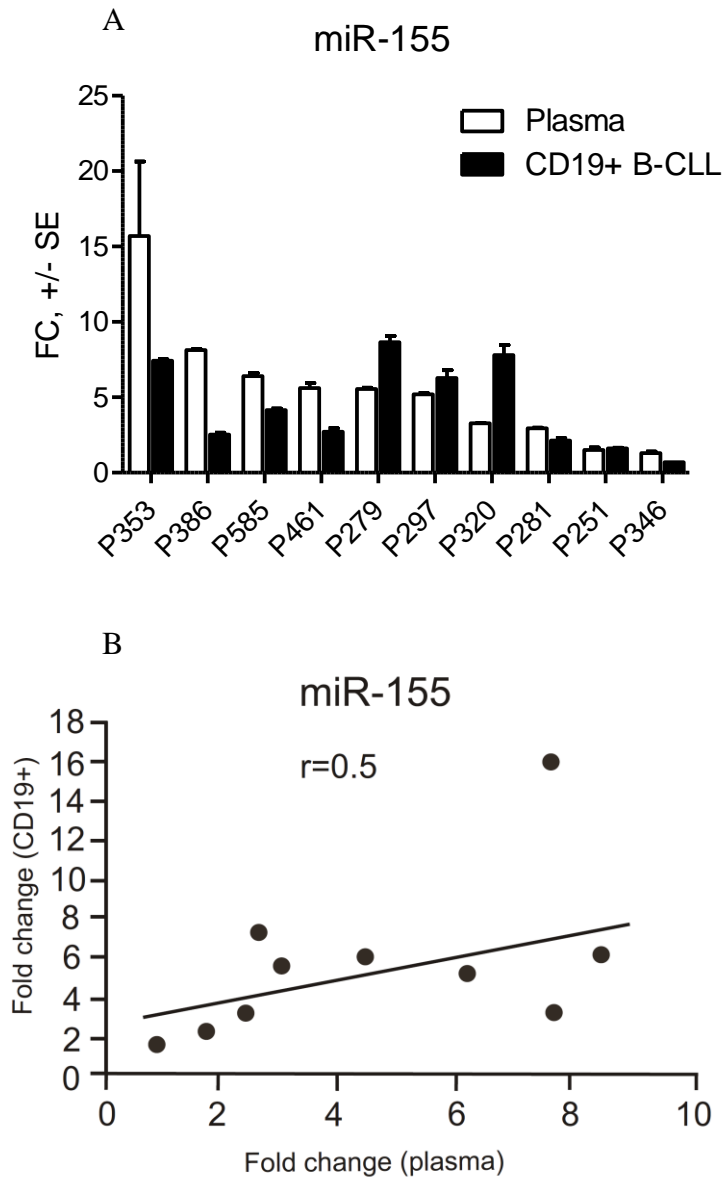


Figure 4.3: Expression of mature miR-155 in plasma samples (N=10) derived from B-CLL patients.

Graph A shows results of TaqMan based RT-qPCR of mature miR-155 measured in plasma samples (white box plots) and in separated CD19+ B-CLL samples (black box plots). Expression data are shown as fold change (FC) and in the case of B-CLL CD19+ cells normalized to the RNA levels of controls (control measurements were set equal to 1). Expression data of miR-155 in plasma were normalized on an average of RNA levels of let-7a and miR-24. Error bars represent standard error of the mean (SEM). Graph B shows correlation of miR-155 expression in plasma and separated CD19+ B-CLL cells. We analyzed plasma and peripheral blood samples of 10 B-CLL patients: P353, P386, P585, P461, P279, P297, P320, P281, P251 and P346.

The key hematopoietic transcription factor PU.1 is validated direct target of miR-155 that blocks both RNA stability as well as translation of PU.1¹⁵⁷. miR-155-directed inhibition of PU.1 expression may leads to leukemogenesis¹⁵⁶. We herein detected 4-32 fold downregulation of mRNA PU.1 in PBMCs (Figure 4.4A) and as well in the purified CD19+ B-CLL separated population (Figure 4.4B). Decreased amount of mRNA PU.1 coincided with decreased PU.1 protein levels (Figure 4.5). The established target of *PU.1* gene is early transcription gene *FOS* (FBJ murine osteosarcoma viral oncogene homolog). Indeed, the mRNA of *FOS* in B-CLL patient samples was significantly 4-10 fold decreased as shown in Figure 4.6.

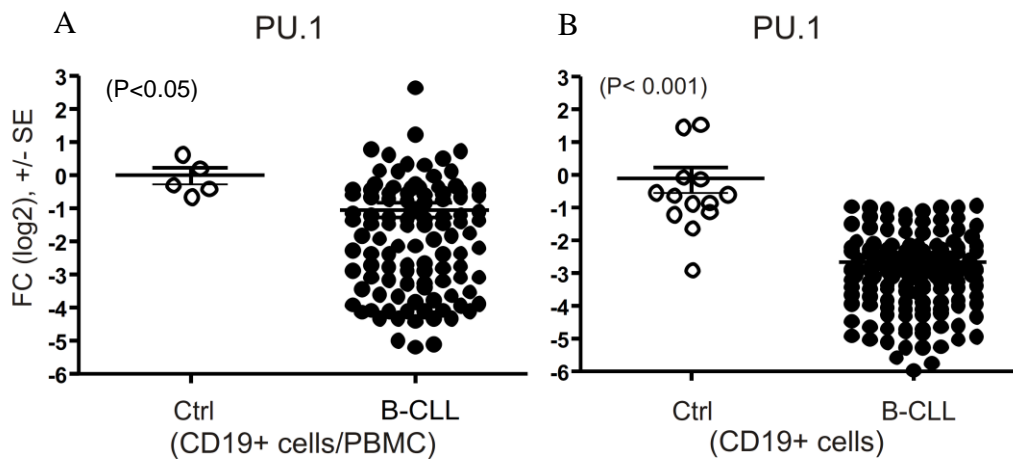


Figure 4.4: Expression of PU.1 in B-CLL patient B-cell samples.

Results of TaqMan based RT-qPCR show decrease in mRNA PU.1 in our B-CLL patient samples ($N_{CD19+/PBMC}=119$; $N_{CD19+}=157$) in comparison with the PU.1 levels in the control CD19+ B-cells ($N_{ctrl\ CD19+}=5$; $N_{ctrl\ CD19+}=13$). Y-axis represents relative abundance of PU.1 normalized to the levels of the reference gene - GAPDH. Data are shown as fold change (FC) in log₂ scale and normalized to mRNA levels of controls (control measurements were set equal to 1). Error bars represent standard error of the mean (SEM). Statistics was done by Student's t-test (A) or Mann-Whitney test (B). P values are shown in parentheses.

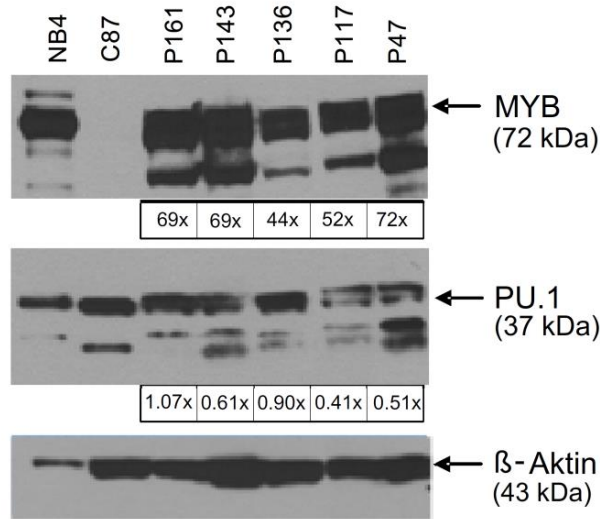


Figure 4.5: Protein levels of PU.1 and MYB in primary B-CLL cells.

Immunoblot analysis of MYB and PU.1 in B-CLL (P161, P143, P136, P117, P47) and control B-cells (C87). The NB4 cell line was used as a positive control. The optical density (below each lane) of each protein band was measured by Scion Image software Beta 4.0.2. The values below the blots represent a fold change of densities of the specific bands (or their expected positions) in the patient samples. Control lanes (NB4 and C87) were normalized to the levels of reference protein - β -actin.

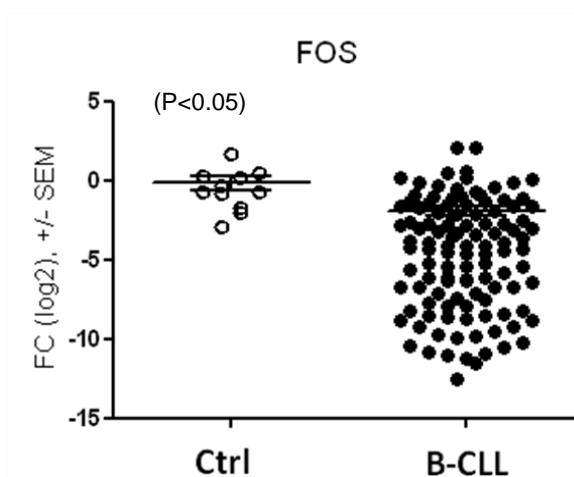


Figure 4.6: Expression of FOS in B-CLL patient B-cell samples. Results of TaqMan based RT-qPCR show decrease of mRNA FOS in separated CD19+ B-CLL cells (N=113) in comparison with the levels of FOS in normal CD19+ B-cells (N=11). The Y-axis represents relative abundance of FOS normalized to the levels of reference gene - GAPDH. Data are shown as fold change (FC) in log₂ scale and normalized to mRNA levels in controls (control measurements were set equal to 1). Error bars represent standard error of the mean (SEM). Statistics was done by Student's t-test. P values are shown in parentheses.

In addition to miR-155, miR-150 is likely related to the pathogenesis of B-CLL¹⁷. In addition, reviewers of our manuscript¹⁴⁴ asked to determine also miR-150 levels. Our data show, that around 30% of B-CLL samples have elevated miR-150 levels while similar proportion of patients have down- & un-regulated miR-150 levels in both peripheral blood B-CLL cells (Figure 4.7A) and CD19+ B-CLL cells (Figure 4.7B).

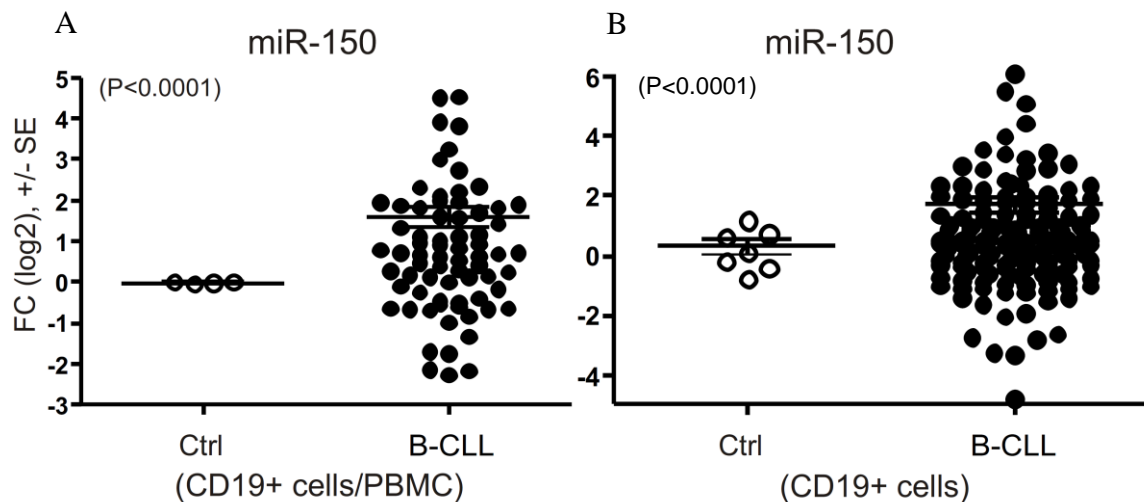


Figure 4.7: Expression of miR-150 in B-CLL patient B-cell samples.

Results of TaqMan based RT-qPCR showed expression of miR-155 in B-CLL patient samples ($N_{CD19+/PBMC}=71$; $N_{CD19+}=151$) and normal CD19+ B-cells ($N_{ctrl\ CD19+}=4$; $N_{ctrl\ CD19+}=7$). The Y-axis represents relative abundance of miR-150 relative to reference gene - RNU44. Data are shown as fold change and are normalized to RNA levels in controls (control measurements were set equal to 1) and shown in log2 scale. Error bars represent standard error of the mean (SEM). Statistics was done by Student's t-test (A) or Mann-Whitney test (B). P values are shown in parentheses.

Validated target of both miR-150 and miR-155 is a proto-oncogene and transcription factor *MYB* (*v-myb* myeloblastosis viral oncogene homolog) that is important for normal B-cells development^{72,73,227}. The mRNA levels of MYB showed wide interval of expression levels, starting from 2-32 fold in comparison to normal B-cells. In subset (~40%) of B-CLL patient samples we detected over-produced MYB mRNA and in 20% its down-production (Figure 4.8). The production of MYB protein was in some B-CLL patient samples ($N=5$) up to 72-fold higher as in the lysates from normal B-cells. MYB protein normally migrates at ~72 kDa (wt form) (Figure 4.5). Additionally, we determined the MYC mRNA levels – an another member of E-box family. In our B-CLL patient samples the MYC mRNA levels were above the levels of normal CD19+ B-cells (Figure 4.9).

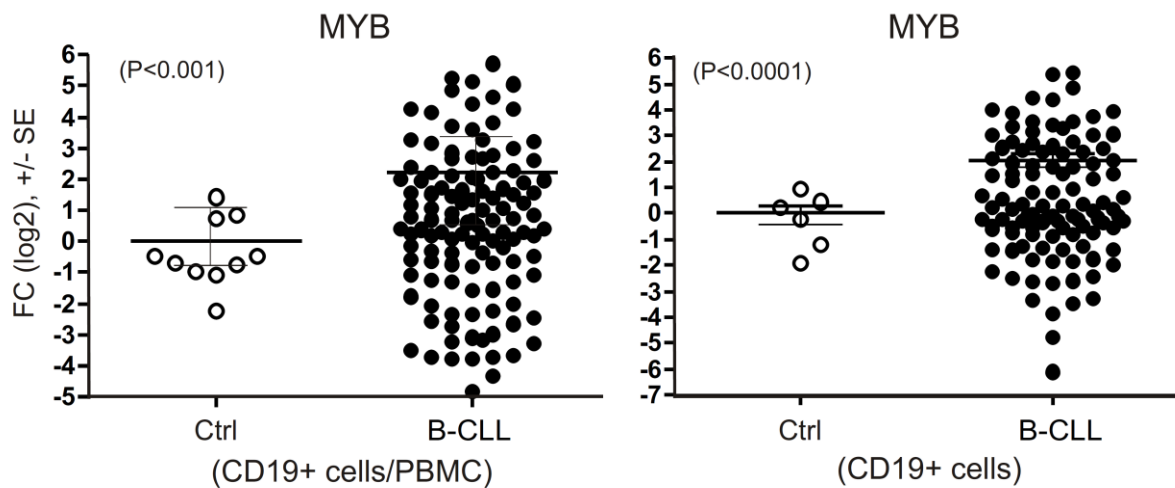


Figure 4.8: Expression of MYB in B-CLL patient B-cell samples.

Results of TaqMan based RT-qPCR showed expression of MYB in B-CLL patient samples ($N_{CD19+/PBMC}=109$; $N_{CD19+}=108$) and in normal CD19+ B-cells ($N_{ctrl\ CD19+}=10$; $N_{ctrl\ CD19+}=6$). The Y-axis represents relative abundance of MYB relative to reference gene - GAPDH. Data are shown as fold change (in log2 scale) and baseline normalized to the levels of MYB in controls (control measurements were set equal to 1). Error bars represent standard error of the mean (SEM). Statistics was done by Student's t-test (left graph) or Mann-Whitney test (right graph). P values are shown in parentheses.

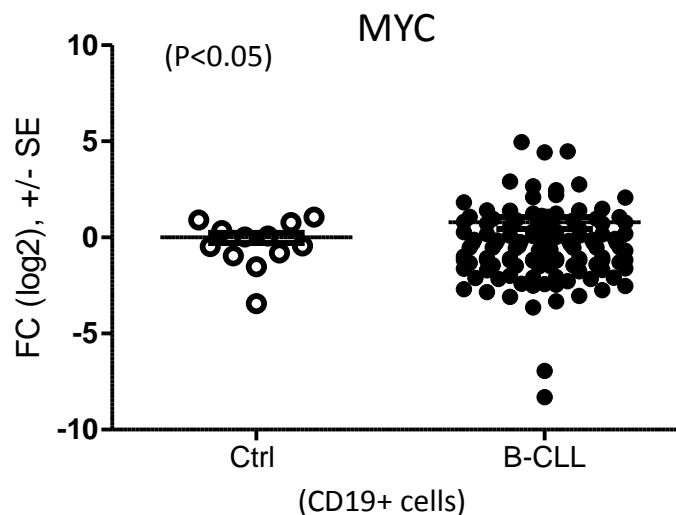


Figure 4.9: Expression of MYC in B-CLL patient B-cell samples.

Results of TaqMan based RT-qPCR shown decrease of MYC in separated CD19+ B-CLL cells ($N_{CD19+}=122$) and in normal CD19+ B-cells ($N_{ctrl\ CD19+}=12$). The Y-axis represents relative abundance of MYC relative to GAPDH. Data are shown as fold change (in log2 scale) and baseline normalized to the levels of MYB in controls (control measurements were set equal to 1). Error bars represent standard error of the mean (SEM). Statistics was done by Mann-Whitney test. P values are shown in parentheses.

4.1.2 Relationship between expressions of miR-155 – miR-150 - PU.1 – MYB in B-CLL patient samples

Based on our previous expression data (section 4.1.1) we asked if there exists functional relationship between expression patterns of oncomiR miR-155, miR-150, oncogenic transcription factor MYB and tumor suppressor transcription factor PU.1 in B-CLL patient samples (N=156). All possible pair combinations within this four molecules demonstrate figures 4.10-12. In the B-CLL patient samples with elevated miR-155 the PU.1 mRNA was decreased (Figure 4.10). Interestingly, expression of miR-155 and miR-150 showed very similar pattern and expression of MYB mRNA in ~30% of B-CLL patients samples overlapped with the elevated expression levels of miR-155 (Figure 4.10).

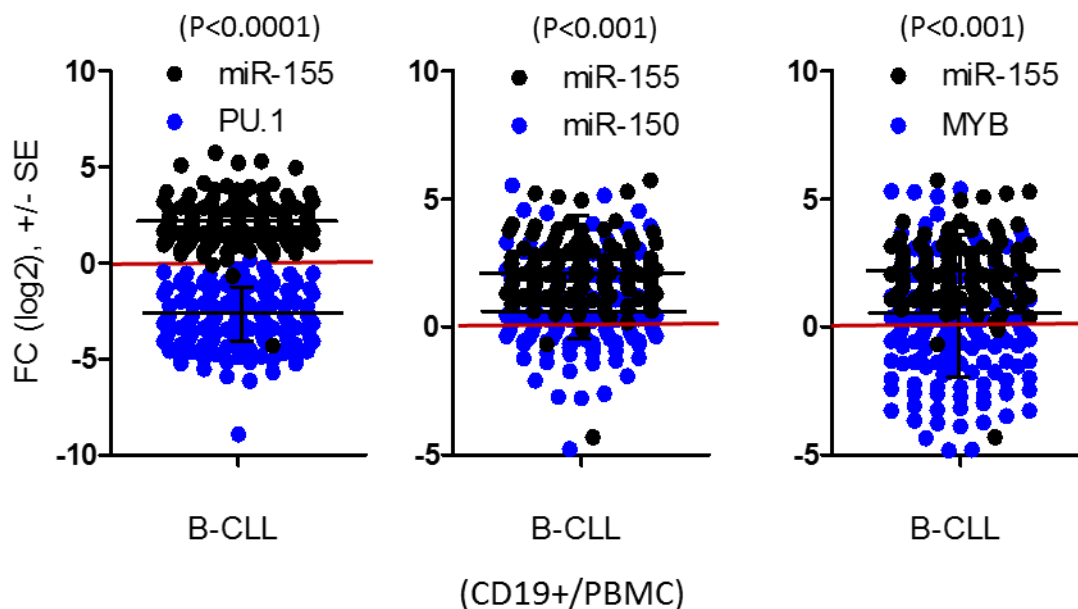


Figure 4.10: Expression of miR-155, PU.1, MYB and miR-150 in B-CLL patient B-cell samples.

Graphs show results of TaqMan based RT-qPCR of B-CLL ($N_{CD19+/PBMC}=156$) patient samples. The dataset contains data that expressed all four genes. The Y-axis represents expression of miR-155 - PU.1, miR-155 – miR-150 and miR-155 – MYB. The expression of miR-155 and miR-150 is relative to the reference gene RNU44 and of PU.1 and MYB relative to the reference gene GAPDH. Data are shown as fold change (in log2 scale) and normalized to the levels of controls (control measurements were set equal to 1 – represented by red line). Error bars represent standard error of the mean (SEM). P values (in parentheses) represent results of Student's t test.

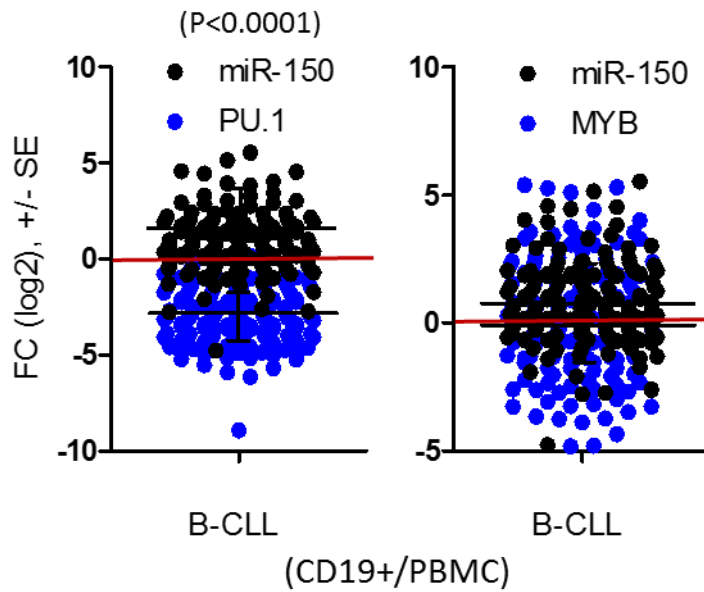


Figure 4.11: Expression of miR-155, miR-150, PU.1 and MYB in B-CLL patient B-cell samples.

Graphs show results of TaqMan based RT-qPCR of B-CLL ($N_{CD19+/PBMC}=156$) patient samples. The dataset contains data that expressed all four genes. The Y-axis shows expression of miR-155 - PU.1, miR-155 - miR-150 and miR-155 - MYB. The expression of miR-155 and miR-150 is relative to reference gene RNU44 and PU.1 and MYB relative to reference gene GAPDH. Data are shown as fold change (in log₂ scale) and normalized to the levels of controls (control measurements were set equal to 1 – red line). Error bars represent standard error of the mean (SEM). P values (in parentheses) represent results of Student's t test.

To see how differed the expression pattern of miR-150 and its target MYB in the same subset of B-CLL patient samples we showed both expression data in one graph (Figure 4.11). Interestingly, MYB and miR-150 showed similar expression pattern (statistically non-significant) and not reciprocal as it would be expected²²³. The expression of PU.1 mRNA was low as compared to expression of miR-150 (Figure 4.11) and MYB mRNA and indeed high levels of MYB showed reciprocal pattern to PU.1 (Figure 4.12).

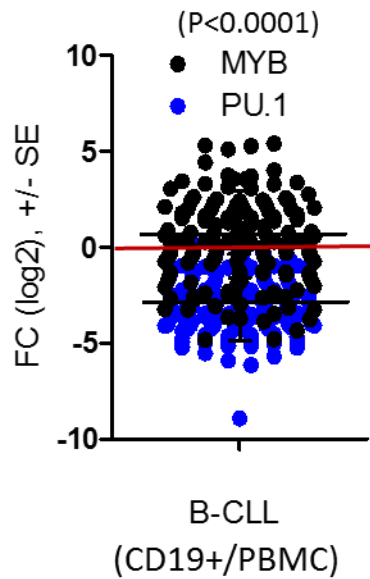


Figure 4.12: Expression of MYB and PU.1 in B-CLL patient B-cell samples.

Graph shows results of TaqMan based RT-qPCR in B-CLL ($N_{CD19+}=156$). The Y-axis represents expression of MYB, PU.1 relative to GAPDH. Data are shown as fold change (in log2 scale) and normalized to the levels of MYB in controls (control measurements were set equal to 1). Error bars represent standard error of the mean (SEM). Statistics was done by Student's t test. P values are shown in parentheses.

4.1.3 Clinical and prognostic importance of miR-155, miR-150, MYB and PU.1 expressions in B-CLL patient samples

The progression of B-CLL characterizes Rai stage ⁴⁴. Here, we showed analysis of expression of miR-155, PU.1, miR-150 and MYB between different Rai staging categories. We also showed the relationship between mRNA/miRNA expression and overall survival, therapy regimen in B-CLL patient samples (Figure 4.13 - 17). Our data show significantly higher expression of MYB mRNA and lower expression of miR-150 in advanced Rai stages II - IV stages while PU.1 and miR-155 appeared to be similar (Figure 4.13).

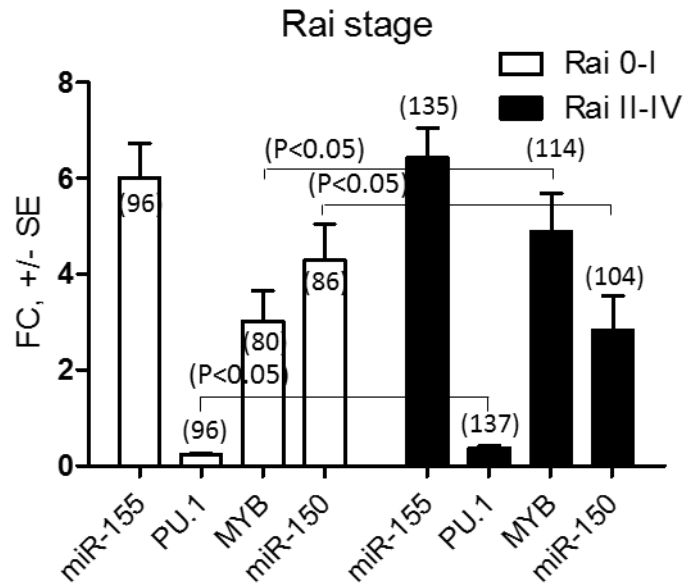


Figure 4.13: Expression of miR-155, PU.1, MYB and miR-150 in B-CLL patient samples based on Rai stage (PBMC/CD19+ B-CLL cells). Graph shows two patient subgroups divided by Rai stage: white bars – represent patients samples with Rai stage 0-I ($N_{\text{miR-155}}=96$, $N_{\text{PU.1}}=96$, $N_{\text{MYB}}=80$, $N_{\text{miR-150}}=86$) and black bars represent patients samples with Rai stage II-IV ($N_{\text{miR-155}}=135$, $N_{\text{PU.1}}=137$, $N_{\text{MYB}}=114$, $N_{\text{miR-150}}=104$). Expressions of miR-155, PU.1, MYB and miR-150 are shown as fold change, each bar represents average of expression. Error bars represent standard error of the mean. P values are shown in parentheses, Student's t test was used.

We compared expression of miR-155, PU.1, MYB and miR-150 with different Rai stages, therapy regimens and overall survival (from diagnosis to the last follow-up) in B-CLL patients. We divided patient samples primarily into two groups based on the Rai staging system – Rai stage 0-I and Rai stage II-IV and secondarily into three subgroups based the number of therapy regimens (1 or more than 1) and on the overall survival status (OS) (0 (live) or 1 (dead)). In the advanced Rai stages (II-IV) we detected an increased expression of MYB compared with Rai 0-I (Figure 4.14). We observed elevated levels of miR-155 in a patient group with advanced Rai stages (II-IV) that underwent at least one line of therapy and are still alive (Figure 4.15; grey bar). The PU.1 mRNA levels increased in the group of patients that underwent more therapy lines (Figure 4.16). The expression of miR-150 decreased in the advanced Rai stages (II-IV) and especially in the group of patients that underwent more therapy lines (Figure 4.17).

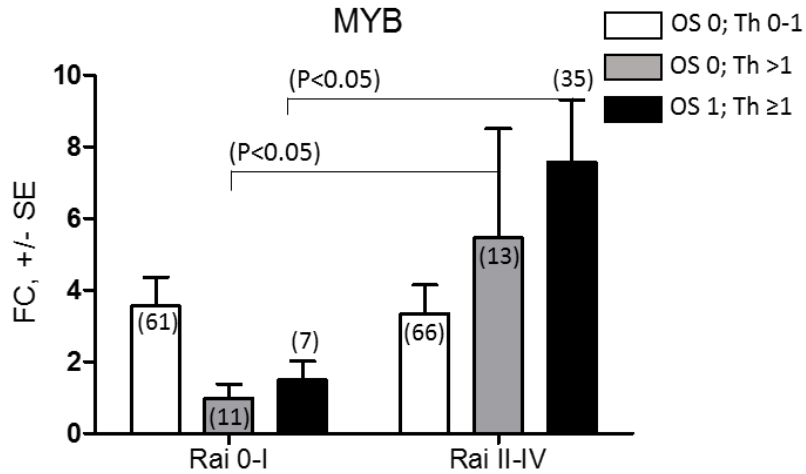


Figure 4.14: Expression of MYB based on the Rai stage, overall survival and therapy regimen in B-CLL patient B-cell samples (PBMC/CD19+ B-CLL cells). Graph shows expression of MYB based on the Rai stage. Three patient subgroups are shown: white bars: currently investigated patients (OS 0) either treated by first line of therapy or untreated (Th 0-1), $N_{\text{Rai 0-I}}=61$, $N_{\text{Rai II-IV}}=66$; grey bars: currently investigated patients (OS 0) who received second or more lines of therapy (Th>1), $N_{\text{Rai 0-I}}=11$, $N_{\text{Rai II-IV}}=13$; black bars: previously investigated patients that already died (OS 1) and received at least one line of therapy (Th \geq 1), $N_{\text{Rai 0-I}}=7$, $N_{\text{Rai II-IV}}=35$. Error bars represent standard error of the mean. P values are shown in parentheses, Student's t test was used.

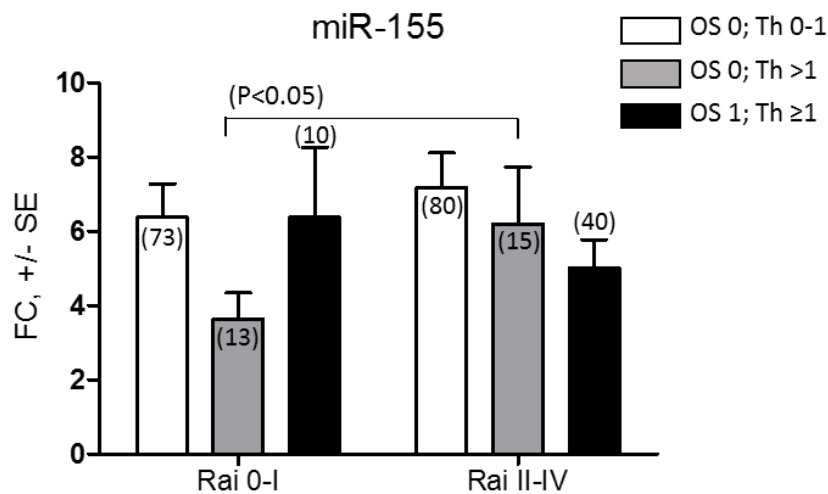


Figure 4.15: Expression of miR-155 based on the Rai stage, overall survival and therapy regimen in B-CLL patient B-cell samples (PBMC/CD19+ B-CLL cells). Graph shows expression of mR-155 based on the Rai stage. Three patient subgroups are shown: white bars: currently investigated patients (OS 0) either treated by first line of therapy or untreated (Th 0-1), $N_{\text{Rai 0-I}}=73$, $N_{\text{Rai II-IV}}=80$; grey bars: currently investigated patients (OS 0) who received second or more lines of therapy (Th>1), $N_{\text{Rai 0-I}}=13$, $N_{\text{Rai II-IV}}=15$; black bars: previously investigated patients that already died (OS 1) and received at least one line of therapy (Th \geq 1), $N_{\text{Rai 0-I}}=10$, $N_{\text{Rai II-IV}}=40$. Error bars represent standard error of the mean. P values are shown in parentheses, Student's t test was used.

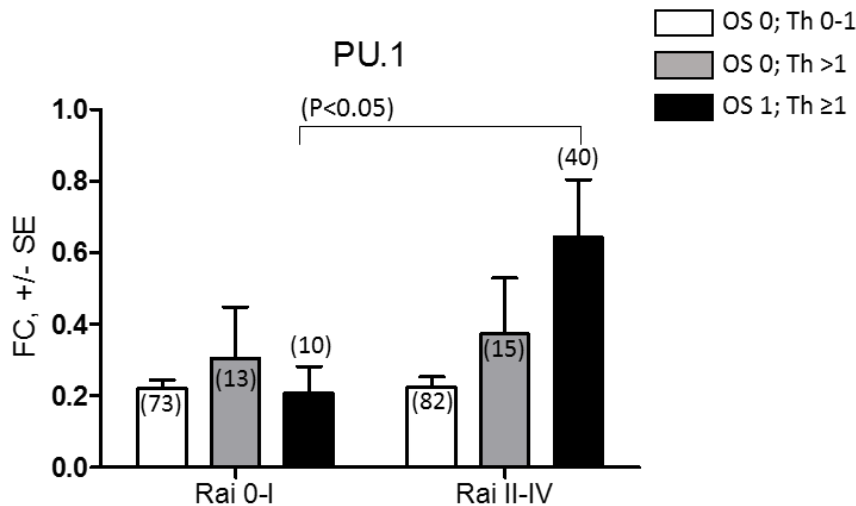


Figure 4.16: Expression of PU.1 based on the Rai stage, overall survival and therapy regimen in B-CLL patient B-cell samples (PBMC/CD19+ B-CLL cells). Graph shows expression of PU.1 based on the Rai stage. Three patient subgroups are shown: white bars: currently investigated patients (OS 0) either treated by first line of therapy or untreated (Th 0-1), $N_{\text{Rai 0-I}}=73$, $N_{\text{Rai II-IV}}=82$; grey bars: currently investigated patients (OS 0) who received second or more lines of therapy (Th>1), $N_{\text{Rai 0-I}}=13$, $N_{\text{Rai II-IV}}=15$; black bars: previously investigated patients that already died (OS 1) and received at least one line of therapy (Th \geq 1), $N_{\text{Rai 0-I}}=10$, $N_{\text{Rai II-IV}}=40$. Error bars represent standard error of the mean. P values are shown in parentheses, Student's t test was used.

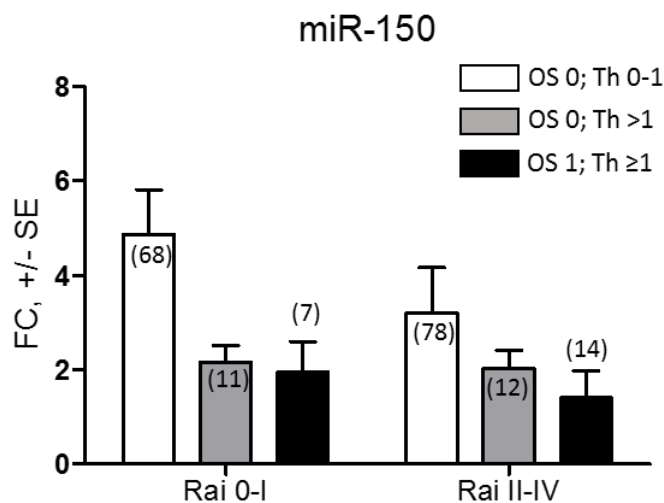


Figure 4.17: Expression of miR-150 based on the Rai stage, overall survival and therapy regimen in B-CLL patient B-cell samples (PBMC/CD19+ B-CLL cells). Graph shows expression of miR-150 based on the Rai stage. Three patient subgroups are shown: white bars: currently investigated patients (OS 0) either treated by first line of therapy or untreated (Th 0-1), $N_{\text{Rai 0-I}}=68$, $N_{\text{Rai II-IV}}=78$; grey bars: currently investigated patients (OS 0) who received second or more lines of therapy (Th>1), $N_{\text{Rai 0-I}}=11$, $N_{\text{Rai II-IV}}=12$; black bars: previously investigated patients that already died (OS 1) and received at least one line of therapy (Th \geq 1), $N_{\text{Rai 0-I}}=7$, $N_{\text{Rai II-IV}}=14$. Error bars represent standard error of the mean. P values are shown in parentheses, Student's t test was used.

Prognosis and stage of B-CLL is characterized by Rai staging and also by expression of prognostic markers: ZAP-70, CD38 and IgVH gene mutation status. We further analyzed the expression of miR-155, PU.1, MYB and miR-150 in the relationship with mRNA/miRNA expression with positivity of different prognostic markers (Figures 4.18-20). IgVH – unmutated IgVH versus IgVH mutated B-CLL cells expressed more miR-155 and MYB (not significantly), similar amounts of PU.1 and less miR-150 (Figure 4.18). As pointed in the Introduction, the presence of surface marker ZAP-70 on B-CLL cells categorizes patient into more advanced stage with unfavorable prognosis. We found that patient overexpressing ZAP-70 also expressed more MYB mRNA, less miR-150, while miR-155 and PU.1 expression remained unchanged (Figure 4.19). Presence of another B-CLL prognostic marker, CD38 categorizes patient into more advanced stage with unfavorable prognosis. B-CLL cells expressing the CD38 expressed more miR-155 and mRNA of MYB (significantly), less miR-150, PU.1 remained unchanged (Figure 4.20).

Additionally we divided the patient samples by clinical outcome into two patient groups: 1) favorable (ZAP-70-/CD38-/IgVH mutated) and 2) unfavorable (ZAP-70+/CD38+/IgVH unmutated). This creates highly specifically and robustly two very distinct groups whose prognosis is different and that is not influenced by false positive or false negative outcomes from prognostic determinations. Hence, the expression of miR-155 in group of patients with unfavorable clinical outcome was significantly higher than in group with favorable clinical outcome (Figure 4.21). Furthermore, the expression of PU.1 mRNA was overall decreased in both clinical groups without significant change. Expression of MYB mRNA in unfavorable group of patients was significantly increased (~3-fold). On the other hand, expression of miR-150 decreased (~2-fold) with disease progression (Figure 4.21). Our data points to the relationship between expression of miR-155, PU.1, MYB and miR-150 during the progression of B-CLL. This notion is supported by our data indicating that the studied molecules are likely involved during disease progression and therapy requirement (unpublished, Manuscript *in preparation*).

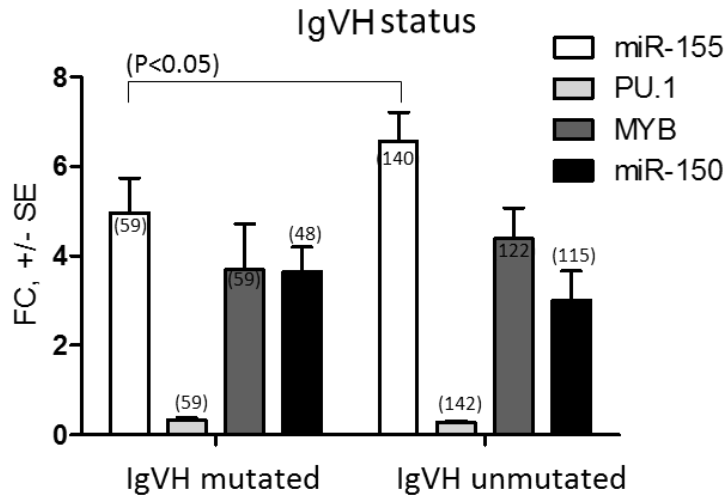


Figure 4.18: Expression of miR-155, PU.1, MYB and miR-150 based on IgVH status of B-CLL (PBMC/CD19+ B-CLL cells). Graph shows expression (shown in fold change) of miR-155, PU.1, MYB and miR-150. B-CLL patient samples were divided into two groups based on the IgVH status: 1, IgVH mutated ($N_{miR-155}=59$, $N_{PU.1}=59$, $N_{MYB}=59$, $N_{miR-150}=48$) and 2, IgVH unmutated ($N_{miR-155}=140$, $N_{PU.1}=142$, $N_{MYB}=122$, $N_{miR-150}=115$). Error bars represent standard error of the mean. Number of patients in each group is shown in parentheses above/or in the each bar respectively. P values are shown in parentheses, Student's t test was used.

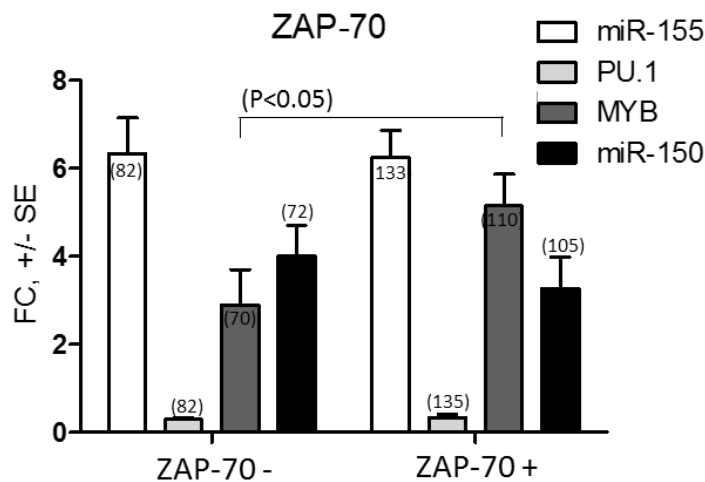


Figure 4.19: Expression of miR-155, PU.1, MYB and miR-150 based on presence of surface marker ZAP-70 in B-CLL patient samples (PBMC/CD19+ B-CLL cells). Graph shows expression (shown in fold change) of miR-155, PU.1, MYB and miR-150. B-CLL patient samples were divided into two groups based on presence of ZAP-70 (sample was assessed as ZAP-70 positive when >20% of cells expressed ZAP-70): 1, ZAP-70 negative ($N_{miR-155}=82$, $N_{PU.1}=82$, $N_{MYB}=70$, $N_{miR-150}=72$) and 2, ZAP-70 positive ($N_{miR-155}=133$, $N_{PU.1}=135$, $N_{MYB}=110$, $N_{miR-150}=105$). Error bars represent standard error of the mean. Number of patients in each group is shown in parentheses above/or in the each bar respectively. P values are shown in parentheses, Student's t test was used.

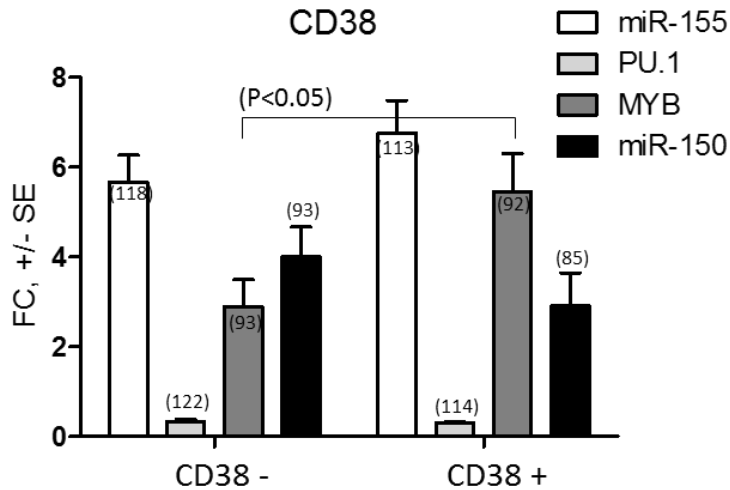


Figure 4.20: Expression of miR-155, PU.1, MYB and miR-150 based on presence of surface marker CD38 in B-CLL patient samples (PBMC/CD19+ B-CLL cells). Graph shows expression (shown in fold change) of miR-155, PU.1, MYB and miR-150. B-CLL patient samples were divided into two groups based on presence of CD38 (sample was assessed as CD38 positive when >30% of cells expressed CD38): 1, CD38 negative ($N_{\text{miR-155}}=118$, $N_{\text{PU.1}}=122$, $N_{\text{MYB}}=93$, $N_{\text{miR-150}}=93$) and 2, CD38 positive ($N_{\text{miR-155}}=113$, $N_{\text{PU.1}}=114$, $N_{\text{MYB}}=92$, $N_{\text{miR-150}}=85$). Error bars represent standard error of the mean. Number of patients in each group is shown in parentheses above/or in the each bar respectively. P values are shown in parentheses, Student's t test was used.

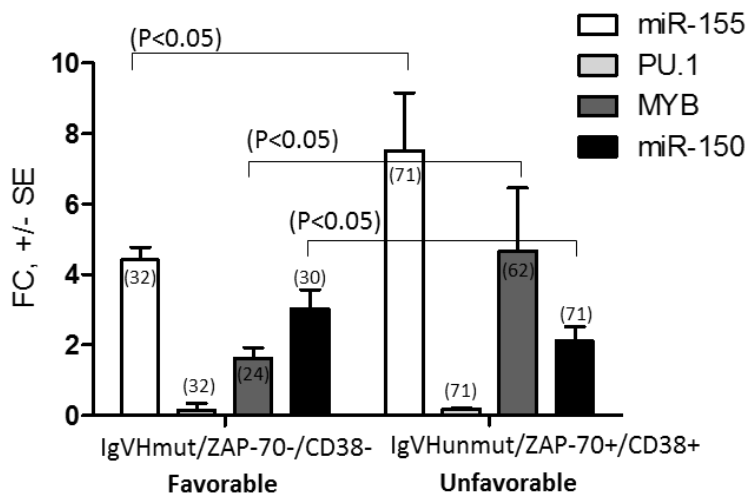


Figure 4.21: Expression of miR-155, PU.1, MYB and PU.1 based on prognostic markers (PBMC/CD19+ B-CLL cells). Graph shows expression (shown in fold change) of miR-155, PU.1, MYB and miR-150. B-CLL patient samples were divided into two groups based on the clinical outcome: 1) favorable clinical outcome = IgVH mutated/CD38-/ZAP-70- ($N_{\text{miR-155}}=32$, $N_{\text{PU.1}}=32$, $N_{\text{MYB}}=24$, $N_{\text{miR-150}}=30$) and 2) unfavorable clinical outcome = IgVH unmutated/CD38+/ZAP-70+ ($N_{\text{miR-155}}=71$, $N_{\text{PU.1}}=71$, $N_{\text{MYB}}=62$, $N_{\text{miR-150}}=71$). Error bars represent standard error of the mean. Number of patients in each group is in parentheses above/or in the each bar respectively. P values are shown in parentheses, Student's t test was used.

Besides Rai stage and expression of prognostic surface markers, the progression of B-CLL is additionally evaluated by chromosomal aberrations. Among such aberrations belongs deletions of chromosome 13q14, 11q22-23, 17p13 and trisomy of chromosome 12 (see Introduction section). The most frequent aberration of B-CLL cells is deletion of chromosome 13q14 that is also described as a low risk factor of B-CLL. The intermediate risk represents trisomy of chromosome 12 while high risk are deletions of 11q22-23 and 17p13¹³. We divided our B-CLL patient samples into 5 groups based on the chromosomal aberrations (Figure 4.22-25).

Based on cytogenetics we observed that, expression of miR-155 in the patient samples with deletion of 13q14 or 11q22-23 was interestingly higher as compared to B-CLL patient samples with negative FISH analysis (Figure 4.22). In the B-CLL patient samples with trisomy of chromosome 12 and with the deletion of 17p13 the expression of miR-155 was unchanged (Figure 4.22).

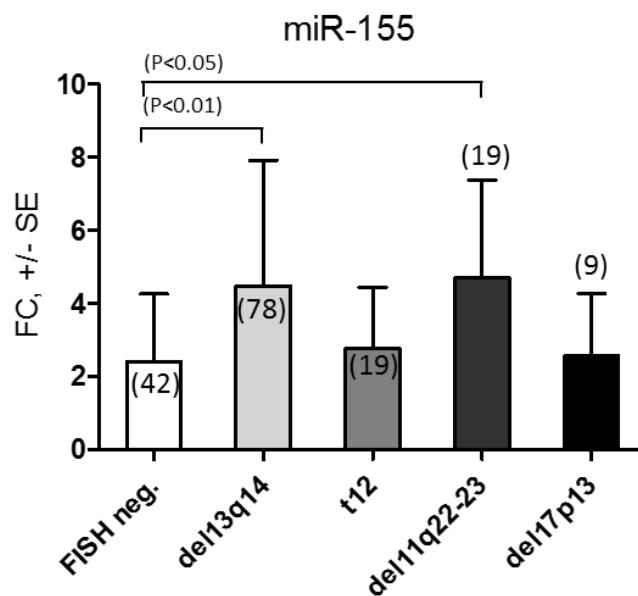


Figure 4.22: Expression of miR-155 in B-CLL samples based on cytogenetics. Graph shows expression (shown in fold change) of miR-155 in the primary B-CLL patient samples (PBMC/CD19+ B-CLL cells) based on the cytogenetics: FISH (B-CLL) negative (N=42); 13q14 (N=78); t12 (N=19); del11q22-23 (N=19); del17p13 (N=9). Error bars represent standard error of the mean. Number of patients in each group is in parentheses above/or in the each bar respectively. P values are shown in parentheses, Student's t test was used.

Based on cytogenetics we observed that, expression of PU.1 mRNA is not reciprocal to miR-155 (according cytogenetics). In the B-CLL patient samples with deletion of 13q14 and 11q22-23 PU.1 level remains unchanged relative to FISH negative B-CLL, while in the trisomy of chromosome 12 PU.1 was increased (2-fold) and in deletion of 17p13 was significantly decreased (Figure 4.23).

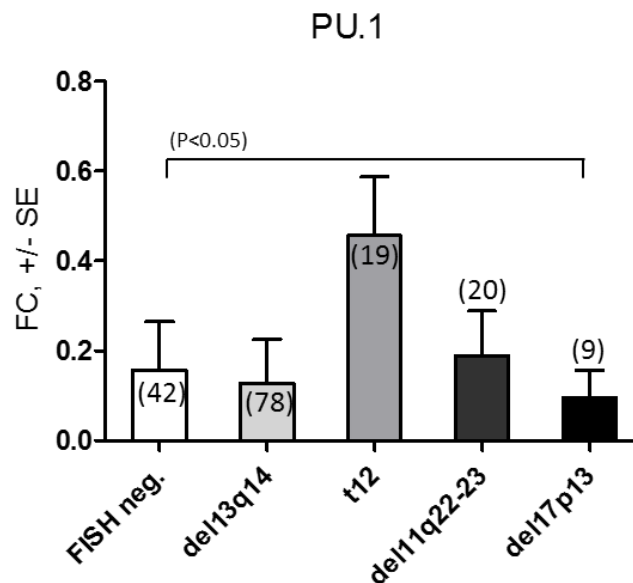


Figure 4.23: Expression of PU.1 in B-CLL samples based on the cytogenetics. Graph shows expression (shown in fold change) of PU.1 in the primary B-CLL patient samples (PBMC/CD19+ B-CLL cells) based on the cytogenetics: FISH (B-CLL) negative (N=42); 13q14 (N=78); t12 (N=19); del11q22-23 (N=20); del17p13 (N=9). Error bars represent standard error of the mean. Number of patients in each group is in parentheses above/or in the each bar respectively. P values are shown in parentheses, Student's t test was used.

Based on cytogenetics we observed that, levels of MYB mRNA were increased (not significantly) in the B-CLL patient samples with trisomy of chromosome 12 and with 13q14 deletion, but unchanged in the patient samples with deletions 11q22-23, 17p13 (Figure 4.24).

Based on cytogenetics we observed that, interesting expression pattern showed miR-150 regarding to several cytogenetic aberrations (Figure 4.25). Expression of miR-150 was significantly decreased in both high risk groups of B-CLL patient samples: del11q22-23 and del17p13. This suggests potential role of miR-150 in aggressive B-CLL as tumor suppressor. The low risk and intermediate group of B-CLL patient samples was similar to the group of B-CLL patient samples with negative FISH analysis (Figure 4.25).

Cytogenetic data unlike data from prognostic markers as ZAP-70/CD38/IgVH did not overall support conclusion of association between subgroups and expression of miR-155 and its upstream and downstream regulators.

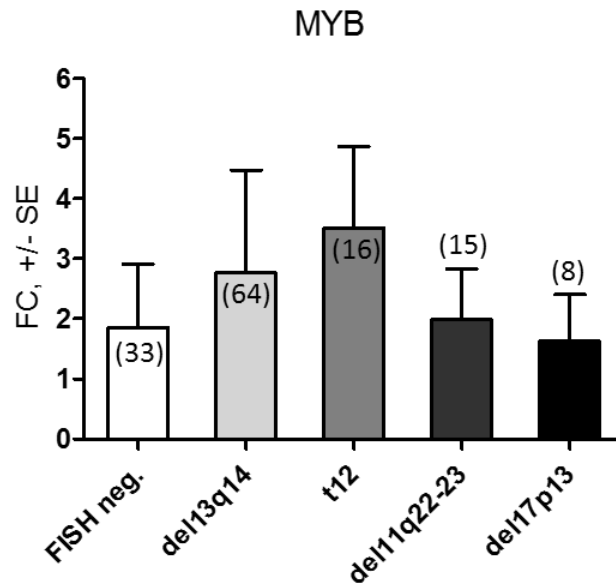


Figure 24: Expression of MYB in B-CLL samples based on the cytogenetics. Graph shows expression (shown in fold change) of MYB in the primary B-CLL patient samples (PBMC/CD19+ B-CLL cells) based on the cytogenetics: FISH (B-CLL) negative (N=33); 13q14 (N=64); t12 (N=16); del11q22-23 (N=15); del17p13 (N=8). Error bars represent standard error of the mean. Number of patients in each group is in parentheses above/or in the each bar respectively. P values are shown in parentheses, Student's t test was used.

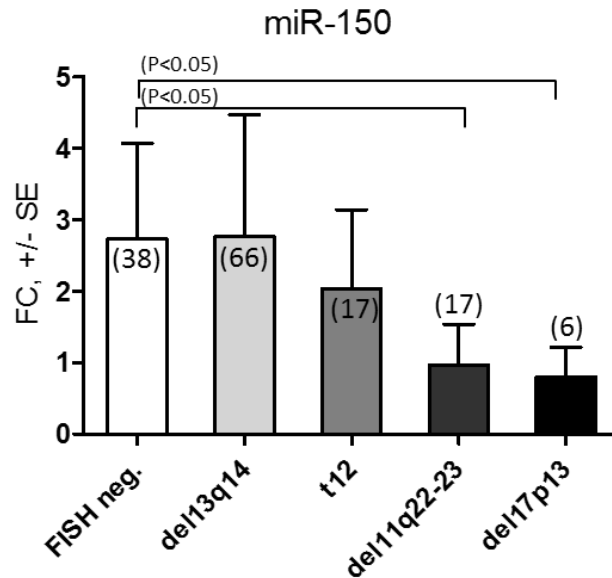


Figure 4.25: Expression of miR-150 in B-CLL samples based on the cytogenetics. Graph shows expression (shown in fold change) of miR-150 in the primary B-CLL patient samples (PBMC/CD19+ B-CLL cells) based on the cytogenetics: FISH (B-CLL) negative (N=38); 13q14 (N=66); t12 (N=17); del11q22-23 (N=15); del17p13 (N=6). Error bars represent standard error of the mean. Number of patients in each group is in parentheses above/or in the each bar respectively. P values are shown in parentheses, Student's t test was used.

To determine whether there is relationship between expression of MYB mRNA and expressions of miR-155, miR-150 and PU.1, we divided B-CLL patient samples into two groups: 1) samples over expressing MYB (>1.5-fold change) and 2) samples with low expression of MYB (<1.5-fold change). In the MYB-high group we detected increased levels of miR-155 (statistically significant), miR-150 (not significant) and surprisingly also of PU.1 (statistically significant) as compared to MYB-low group (Figure 4.26). Our data indicate on the relationship (yet not always in expected direction) between the levels of studied molecules. We further split B-CLL patient samples into another two groups based on the expression of miR-155 to see the relationship between expression of miR-155 compared to PU.1, MYB and miR-150 (Figure 4.28). In patient group with miR-155 > 2.5-fold change, the expression of miR-150 was significantly higher than in second group (miR-155 < 2.5-fold change). The mRNA of MYB was also increased in group with higher miR-155, but not statistically significant. The PU.1 mRNA level was unchanged (Figure 4.27). Again, this data indicate on the relationship between the four studied molecules.

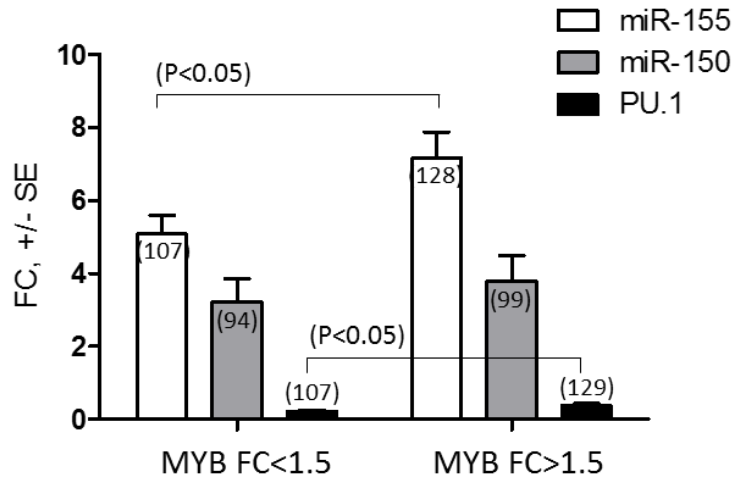


Figure 4.26: Levels of miR-155, miR-150 and PU.1 in B-CLL based on expression of MYB (PBMC/CD19+ B-CLL cells). Graph shows expression of miR-155 (white bars) and miR-150 (grey bars) in MYB under-expressors (<1.5 fold decrease, left) and MYB over-expressors (>1.5 fold increase; right) relative to control B-cells. Error bars represent standard errors of the mean (SEM). Number of patients in each group is in parentheses above/or in the each bar. Statistics was done by Student's t test and P values are shown in parentheses on top of the brackets indicating the groups being compared.

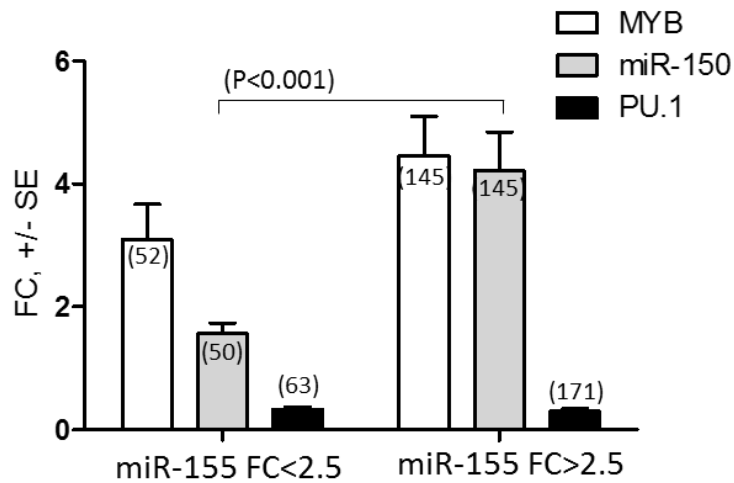


Figure 4.27: Levels of MYB, miR-150 and PU.1 in B-CLL based on the expression of miR-155 (PBMC/CD19+ B-CLL cells). Graph shows expression of miR-155 (white bars) and miR-150 (grey bars) in miR-155 underexpressors (<2.5 fold decrease, left) and miR-155 overexpressors (>2.5 fold increase; right) relative to control B-cells. Error bars represent standard errors of the mean (SEM). Number of patients in each group is in parentheses above/or in the each bar. Statistics was done by Student's t test and P values are shown in parentheses on top of the brackets indicating the groups being compared.

We hypothesize, the analysis of expression patterns of miR-155, PU.1 and MYB may reflect the therapy efficacy. Our B-CLL patient cohort include 40% of patients that undergoes therapy prior the study and 49% of patients that undergoes therapy during the study (see Table 3.2 in the section 3. Material and Methods). The therapy as well comprised from Rituximab (anti-CD20 antibody) treatment (375mg/m² intravenous infusion D1 of each cycle). To test our hypothesis, the peripheral blood of B-CLL patient was collected before, 24 hrs and 48 hrs after Rituximab treatment. We observed the following expression pattern: miR-155 and MYB were markedly decreased after Rituximab (11- and 6-folds respectively). In contrast, the level of PU.1 mRNA was increased 1-fold (Figure 4.28). Based on these results, the measuring of the levels of miR-155, PU.1 and MYB before and after therapy could help to control success of therapy and can be used as molecular biomarkers of Rituximab efficacy.

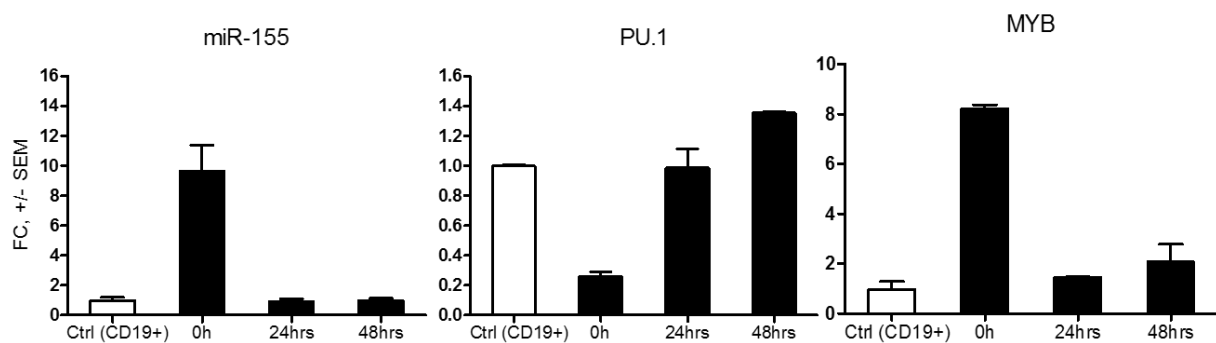


Figure 4.28: Expression of miR-155, PU.1 and MYB in B-CLL patient samples after therapy.

Graphs show expression of miR-155, MYB and PU.1 in the peripheral blood mononuclear cells of B-CLL patient (P143) before and after B-cell specific treatment by Rituximab (antiCD20 antibody) at 0, 24 and 48 hours (X-axis). Expression levels are shown as fold change (Y-axis). Error bars represent standard errors of the mean (SEM).

To summarize this part of results *point-by-point*, we found that expression of miR-155 and MYB increased, PU.1 remains unchanged and miR-150 decreased with disease progression in the advanced Rai stages. Moreover, in the B-CLL patient samples with unfavorable clinical outcome (ZAP-70+/CD38+/IgVH unmutated) the levels of miR-155 and MYB were increased; PU.1 was unchanged and miR-150 significantly decreased. Based on the cytogenetic data, we could conclude that expression of miR-155 increased in the B-CLL patient group with deletion 13q14 and 11q22-23 while in other cytogenetic subgroups it was unchanged. The PU.1 expression was significantly low in a group of patients with

deletion of 17p13, and high in a group with trisomy of chromosome 12. Expression of MYB mRNA was higher in patient group with deletion of 13q14 and with trisomy of 12 chromosome. Significantly low expression of miR-150 correlates with high risk of B-CLL based on cytogenetics. The measurement of levels of miR-155, PU.1 and MYB in B-CLL cells after Rituximab therapy underlines the importance of these molecules as potential biomarkers for disease progression and therapy efficacy.

4.2 The occupancy of MYB and epigenetic features at *MIR155HG* promoter region in B-CLL

4.2.1 Occupancy of transcription factor MYB at *MIR155HG* promoter region

To determine whether MYB recruits the *MIR155HG* promoter region we performed the chromatin immunoprecipitation (ChIP) within the *MIR155HG* locus. ChIP was followed by qPCR, where the amplicons ranged from -3.2 kb to 4.4 kb relative to the transcriptional start site (TSS) of *MIR155HG* promoter. Our data shows that in the primary B-CLL cells (N=6) MYB is consistently occupied at the *MIR155HG* promoter region, exclusively at three loci: -0.5kb; +0.1kb and +1.6kb relatively to TSS in comparison to the normal healthy CD19+ B-cells (N=6). While the recruitment was not detected anywhere else upstream or downstream of the studied *MIR155HG* promoter region, we could conclude that MYB recruitment at the *MIR155HG* gene is TSS specific (Figure 4.30A).

To answer the question if MYB has ability to stimulate the transcription of *MIR155HG* we generated luciferase reporter constructs either with mutation in MYB binding site (mut; E-box 1) or without mutation (wt; E-box 1). The putative MYB binding sites (E-box 1-3) sequence A A C T/G G are localized at the positions: 1) -399 – 394bp; 2) +209bp +215bp; 3) +1201bp +1207bp adjacent to the CpG island at *MIR155HG* (Figure 3.3 Section 3. Material and Methods; and schematically Figure 4.29). The figure 4.30B shows the relative luciferase activity of HeLa cells measured after 48 hours post transfection with reporter vectors: 1) with mutation in MYB binding site (mut; E-box 1); 2) without mutation in MYB binding site (wt; E-box 1) and 3) control vector (empty vector pGL4.17). Based on our results, only the construct without mutation in MYB binding site was able to bind and stimulate the luciferase activity in HeLa cells. Additionally, we transiently transfected HeLa cells with construct that either carries or not the mutation in MYC binding site. In this case the

luciferase activity was not stimulated neither with unmutated nor mutated binding sites for MYC (data not shown). Based on the ChIP and luciferase assay data we could conclude that MYB specifically binds and stimulates the transcription of *MIR155HG* in B-CLL cells (Figure 4.30).

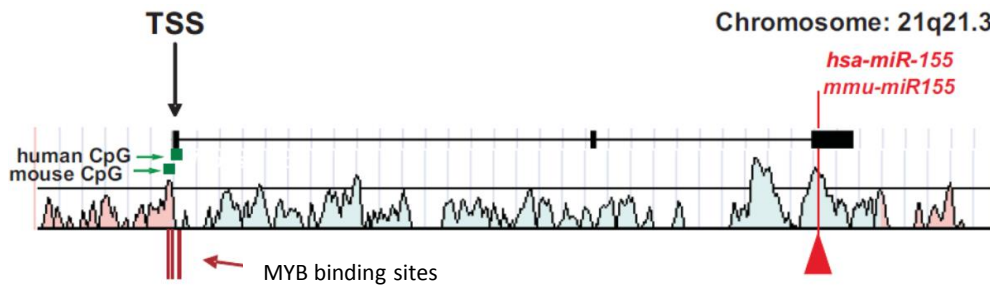


Figure 4.29: Vista plot of MYB binding sites and CpG at *MIR155HG* promoter region. Figure shows presence of MYB binding sites at the *MIR155HG* promoter near the CpG region (-33 – 339bp).

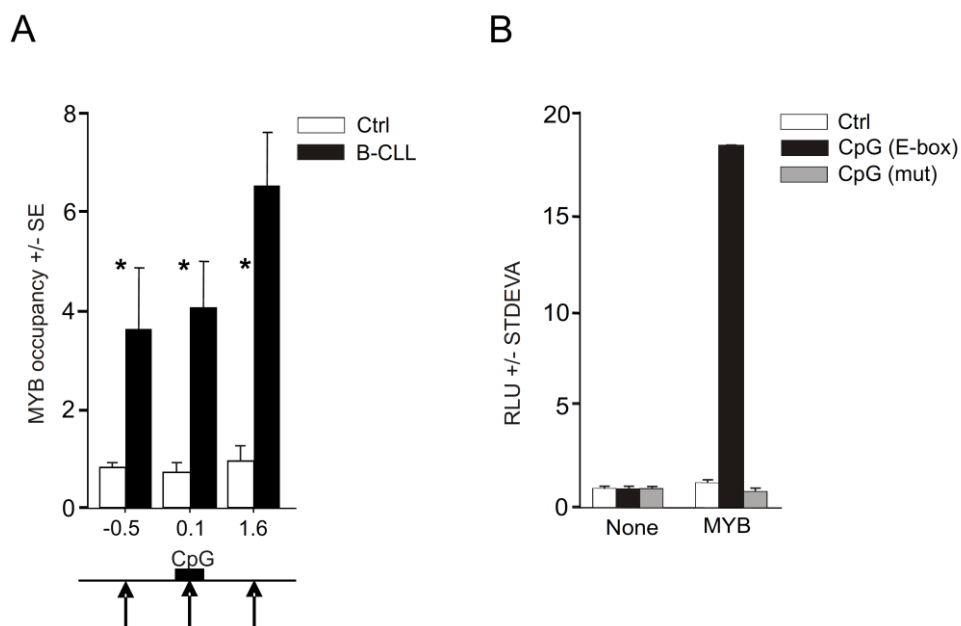


Figure 4.30: Transcription factor MYB binds and stimulates activity of *MIR155HG* promoter.

(A) MYB occupancy within *MIR155HG* was determined by ChIP by using anti-Myb or control antibodies. The ChIP was carried out on cross-linked chromatin isolated from B-CLL cells (N=6, average value indicated by black bars, P40, P39, P47, P250, P254, P255) and normal B-cells (N=6, white bars). The Y-axis indicates the relative occupancy of MYB. The X-axis marks the positions (in kb) of PCR amplicons relative to TSS of *MIR155HG*. The arrows indicate the positions of MYB DNA binding motifs; black box indicates position of the CpG island. Error bars represent standard error of the mean (SEM) of three independent experiments. P values <0.05 are indicated by one asterisk. (B) HeLa cells were transiently transfected with 0.5 μ g pGL4.17 reporter vector containing the MYB binding site (-399 bp to -394 bp) (black bars), its deletion mutant (grey bars) or a control reporter plasmid (None (pGL4.17); white bars). Reporter vectors were transfected either alone (left) or co-transfected with 1 μ g MYB cDNA expression vector (right). Luciferase activity measured at 48 hrs is shown relative to a control vector, the background activity was subtracted, and data were equalized to the protein content. Average values and standard deviation (STDEVA) of at least two independent experiments are plotted.

4.2.2 Active chromatin marks associates with elevated levels of miR-155 in B-CLL cells

The histone modifications are marks of the transcription activity of chromatin. The actively transcribed DNA contains chromatin (euchromatin) with active epigenetic markers on histones as e.g. H3K9Ac, H3K4Me3. These post translational histone modifications play important role in gene expression, especially in the leukemic cells.

To determine the acetylation status of lysine 9 of histone H3 (H3K9Ac) we performed the ChIP assay on the chromatin of B-CLL patient samples (N=9) and healthy control B-cell samples (N=8). Within the *MIR155HG* promoter region we observed the acetylation peak of H3K9Ac at the loci +0.1kb as compared with healthy control B-cell samples (Figure 4.31; upper graph). In the B-CLL cells we detected spread chromatin H3K9acetylation within the region from -3.2kb to +1.6kb. This epigenetic pattern of H3K9Ac overlapped with the occupied loci (-0.5 - +1.6kb) by MYB at CpG region of *MIR155HG* (Figure 4.31). Moreover, we detected a positive correlation between the MYB mRNA expression and acetylation status (H3K9Ac) at the same three loci: -0.5 kb ($r=0.5$), +0.1kb ($r=0.6$) and +1.6 kb ($r=0.5$) of the *MIR155HG* promoter region (Figure 4.32). Based on our data we could conclude that, transcription factor MYB activates the *MIR155HG* transcription in B-CLL cells.

We further determined another epigenetic mark, the H3K4Me3 at *MIR155HG* promoter region in B-CLL (N=9) and in the normal healthy controls (N=8). The presence of H3K4Me3 mark associated with active gene transcription by DNA dependent RNA polymerase II. Significant enrichment of H3K4Me3 methylation occurred at loci -0.5kb – +1.6kb of *MIR155HG* promoter region (Figure 4.31; lower graph). Taken together, ChIP data: acetylation of H3K9 and methylation (H3K4Me3) at the loci +0.1kb – +1.6kb and positive correlation with expression of MYB mRNA indicates on direct relationship between MYB and transcription activity of *MIR155HG* in B-CLL (Figure 4.31 and 4.32).

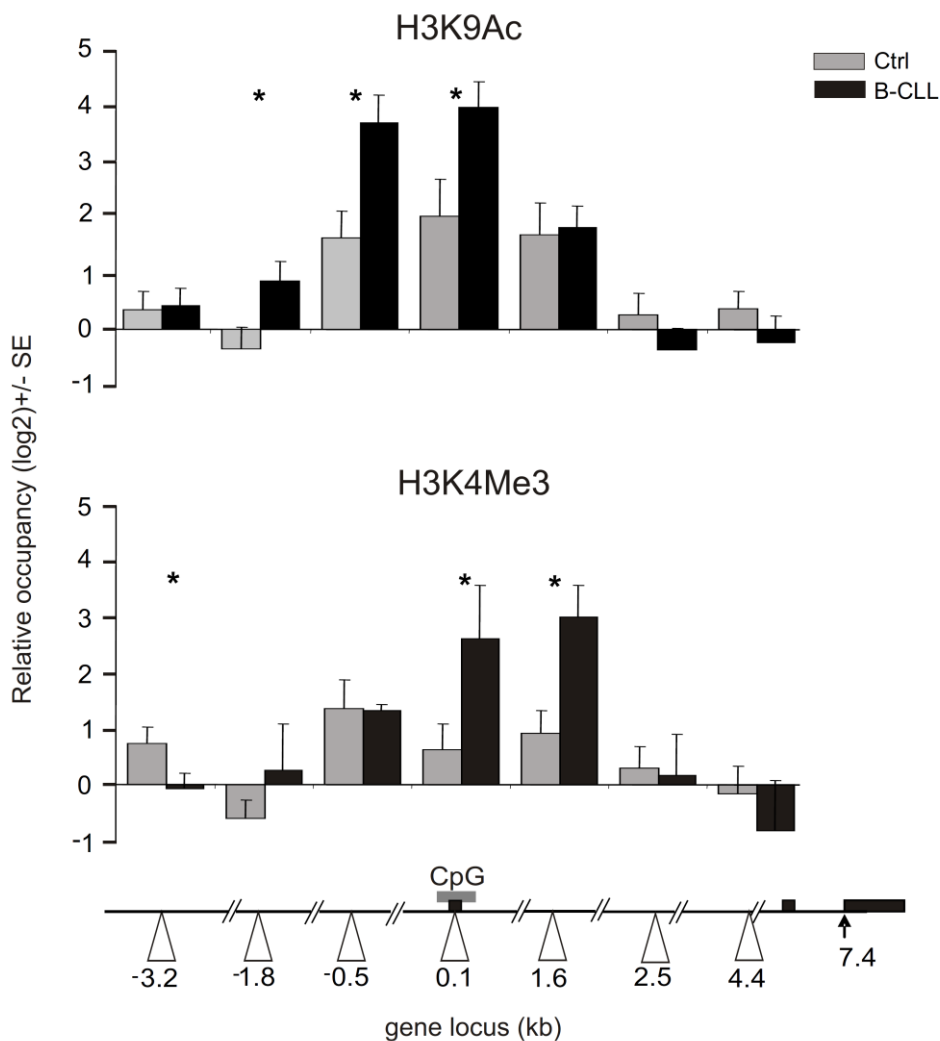


Figure 4.31: Histone modifications at *MIR155HG* promoter in B-CLL cells.

ChIP was performed on cross-linked chromatin from primary B-CLL (N=9: P250, P254, P255, P143, P130, P161, P162, P164, P179, P135, black bars) and control B-cells (N=8, grey bars) using the anti-H3K9Ac, H3K4Me3 and control anti-rabbit IgG antibody as described in section 3. Material and Methods. The level of H3K9 acetylation and H3K4 trimethylation (Y-axis) is expressed relative to control IgG antibody and relative to upstream -4.7kb locus with consistent low H3K9 acetylation and H3K4 methylation patterns. X-axis indicates positions of amplicons (in kb) relative to TSS. Position of CpG is indicated by grey box, exons of human *MIR155HG* indicated as black boxes. Error bars represent standard error of the mean. P values below 0.05 are indicated by asterisks.

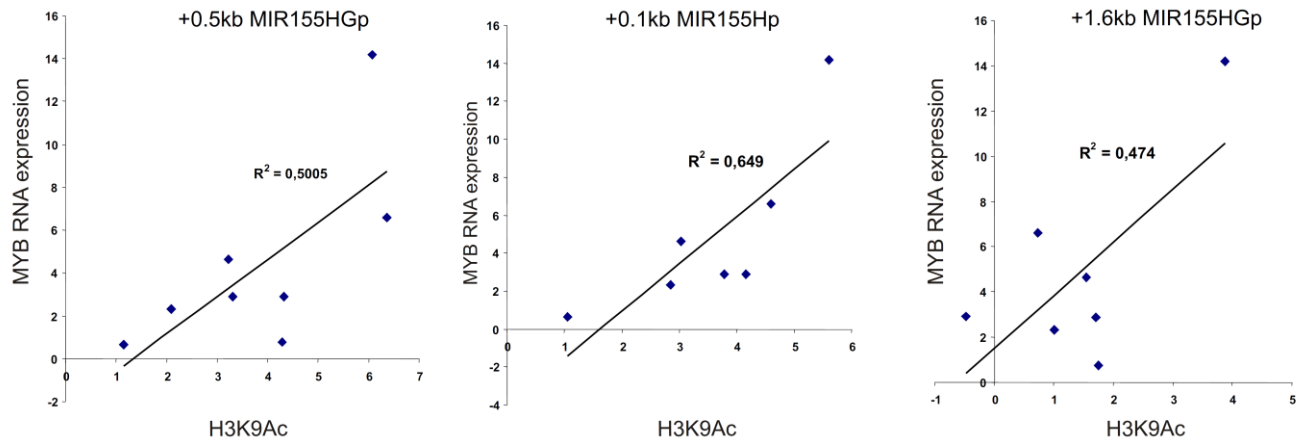


Figure 4.32: Correlation between MYB mRNA level and active chromatin marker H3K9Ac at *MIR155HG* promoter region in B-CLL cells. Scatter plots shows the correlation between MYB mRNA expression (Y-axis) and the levels of H3K9Ac (X-axis) at three amplicons at the CpG island of *MIR155HG*. Correlation coefficient R is shown at the centre of each graph.

4.3 Mutation status of canonical MYB binding motive at the *MIR155HG* in B-CLL

MIR155HG gene contains highly conserved CpG islands localized -33bp to -349bp upstream of TSS (Figure 3.3 in Section 3. Material and Methods). ChIP data indicated that *MIR155HG* promoter is occupied by MYB (Figure 4.30A) and by luciferase assay we confirmed that MYB by its binding stimulates *MIR155HG* transcription (Figure 4.30B). To test whether are some mutations within the promoter region of *MIR155HG*, we prove the sequence of MYB binding site (E-box 2). Our sequential data showed that the canonical MYB binding motif (E-box 2) within the CpG region at *MIR155HG* is not mutated in B-CLL samples (P89 and P143) in comparison with the sequence from normal healthy control sample (Ctrl; C41) (section 10. Supplement; Supplemental data 10.3).

4.4 Global gene expression profile in B-CLL unrolls deregulation of MYB and miR-155 targets

The mRNA microarray (Affymetrix Human Genome HG-U133 Plus 2.0 Array) was performed on mRNAs isolated from twelve peripheral blood B-CLL samples and five normal healthy donor blood samples (CD19+ separated B-cells) (Figure 4.33).

The clinical characterization of the patients samples is as follows: the most patient samples are in the advanced Rai stage II-IV (P33, P86, P136, P143, P249, P250, P254, 255); more than half of patients express the CD38 surface marker (P33, P86, P137, P138, P143, P155, P250), ZAP-70 (P33, P86, P138, P143, P249, P254, P255) and majority of samples have unmutated IgVH gene (P33, P86, P38, P143, P155, P250, P255, P271). Half of patients have deleted chromosome 11q22-23 (*ATM* gene) (P33, P138, P143, P155, P255, P271) and two patient samples have deleted 17p13 chromosome (*TP53* gene) (P86, P136). The majority of patients have deleted 13q14 chromosome (P33, P86, P136, P143, P155, P249, P250, P255, P271). Transformation into the Richter's syndrome (DLBCL) occurred in two patient samples (P86 and P143). To summarize this information, the analyzed patients are in the advanced Rai stage (II-IV) and have poor prognosis that is underlined by elevated levels of miR-155 and MYB and decreased level of PU.1. We detected low expression of miR-155 in only one patient (P155) however this is due to remission.

The target genes of miR-155 and MYB programs in B-CLL is one of our research interest. We used six prediction tools for *in silico* data analysis to determine the miR-155 target genes (for details see section 3. Materials and Methods). The lower part of heat map miR-155 target genes shows significantly decreased levels of many miR-155 targeted mRNAs, among them also a key hematopoietic transcription factor PU.1 (SPI1) and its target *FOS* gene (Figure 4.33A). This is in accordance with our data from RT-qPCR (Figures 4.4 and 4.6). Another important transcription factors that were targeted by miR-155 in B-CLL were: 1) *FOS* (FBJ murine osteosarcoma viral oncogene homolog); 2) *HIF1A* (hypoxia inducible factor 1); 3) *MAFB* (v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian)) transcription factor regulates hematopoiesis); 4) *SATB1* (special AT-rich sequence binding protein 1).

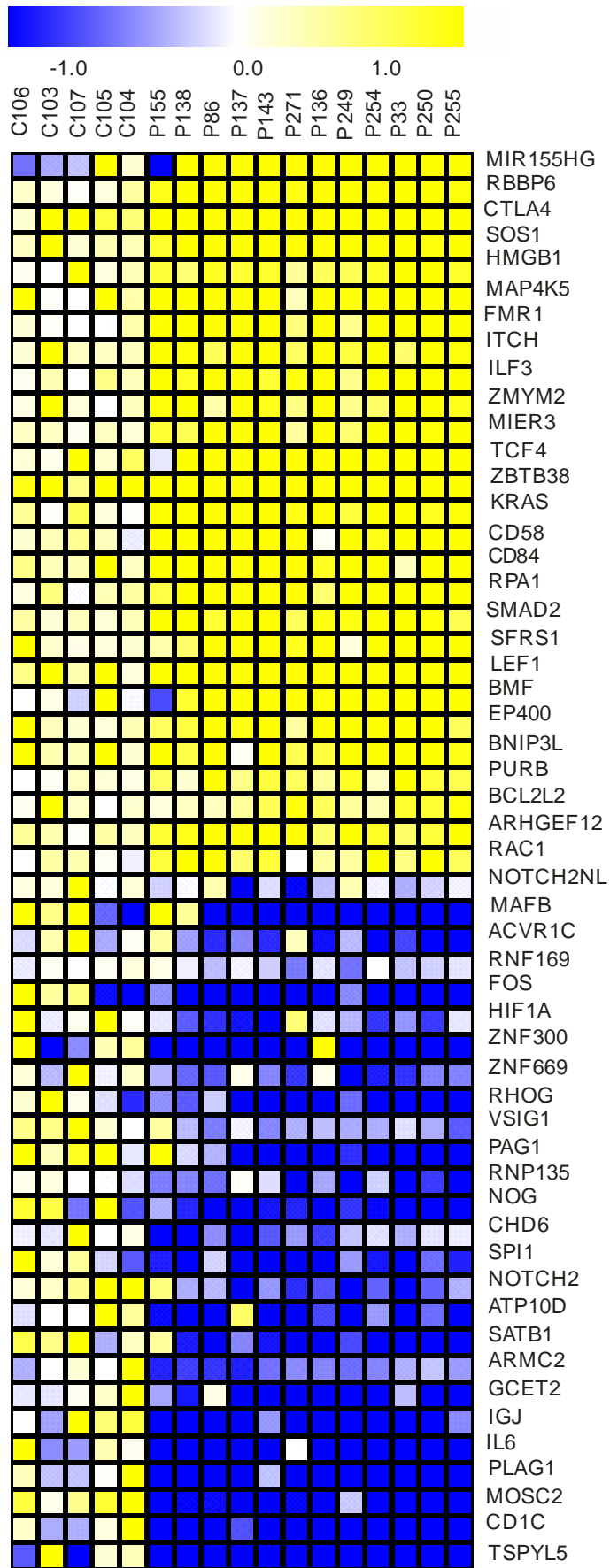
We found several genes whose mRNA was up regulated in B-CLL samples and at the same time were targets of miR-155 such as *KRAS* and *SMAD2*. Expression of these genes is

probably regulated by other compensatory mechanism that is dependent or independent on the presence of miR-155.

Three prediction tools were used for construction of MYB target gene list (for details see section: 3. Material and Methods). The MYB target gene list included also typical B-cells and B-CLL genes as *BCL2*, *CD5*, *AICDA* (Figure 4.33B). The over production of Bcl2, an anti-apoptotic protein is due to negative regulation by miR-15/16 cluster that is localized at chromosome 13q14. The surface marker CD5 is expressed by B-CLL cells, therefore it is used for confirmation of B-cell malignancies. Product of gene *AICDA*, the AID protein is involved in two processes that occur during maturation of B-cells: somatic hypermutation and class-switch recombination.

From microarray profiling we conclude, that MYB and miR-155 programs are in B-CLL deregulated.

A



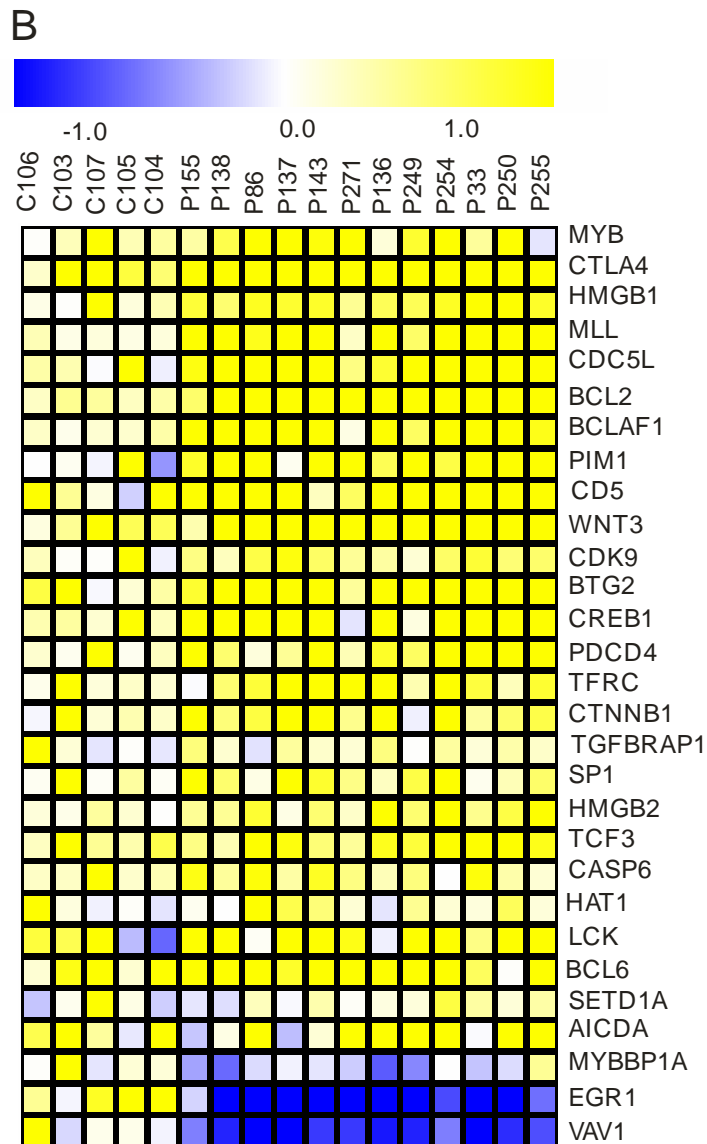


Figure 4.33: Targets of miR-155 and MYB are deregulated in B-CLL.

(A) Heat map shows selected miR-155 target genes which are differentially expressed between B-CLL (N=12; patient codes are indicated on top of the heat maps) and normal B-cells samples (P value of t-test <0.05). Genes were selected from union of six lists of either predicted or experimentally validated target genes of miR-155 (see section 3. Material and Methods/GSEA). (B) Heat map shows selected MYB target genes which are differentially expressed between B-CLL (N=12; patient codes are indicated on top of the heat maps) and normal B cell samples (P value of t-test <0.05). Genes were selected from three independent sources of MYB target genes (see section 3. Material and Methods/GSEA).

4.5 *In vitro* changes at levels of MYB, miR-155 and PU.1 in B-CLL cells show on the tight relationship between these molecules

Our previously published data show, that miR-155 and its validated target gene *PU.1* are in B-CLL are differently expressed¹⁴⁴. Our data from CHIP and luciferase assay showed that MYB binds and stimulates transcription of *MIR155HG* gene (Figure 4.30). Therefore we performed functional assays where we manipulated the levels of miR-155, PU.1 and MYB to assess the existence of relationship between these molecules *in vitro* (Figures 4.34 – 4.38).

For downregulation of MYB levels we transfected Raji cells and primary B-CLL cells with siRNA MYB. Figure 4.34 indicates on the downregulation of MYB in both Raji and primary B-CLL cells (P88) at 48 hours post transfection. Downregulation of MYB was followed by a decreased level of miR-155 in Raji cell line and in primary B-CLL cells. Reduction of MYB mRNA and afterwards decreased level of miR-155 were concentration dependent (Figure 4.34).

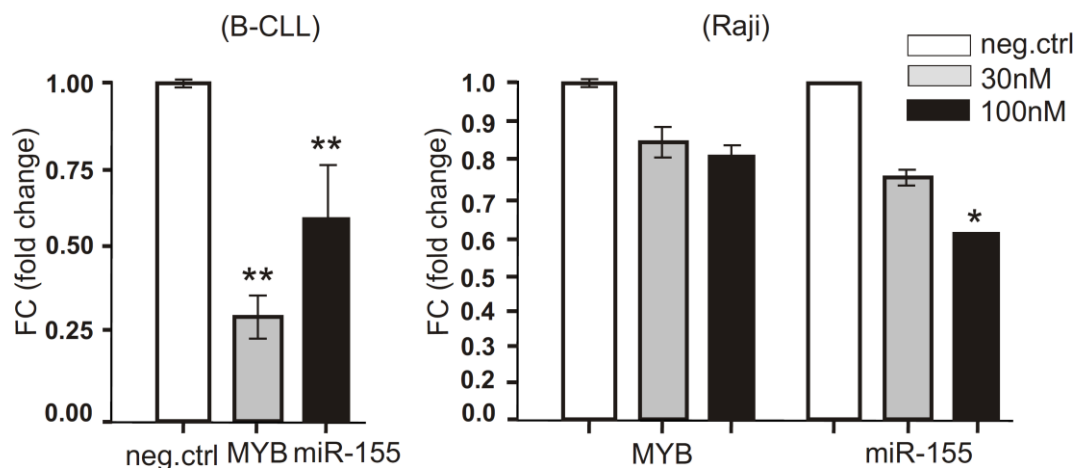


Figure 4.34: Transfection of primary B-CLL cells and Raji cell line with siRNA MYB.

Transfection of primary B-CLL cells (P88) and Raji cell line with siRNA oligonucleotides inhibiting expression of MYB or with negative control oligonucleotides (final concentrations 30 - 100nM). After 48 hours the total RNA was purified, reverse transcribed and measured by quantitative PCR as described in section 3. Material and Methods. The Y-axis represents the relative expression of mRNA of indicated genes relative to reference gene: *GAPDH* (for MYB, PU.1) or *RNU44* (for miR-155). Data are shown as a fold change (FC) and normalized to mRNA levels obtained in negative control transfection experiments (control measurements were set equal to 1). Error bars represent standard errors of the mean (SEM). For statistical analysis we used Student's t-test, where the P values <0.05 are indicated by one and <0.001 by two asterisks respectively.

To see the effect of *in vitro* increasement of the MYB mRNA and protein level we transiently transfected Raji cells and HeLa cells by expression plasmid that contains MYB

cDNA. After 48 hours post transfection of HeLa cells with MYB pcDNA we detected by western blot 5-fold overproduction of MYB protein (Figure 4.35A) and dose dependent increase of MYB mRNA in Raji cells (Figure 4.35B).

The *in vitro* transient changes of MYB levels leads to corresponding changes of miR-155 expression levels in Raji, HeLa cell line and in primary B-CLL cells (Figure 4.34 and 4.35).

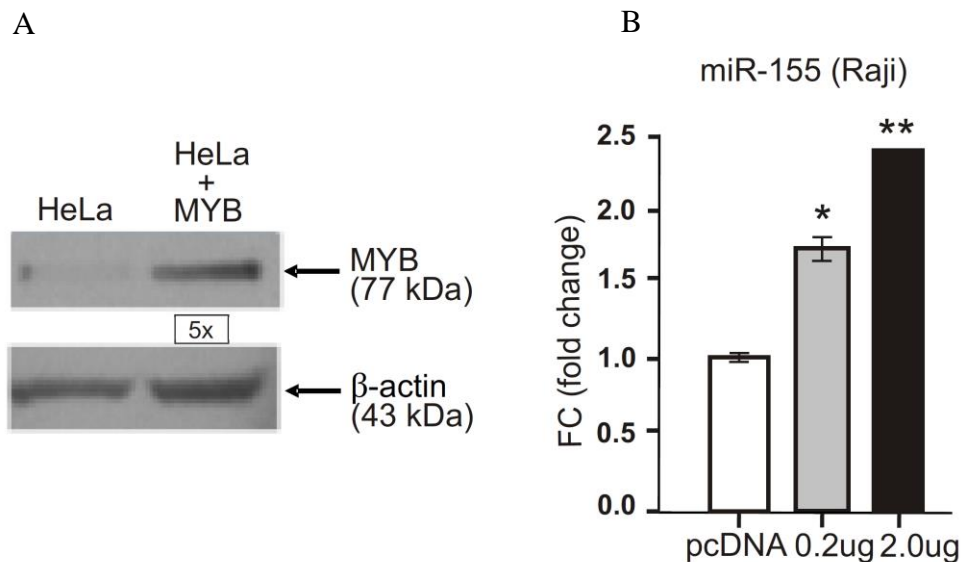


Figure 4.35: Transfection of primary B-CLL cells and Raji cell line with MYB expression plasmid.

(A) The figure shows immunoblotting of MYB protein 48 hours post transfection (HeLa cell line) with MYB cDNA encoding plasmid. Protein level was normalized on the levels of β -actin protein. The optical density of both proteins is indicated below the blots. (B) The Raji cells were transfected with MYB cDNA encoded by expression plasmid (in two final concentrations: 0.2 $\mu\text{g}/\mu\text{L}$ and 2.0 $\mu\text{g}/\mu\text{L}$) or with a negative control plasmid (pcDNA 3.1). Cells were harvested 48 hours post transfection and total RNA was extracted and measured by RT-qPCR. Y-axis represents the relative expression of miRNA-155 relative to RNU44 (negative control measurements were set equal to 1). Error bars represent standard error of the mean. We used Student's t-test, the P values <0.05 are indicated by 1 and <0.001 by 2 asterisks respectively.

Silencing of MYB or exogenous over production of MYB mRNA influences expression level of miR-155. Based on this knowledge we silenced or overproduced levels of miR-155 *in vitro* (Figure 4.36 and 4.37).

We transfected Raji cell line and primary B-CLL cells (P161) by anti-miR-155 oligonucleotides at final concentrations 40nM and 100nM. The figure 4.37 indicates on the significant decrease of miR-155 mediated by anti-miR-155 oligonucleotides. This decrease of miR-155 led to a significant PU.1 rescue (3-fold). These results show on the direct effect of miR-155 on the mRNA level of its target gene PU.1 in B-CLL (Figure 4.36).

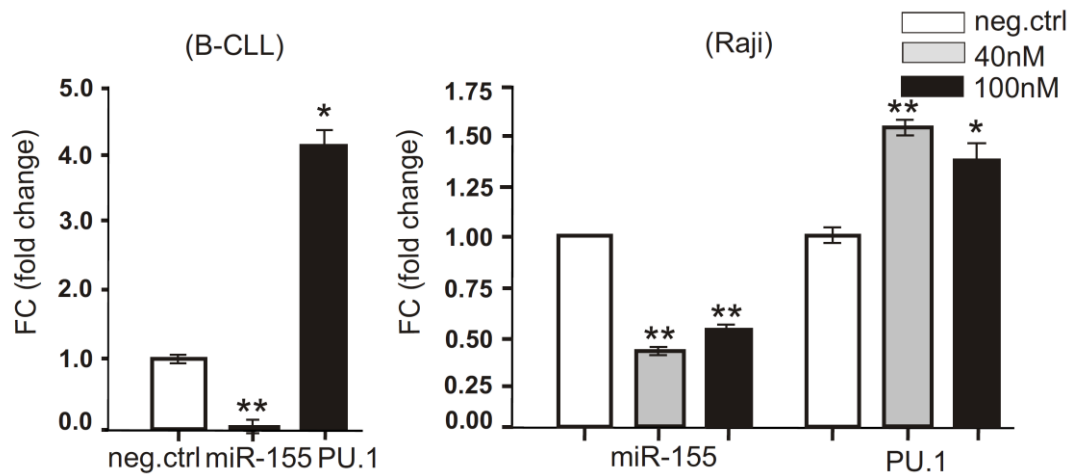


Figure 4.36: Transfection of primary B-CLL cells and Raji cell line with anti-miR-155 oligonucleotides. B-CLL cells (P161) and Raji cell line were transfected with anti-miR-155 oligonucleotides (in the final concentration 40nM, 100nM) and parallel with negative control oligonucleotides (in the final concentration 40nM, 100nM). After 96 hours total RNA was purified, reverse transcribed and measured by quantitative PCR as described in Methods, Y-axis represents the relative expression of mRNA of indicated genes relative to a reference gene *GAPDH* (for PU.1) or *RNU44* (for miR-155). Data are normalized to mRNA levels obtained in negative control transfection experiment (negative control measurements were set equal to 1). Error bars represent standard error of the mean. We used Student's t-test, the P values <0.05 are indicated by one and <0.01 by two asterisks respectively.

We further tested the effect of aberrant expression of miR-155 on its target gene *PU.1*. The Raji cells were transiently transfected with oligonucleotides of mimic-miR-155 in the final concentrations 40nM and 100nM. After 24 hours post transfection we detected significant increase (~12-32 fold) of miR-155 expression level and corresponding decrease of *PU.1* mRNA. Expression changes of miR-155 and *PU.1* were concentration dependent (Figure 4.36).

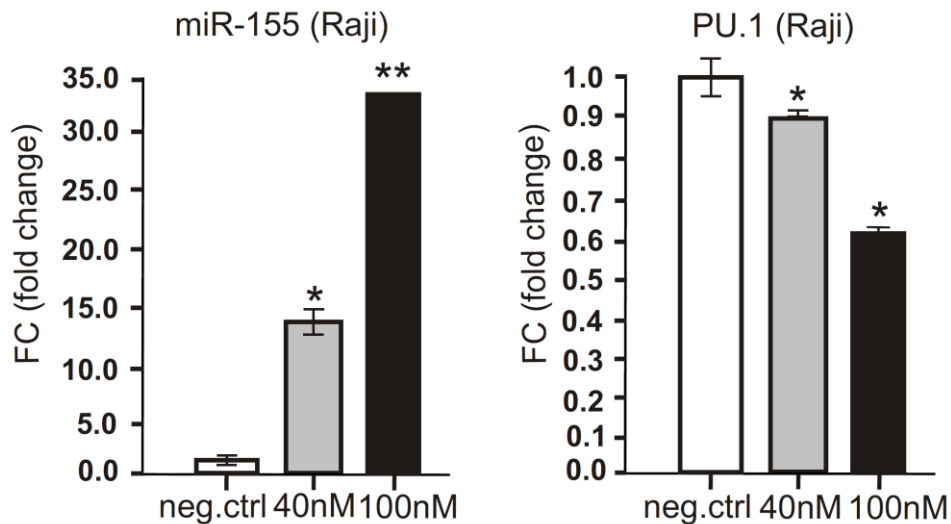


Figure 4.37: Transfection of primary B-CLL cells and Raji cell line with mimic-miR-155 oligonucleotides. Raji cell line was transfected with miR-155 oligonucleotides (in the final concentration 40nM, 100nM) or with negative control oligonucleotides. After 24 hours total RNA was purified, reverse transcribed and measured by quantitative PCR as described in Methods, Y-axis represents the relative expression of mRNA of indicated genes relative to a reference gene *GAPDH* (for PU.1) or *RNU44* (for miR-155). Data are normalized to mRNA levels measured in negative control transfection experiments (negative control measurements were set equal to 1). Error bars represent standard error of the mean. We used Student's t-test, the P values <0.05 are indicated by one and <0.001 by two asterisks respectively.

The hematopoietic transcription factor PU.1 acts as dose-dependent mediator in maturation of myeloid and lymphoid progenitor cells. Extremely low levels of PU.1 in blood cells leads to leukemia. From literature is known that in mouse progenitor cells the PU.1 expression stimulates production of miR-155 thus initiates differentiation of progenitor cells¹⁵⁷. We asked whether PU.1 regulates expression of miR-155 in the primary B-CLL cells. B-cells express low levels of PU.1, we next asked: What cause an exogenous increase of PU.1 in B-CLL cells? To answer this question, we transduced the primary B-CLL cells (P40) with lentiviral vector carrying PU.1 and GFP: PU.1-IRES-GFP lentiviral vector. After 72 hours post transduction we sorted out the GFP positive infected cells that contained PU.1-IRES-GFP lentiviral vector (MOI=3) and afterwards we measured changes at mRNA level. The figure 4.38 shows that rescued levels of PU.1 caused an increase of the mRNA of PU.1 (>2-fold) and resulted in decrease of miR-155 and MYB levels. We could conclude: manipulation with PU.1 level significantly decrease miR-155 expression in primary B-CLL cells.

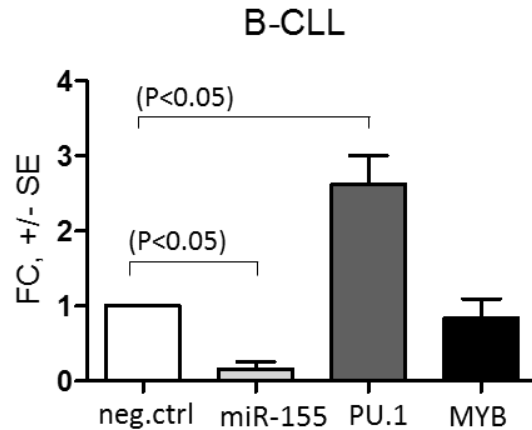


Figure 4.38: Transfection of primary B-CLL cells with PU.1-IRES-GFP lentivirus.

Graph shows results of transduction of primary B-CLL cells (P40) with PU.1-IRES-GFP lentiviral vector. After 72 hours the GFP positivity was measured, GFP positive cells with PU.1-IRES-GFP lentiviral vector were sorted out by FACS Ariallu cell sorter. From GFP positive sorted cells were purified total RNA, reverse transcribed and measured by quantitative PCR as described in section 3. Material and Methods. The Y-axis represents the relative expression (shown in fold change) of mRNA/miRNA of indicated genes relative to reference gene GAPDH (for PU.1) or RNU44 (for miR-155). Data were normalized to mRNA levels measured in GFP positive sorted cells that do not contain PU.1-IRES and were set equal to 1. Error bars represent standard error of mean. For statistical analysis we used Student's t-test. The P values lower than 0.05 are indicated by one asterisk.

Based on our data, we suppose that regulatory pathway: MYB → miR-155 —| PU.1 may play a role in reprogramming of gene expression pattern in B-CLL cells. Taken together, in our cohort of B-CLL patients the expression profile of miR-155 and MYB are significantly elevated in subset of patient samples. On the other hand, the production of mRNA and protein of PU.1 were decreased likely by elevated levels of miR-155. We created our working model of B-CLL: the transcription factor MYB stimulates the expression of miR-155 and over production of miR-155 inhibits the production of PU.1 (Figure 4.39). Based on data from PU.1 rescue experiment we could add to our model additional finding: increased level of PU.1 inhibits miR-155 and MYB expression. To this model we could not add miR-150 as we do not have the data from *in vitro* functional assays. Finally, the 20% of our B-CLL patient samples matched the criteria: elevated miR-155 (>2.5-fold), elevated MYB (>1.5-fold) and decreased PU.1 (<0.5-fold) (Figure 4.40).

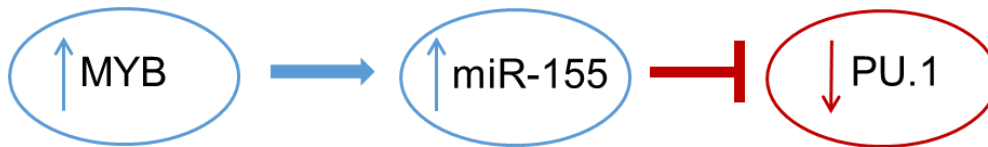


Figure 4.39: Scheme of our working model in B-CLL. The elevated level of MYB enhances the production of miR-155 that inhibits mRNA and protein production of PU.1 in B-CLL patient samples.

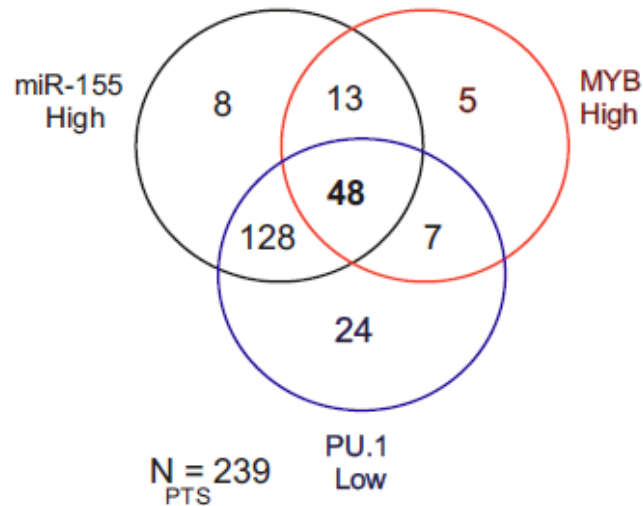


Figure 4.40: Venn diagram of B-CLL patient samples divided accordingly expressions of MYB, PU.1 and miR-155. The patient samples ($N_{CD19+/PBMC}=239$) were divided into three groups – accordingly mRNA/miRNA expression of miR-155 High ($FC>2.5$), MYB High ($FC>1.5$) and PU.1 Low ($FC<0.5$). The 48 patient samples expressed high levels of miR-155, MYB and low PU.1 levels. The 20% of our B-CLL patient samples (20% (48/239)) fit to our working model (Figure 4.39). The Venn diagram was done using the program by Oliveros, J.C. (2007) VENNY. An interactive tool for comparing lists with [VennDiagrams.http://bioinfogp.cnb.csic.es/tools/venny/index.html](http://bioinfogp.cnb.csic.es/tools/venny/index.html).

5. DISCUSSION

Chronic lymphocytic leukemia (**B-CLL**) belongs to the most frequent leukemia with recurrent relapses and heterogeneous course. There arise questions as: How to predict in time the disease progression? How to control patient response to the therapy? When to start with therapy? How the normal B-cell transforms into the leukemic cell is under intensive study. *In vitro* studies on the primary leukemic B-cells help us to understand pathogenesis of leukemia in more detail.

5.1 Expression profile of miR-155, PU.1, MYB and miR-150 in relation to the clinical data and epigenetic markers

This Thesis focuses on the role of **miR-155** in B-CLL and on causes of its aberrant expression in B-CLL. The miR-155 was identified by Tam *et al.*, as a B-cell integration cluster, while it is activated by proviral insertions in avian leucosis virus-induced lymphomas¹⁴⁷. The first report about the elevated level of miR-155 in human leukemia was in Hodgkin's lymphoma cells, described by group of van den Berg¹⁴¹ and later in B-CLL by Callin *et al.*¹⁴⁵. Here we found 4-32 fold increase of mature miR-155 in primary B-CLL cells as well as in separated CD19+ B-CLL cells (Figure 4.1A, B). Similar expression pattern described Fulci *et al.*¹⁷. We ask if there is an association between the expression of primary transcript of miR-155 (pri-miR-155) and its mature form (miR-155). We detected elevated levels of both forms in the primary B-CLL cells, even the primary transcript showed higher expression (Figure 4.2A, B). Our data are in accordance with others¹⁴⁹. We measured in parallel the levels of miR-155 in plasma of 10 B-CLL patient samples and observed that the levels of miR-155 in plasma and in separated CD19+ B-CLL cells were similar (Figure 4.3A, B). In plasma of B-cell lymphoma patients were described elevated levels of miRNAs as miR-155, miR-210, miR-21 that associated with disease progression²⁴⁴. Thus, the measurement of microRNAs in plasma could be used as biomarker of disease and its progression. Callin *et al.*, found a positive association between the high expression of specific miRNAs (miR-155, miR-181a, miR-146, miR-4-2, miR-23b, miR-23a, miR-222, miR-221) and shorter interval from diagnosis to the first therapy in B-CLL patient samples¹⁴⁵. These findings strongly support importance of miR-155 in B-CLL as a new prognostic marker. Furthermore, others determined the miR-

155 levels in plasma of patients with monoclonal B-lymphocytosis (MBL) that developed into B-CLL and concluded that miR-155 represents prognostic marker of MBL to B-CLL progression²⁰. Similarly to this report, we observed that expression of miR-155 during the disease progression fluctuates thus we hypothesize that it could be a useful prediction marker of disease progression and staging²⁴⁵. Different levels of miR-155 during disease progression document its higher level in advanced Rai stage (II-IV) (Figure 4.13). In B-CLL patient samples with advanced Rai stage and after therapy, the expression of miR-155 was decreased in dependence on the number of therapy cycles and overall survival (Figure 4.14). Moreover, B-CLL patient samples after Rituximab treatment displayed rapid decrease of miR-155 expression (Figure 4.28). Among the prognostic parameters of B-CLL belong mutation status of IgVH and presence of surface markers ZAP-70 and CD38²⁴⁶. B-CLL cells with unmutated IgVH gene underwent final development prior their entry into the germinal center that correlates with poor prognosis and shorter overall survival of B-CLL patients²⁴⁶. We measured the levels of miR-155 in both groups of B-CLL patient samples: unmutated vs. mutated IgVH status (Figure 4.18). B-CLL cells with unmutated IgVH genes expressed significantly higher levels of miR-155 (Figure 4.18). We divided B-CLL patient samples into two groups (favorable and unfavorable) based on the presence of surface markers ZAP-70 and CD38 and on the IgVH status (Figure 4.21). The favorable group is characterized by absence of prognostic surface markers ZAP-70 and CD38 and as well by mutated status of IgVH gene in contrast to unfavorable group (where the surface markers are present and IgVH gene are unmutated). We detected in the B-CLL cells of patient group with unfavorable prognosis IgVH unmutated/CD38+/ZAP-70+ significantly higher expression of miR-155 (Figure 4.21). For additional prognostic evaluation of B-CLL progression the cytogenetic measurement of chromosomal aberrations by FISH is used²⁸. In the B-CLL samples with “high risk”, carrying the deletion of chromosomal region 11q22-23 or 17p13 respectively, we detected increased expression of miR-155 (Figures 4.24, 4.25).

We can conclude that expression of miR-155 reflects the progression of B-CLL as the levels of miR-155 are higher in advanced Rai stage and in the unfavorable prognostic group. The measurement of miR-155 levels in plasma and in peripheral blood correlates, thus it could be used as biomarker of B-CLL. Moreover, the levels of miR-155 after therapy decreased, that underlines its importance in clinical use.

The validated direct target of miR-155 is a key hematopoietic transcription factor - PU.1 (SPI1) ¹⁵⁷. The role of decreased **PU.1** levels in lymphoid malignancies document data showing its down regulation in multiple myeloma and T-cell lymphoma ^{204,247}. Here we detected in the primary PBMCs and in CD19+ separated B-CLL cells the 3-4 fold decrease of PU.1 mRNA (Figure 4.4). This is in accordance with data of Dr. Mankai research group, where authors described low levels of PU.1 in B-CLL cells ²⁴⁸. In the protein lysates of B-CLL patient samples we detected one-fold decrease of PU.1 production (Figure 4.5). An early transcription factor - FOS (FBJ murine osteosarcoma viral oncogene homolog) is a target of PU.1. In our B-CLL patient cohort we found a 4-fold decrease of mRNA FOS (Figure 4.6). The low expressions of FOS and PU.1 support our data from genome-wide arrays, demonstrating their significant down regulation (Figure 4.33A). We detected overall low levels of PU.1 in B-CLL patient samples based on the prognostic markers as IgVH status and expression of ZAP-70, CD38 (Figure 4.18-21). The B-CLL patient samples in advanced Rai stage and after therapy showed increased level of PU.1 as compared with Rai stage 0-I (Figure 4.16). This increase is much stronger when the patients undergo more therapy regimens and displayed more aggressive course of disease (Figure 4.16). Similarly, PU.1 expression decreased 1.5-fold after Rituximab treatment (Figure 4.28). This underlines also fact, that PU.1 expression was significantly decreased in B-CLL patient group with deleted chromosome 17p13 – the patient group with high risk of B-CLL (Figure 4.25). Silenced production of PU.1 mRNA and protein in B-CLL is due to post-transcriptional inhibition mediated by elevated miR-155. Similarly, the mice with low expression of PU.1 (~20%) and with high expression of miR-155 developed more aggressive acute myeloid leukemia in comparison to mice with normal expressions of PU.1 and miR-155 ²⁰⁵. It was also described an opposite interaction between miR-155 and PU.1 in the myeloid progenitor cells, where PU.1 works as an inhibitor of the miR-155 expression ²⁴⁹.

This data demonstrate that expression of PU.1 is in B-CLL cells inhibited by aberrant expression of oncogenic miR-155. Decrease of PU.1 mRNA level correlates with advanced stages and more aggressive course of B-CLL. Thus, measurement of PU.1 showed prognostic and disease progression potential for B-CLL.

Based on above-mentioned information we asked: What causes this increase of miR-155 expression in B-CLL cells? It is known, that proto-oncogenes stimulate cellular growth and what leads to disease progression ²⁵⁰. Increased copy numbers of the proto-oncogen

MYC (v-Myc myelocytomatosis viral oncogene homolog) from the E-box family enhance the transformation of B-CLL into Richter`s syndrome ⁴⁶. The expression of *MYC* mRNA in our patient cohort was not up-regulated as reported by others ²⁵¹. Since the mRNA level of *MYC* in our patient cohort was only slightly above the mRNA *MYC* levels of control CD19+ B-cells (Figure 4.9), we investigated other members of E-box family – *MYB*, *MYBL1* and *MYBL2*. The mRNA levels of *MYBL1* and *MYBL2* did not show any significant expression changes in B-CLL cells as compared with control B-cells (data not shown). Interestingly, we observed that in a subset of B-CLL patients (~40%) there was a 3-fold up-regulation of **MYB** mRNA (Figure 4.8). We also investigated the protein levels of *MYB* in primary B-CLL cells. In protein lysates of B-CLL cells we detected up to 72-fold up-regulation of the *MYB* protein as compared with B-cells from normal healthy donors (Figure 4.5). Here, for the first time we described the role of *MYB* in B-CLL cells ¹⁴⁴. Moreover, we found three putative *MYB* binding sites at the *MIR155HG* promoter region (Figure 4.29 and Figure 3.3). *MYB* binds exclusively at three loci: -0.5kb; +0.1kb and +1.6kb relatively to transcription start site (TSS) of *MIR155HG* gene (Figure 4.30A and section 3. Material and methods, Figure 3.3). Similarly, it was described that *MYC* binds at the *MIR155HG* promoter region close to TSS and induces development of avian lymphomas ²⁵². Based on our data and published data we supposed that *MYB* in B-CLL as *MYC* in chicken B-cell lymphomas enhances the transcription of *MIR155HG* thus leads to disease progression ^{144,252}. To answer the question if *MYB* has ability to stimulate transcription of *MIR155HG*, we generated luciferase reporter constructs either with mutation in *MYB* binding site (E-box 1) or without such mutation (Figure 4.30B and section 3. Material and Methods; Figure 3.3). The mechanism of activation of *MIR155HG* by *MYB* is proposed to include acetylation of histone H3K9, a notion supported by others ²⁵³. By ChIP assay we found that active chromatin marks H3K9Ac and H3K4Me3 are recruited at the *MIR155HG* promoter region (Figure 4.31). The enrichment peak of occupancy by H3K9Ac and H3K4Me3 (Figure 4.31) culminated exclusively at three loci (0.5kb; +0.1kb and +1.6kb relative to TSS) and this enrichment peak correlates with the *MYB* mRNA expression in primary B-CLL cells (Figure 4.32). Next, we asked whether the putative *MYB* binding site (E-box 2) is not mutated in the primary B-CLL cells. After sequencing of this region we did not observe any mutations in the putative *MYB* binding site (see the section 10. Supplement; Supplemental data 10.3). The question arises: What causes an aberrant expression of *MYB* in the leukemic B-cells? The possible answer could be that NFκB by its binding at the first

intron of MYB activates the expression of MYB ²⁵⁴. Moreover it was shown that NFκB stimulates expression of miR-155 in B-cells ¹⁹¹ and that MYB is a direct target of NFκB ²⁵⁴. This let us to speculate that initial step in this leukemic process could be some inflammation stimuli that enhance expression of both MYB and miR-155 and let the leukemic cell accumulate/proliferate. It was shown that the expression of MYBL1 correlates with the proliferation of mature B-cells ²⁵⁵ and with the presence of CD38 surface marker on the germinal center B-cells ²⁵⁶. Presence of surface marker CD38 on B-cells indicates their active proliferation and communication with microenvironment ²⁵⁷. We observed positive correlation between expression of CD38 marker (CD38+) and the elevated expression of MYB mRNA in B-CLL cells (Figure 4.20). Moreover, we detected elevated expression of MYB mRNA in patient samples with unfavorable prognosis – IgVH unmutated/ZAP-70+/CD38+ (Figure 4.21). The prognosis of B-CLL progression defines Rai staging system (0-IV). We found significantly elevated MYB mRNA in the B-CLL samples with advanced Rai stage (Figure 4.13) and with presence of surface marker ZAP-70 (Figure 4.19). The mutation status of IgVH genes does not correlate with expression of MYB in B-CLL cells (Figure 4.18). Taken together, expression of MYB in B-CLL could be prognostic marker for disease progression as well as for control therapy efficacy.

Between the top 10 MYB targets belongs B-cell specific microRNA - **miR-150** ^{72,228}. MiR-150 is selectively expressed by mature resting B- and T-cells, but not by their progenitors ²²⁷. MiR-150 regulates differentiation of B-cells through MYB ²²⁸. In our cohort of B-CLL patients we observed in 30% of patient samples elevated miR-150 (2-3 fold); in 40% above control level and in rest 30% decreased miR-150 (Figure 4.7). Our data are in accordance with data of other research groups ^{17,137,258}. Authors described a negative correlation between expression of miR-150 and prognostic markers IgVH and ZAP-70 (Figures 4.18, 4.19). Wang *et al.*, showed a reciprocal relationship between expressions of miR-155 and miR-150 in the germinal center B-cells, evaluated by *in situ* hybridization ¹³⁷. In contrast, we did not found such reciprocal relationship between the expressions of miR-155 and miR-150 in B-CLL samples (Figure 4.10) but we found a rather positive relationship as it show figures 4.27 and 4.10. Since the MYB is a target of miR-150, we assumed between these molecules any relationship. It was described a reciprocal relationship between expression of miR-150 and MYB in the myelodysplastic syndrome (MDS) ²⁵⁹. We measured

the levels of both, and unfortunately did not find such reciprocal relationship between miR-150 and MYB expressions (Figure 4.11).

5.2 Global gene expression profile: miR-155 and MYB target genes in B-CLL

We performed the global gene expression profiling on the B-CLL patient CD19+ B cell samples (N=12) and the normal healthy control CD19+ B cells (N=5) to assess the behavior of MYB and miR-155 target genes. Our microarray data showed that miR-155 and MYB programs are deregulated in B-CLL (Figure 4.33A, B).

The multifunctional and oncogenic microRNA - miR-155 regulates vast group of targets⁷³. We selected a few miR-155 (N=53) target genes using the gene set enrichment analysis (GSEA)(Figure 4.33A). Among the high score validated target of miR-155 belongs the well known hematopoietic transcription factor **PU.1**, that is extremely low in B-CLL (Figure 4.33A; 4.4). The highest levels of PU.1 mRNA are detected in granulocytes, monocytes/macrophages; the lowest levels are in B-cells and practically no expression in erythrocytes and T-cells^{204,260}. An upstream regulatory element (URE) is located at -14kb and -17kb upstream of the transcriptional start site of the *PU.1* gene and regulates 80% of PU.1 expression²⁶¹. Mouse model with deleted URE region showed decreased expression of PU.1 levels up to 20% that result into acute myeloid leukemia in mice²⁴⁷ and in T-lymphoma and multiple myeloma in humans²⁰⁴. We moreover observed that PU.1 low (*Pu.1^{ure/ure}*) mice with increased miR-155 and MYB mRNA levels developed more aggressive acute myeloid leukemia²⁰⁵. Due to high methylation of the *PU.1* promoter, expression of PU.1 in Hodgkin`s lymphoma cells is decreased. Further decrease of PU.1 in Hodgkin`s lymphoma cells induces cell growth and blocks apoptosis. Exogenous increase of PU.1 levels led to tumor regression and stabilization of disease in xenograft mouse model just like in primary Hodgkin`s lymphoma cells²⁶². This data emphasize that *PU.1* acts as a tumor suppressor in myeloid and lymphoid cells. Rescue of PU.1 is a possible therapeutic option for not only patients with Hodgkin`s lymphoma²⁶² but probably also for patients with B-cell malignancies. We performed similar experiment, where we exogenously increased the levels of PU.1 in the primary B-CLL cells that resulted in significant increase of mRNA PU.1 and decrease of miR-155 and MYB mRNA levels (Figure 4.38). Based on this data, the *in vitro* manipulation with PU.1 levels in B-cell leukemia/lymphoma provide the PU.1 potentially

useful molecule in the future therapy. The mRNA levels of *PU.1* target gene - *FOS* (FBJ murine osteosarcoma viral oncogene homolog) were in B-CLL also significantly decreased (Figures 4.6 and 4.33A). *FOS* is an early transcription factor that by interacting with proteins from the JUN family forms a transcription factor complex called AP-1. The AP-1 complex enhances activity of B-cell receptor signaling and possibly stimulates transcription of *MIR155HG* ¹⁸⁷. Upon activation by the BCR authors showed that *JunB* (major activator) and *FosB* (minor activator) recruit *MIR155HG* promoter. Thus, BCR-mediated signaling leads to the induction of *MIR155HG* expression in B-cell line Ramos ¹⁸⁷. It is known that during tumor growth in the hypoxic environment a hypoxia inducible factor 1 (HIF1A) is aberrantly expressed. **HIF1A** regulates vascular endothelial growth factor (VEGF) and thereby is associated with tumor angiogenesis and vascularization. High levels of VEGF were detected in the microvesicles localized in plasma of B-CLL patients ²⁶³. Microvesicles form an important part of the tumor microenvironment and are released by malignant cancer cells. The amount of microvesicles is higher in cancer cells compared with normal healthy cells. Circulating microvesicles in B-CLL patient plasma samples transfer messages to the targeted cells that could be critical for disease progression ²⁶³. Microvesicles enhance expression of **HIF1A** and stimulates AKT signaling pathway and thus leads to activation of tumor environment ²⁶³. Here we detected decreased expression of HIF1A mRNA (Figure 4.33B). Previous studies have shown that microvesicles released from tumor cells into the blood stream of cancer patients contain also miRNAs. Due to abundant presence of RNases in the blood stream, most of the secreted miRNAs are localized in the apoptotic bodies, microvesicles and exosomes or bound to RNA-binding proteins ²⁶⁴. Zhang Y *et al.*, reported that secreted miR-150 in microvesicles from human blood cells or cultured cells can be taken up by microvascular endothelial cells and these regulate the expression of MYB, which is a known miR-150 target gene ^{228,265}. Furthermore, Pegtel *et al.*, showed that miRNAs released from exosomes of EBV-infected B-cells can be taken up by the peripheral blood mononuclear cells and then can suppress EBV target genes ²⁶⁶. These findings strongly support that at least some of the exported miRNAs are used for cell-to-cell communication. Further research is needed to determine the secretory mechanisms of miRNAs; how miRNAs are recognized for uptake and what kind of information can be transferred via this process. Changes in the miRNA expression contribute to the pathogenesis of hematopoietic malignancies, including B-CLL. However, the mechanisms that cause aberrant miRNA

transcription is still poorly described. Among the miR-155 targets we found differentially expressed **MAFB** (viral musculoaponeurotic fibrosarcoma oncogene homolog B) mRNA. *MAFB* is hematopoietic transcription factor that cooperates with MYB²⁶⁷. We showed that elevated level of miR-155 decreased the level of MAFB mRNA in B-CLL patients samples (Figure 4.33A). Similarly, it was described decreased expression of MAFB in acute and chronic myeloid leukemia (AML, CML) patient samples²⁶⁸ and recently also in the B-cell low grade lymphomas²⁶⁹. Zhang Y *et al.*, showed the relationship between expression of miR-155 and MAFB in the mouse primary B-lymphoma cells and in the MEC1 B-CLL cell line²⁶⁹. Authors moreover showed that after inhibition of miR-155 in the primary B-CLL cells and in the MEC1 cell line cells increased the levels of MAFB mRNA. Thus, miR-155 negatively regulates expression of MAFB in B-cell malignancies^{144,269}. Therefore, we can conclude that miR-155 controls proliferation of B-CLL cells through MAFB, which in this case functions as a tumor suppressor. Among miR-155 target genes we also detected the chromatin remodeling factor SATB1. Han *et al.*, observed over produced proto-oncogene **SATB1** (Special AT-rich Binding protein-1) in the breast cancer cells and associated it with poor prognosis of patients. In the breast cancer was proven the oncogenic potential of miR-155^{167,270}. MiR-155 stimulates expression of the tumor suppressor gene *FOXP3*. In breast cancer *FOXP3* negatively regulates the expression of SATB1 through miR-155. Collective of authors McInnes recently described the existence of so-called “feed-forward regulatory loop” between *FOXP3*, miR-155 and SATB1 in human T-cells²⁷¹. Thus, miR-155 negatively regulates many important genes. We also found some miR-155 target genes up-regulated, such as **KRAS** and **SMAD2**. Expression of these genes is probably regulated by other compensatory mechanism that could be dependent or independent on the presence of miR-155. It was described that TGF- β induces *MIR155HG* transcription through binding of SMAD4 to the promoter region¹⁶⁷.

We detected among the **MYB upstream targets** also the typical B-CLL genes such as *BCL2*, *CD5*, *AICDA*, *BCL6* (Figure 4.33B). The product of the gene B-cell lymphoma 2 (**BCL2**) is the anti-apoptotic protein Bcl2 as it blocks the release of mitochondrial cytochrome c and inhibits the activation of initiator caspase 9. Bcl2 by inhibition of programmed cell death prolongs survival of cells²⁷². Others and we have reported increased mRNA levels of BCL2 in B-cell malignancies^{144,273}. Increased levels of Bcl2 are caused by negative regulation of cluster miR15/16²⁷⁴. Cluster miR-15-16 is localized at 13q14 region, that is often deleted in

B-CLL – therefore the levels of miR-15-16 are very low¹⁶. There is no effect of miR-15a and miR-16-1 on BCL2 mRNA stability, so the expression of Bcl2 is regulated at the post-transcriptional level¹⁶. Elevated levels of BCL2 by miR-15-16 cluster down regulation seems to be the main regulatory mechanism involved in the pathogenesis of the major fraction of B-CLL cells. Because the miR-15-16 cluster miRNAs work as the natural inhibitor of Bcl2, this could be used as therapy in cells that over express BCL2²⁷⁴. We detected in our B-CLL patient samples aberrantly expressed the B-CLL surface marker **CD5**^{144,275,276} (Figure 4.33B). Presence of CD5 together with CD22 and CD23 markers help to distinguish between MCL and B-CLL²⁸. During the maturation process, B-cells undergo somatic hypermutation (SHM) and class-switch recombination (CSR) of immunoglobulin genes²⁷⁷. The activation induced cytidine deaminase (**AID**, also known as **AICDA**) is a RNA-editing enzyme that triggers antibody diversification by the deamination of nucleotides within the immunoglobulin locus²⁷⁷. Germinal center B-cells after CD40 ligand (CD40L) stimulation express AID²⁷⁷. AID plays a key role in maturation of B-cells, where regulates switching between CSR and SHM²⁷⁸, but the exact mechanisms of how the AID acts remains unclear. It was described that B-CLL cells after CSR have *IgVH* genes unmutated and predominately express AID^{279,280}. This is in accordance with our results where the AICDA expression was high in B-CLL patient cells with unmutated *IgVH* genes (Figure 4.33B). Moreover, it was showed in the mouse model that overexpression of *Aicda* mRNA in B-cells result in T- or B-cell leukemia/lymphoma²⁸¹. Interestingly, the splenic B-cells of miR-155 deficient mice do not expressed *Aicda* mRNA¹⁵⁷, thus miR-155 is essential for *Aicda* function. Furthermore, the absence of AID mediated by miR-155 inhibition resulted in imperfect maturation of B-cells¹⁷⁷. Among the up-regulated MYB targets, we detected **BCL6** gene, its mRNA level was increased in B-CLL cells (Figure 4.33B). BCL6 is a multifunctional regulator (mainly repressor) that controls cell cycle, apoptosis, lymphocyte differentiation and immune reactions^{273,282}. BCL6 also participates in the chromosomal translocations that occur in DLBCL and in follicular lymphoma²⁸³. As the highest level of BCL6 was detected in B-cells, its oncogenic properties have been explored mainly in the lymphoid system. Moreover, BCL6 was described as a potential prognostic marker for the diagnosis of B-cell lymphomas²⁸⁴. For terminal B-cell differentiation is typical low expression of BCL6, increased level of BLIMP-1 and low level of MYC. This is regulated by an over expression of cell cycle regulating transcription factors MAD1 and MAD4²⁸². Sandhu SK *et al.*, observed contradictory results in E μ -miR-155 transgenic mice. Authors detected

consistently lower mRNA, protein levels of Bcl6 in mouse splenocytes and purified B-cells in comparison with wild type counterparts. Authors commented such reduction as a result of up-regulation of miR-155 targeted pathway Mxd1/Mad1. Thus transcription of BCL6 is indirectly modulated by miR-155 through the Mxd1/Mad pathway ²⁸⁵. Human DLBCL is divided into two subtypes: the first subtype is germinal center B-cell (GC) DLBCL, where the BCL6 levels were increased and miR-155 was decreased; and the second subtype is the activated B-cell (ABC) DLBCL that displays an opposite mRNA expression profile ²⁸⁶. These data underline the importance of BCL6 in B-cell malignancies; until now mainly detected in B-cell lymphomas. We observed that the target of both miR-155 and MYB - the BCL6 mRNA is over produced in the B-CLL cells (Figure 4.33B).

To conclude this paragraph, the miR-155 and MYB program is in B-CLL deregulated based on the GSEA analysis. Among the miR-155 target genes were half (hematopoietic TF – *PU.1*, *FOS*, *HIF1A*, *SATB1*) down regulated and other half upregulated (Figure 4.33A). Among the MYB targets we detected also the typical B-CLL genes such as CD5 and genes involved in apoptosis as *BCL2* (Figure 4.33B).

5.3 *In vitro* functional assays confirmed relationship between miR-155, PU.1 and MYB in B-CLL cells

We further performed **functional assays** to test the influence of different levels of miR-155, MYB and PU.1 on the primary B-CLL cells and Raji cell line. By *in vitro* manipulations of these molecules we corroborated the existence of a relationship between them (Figures 4.34-4.38). To decrease the elevated levels of miR-155 we transfected Raji and primary B-CLL cells by anti-miR-155 what resulted in significant decrease of miR-155 and increase of PU.1 levels (Figure 4.36). Exogenous increase of miR-155 level by mimic-miR-155 caused an inhibition of PU.1 expression in dose dependent manner (Figure 4.37). The PU.1 rescue experiment showed a direct relationship between levels of PU.1, miR-155 and possibly MYB (Figure 4.38). CHIP data and luciferase assay showed that MYB binds and stimulates *MIR155HG* in B-CLL cells (Figure 4.30A, B). We further asked if there is relationship between MYB and miR-155. Inhibition of MYB mediated by siRNA MYB caused dose dependent decrease of miR-155 levels (Figure 4.34) and transfection of MYB expression plasmid led to increase miR-155 expression in Raji cells (Figure 4.35).

Relationship between expression of miR-155 and MYB further supports significant elevation of miR-155 expression in the B-CLL patients overexpressing MYB (Figures 4.26 and 4.10).

Here we demonstrated that manipulation of miR-155 levels negatively influences PU.1 levels and rescue of PU.1 inhibits expression of miR-155 in B-CLL. We further confirmed that MYB and miR-155 are in tight relationship in B-CLL by either its inhibition or stimulation.

5.4 Working model of B-CLL and future therapy tools

Based on our functional data we designed a **working model for B-CLL**. This model describes relationship between miR-155, MYB and PU.1 (Figure 4.39) verified by our experiments and confronted by published data (Figure 5.1). Here we described for the first time stimulatory role of transcription factor MYB on the *MIR155HG* transcription in primary B-CLL cells (Figures 4.30A, B and 4.39, 5.1). Elevated level of miR-155 inhibits production of both PU.1 mRNA and protein in B-CLL (Figures 4.4, 4.37). Inhibition of miR-155 increased PU.1 mRNA (Figure 4.36). In support of this, Vigorito *et al.*, in miR-155 deficient mice detected elevated levels of PU.1 and after PU.1 rescue the levels of miR-155 decreased in *in vitro* model¹⁵⁷(Figure 5.1). This notion is in accordance with our PU.1 rescue experiment, where we significantly decreased miR-155 (Figures 4.38, 4.39 and 5.1). Interestingly, we found negative relationship between expression of MYB and PU.1, where PU.1 rescue decreased MYB expression in B-CLL (Figure 4.38). Zhao *et al.*, found that MYB inhibits PU.1 expression by CHIP-sequencing method²⁸⁷. Elevated expression of miR-150 inhibits MYB expression²²⁸. Finally, 20% of our patient cohort fit the criteria of described working model: >2.5-fold increase miR-155, >1.5-fold increase MYB and <0.5-fold decrease of PU.1 expression (Figures 4.40; 5.1). Based on these results, therapy of B-CLL could be in future directed to development of synthetic molecules - oligonucleotides that will inhibit production of MYB (siRNA MYB) or miR-155 (anti-miR-155).

Due to aberrant expression of miR-155 in numerous leukemia and cancers and its impact on the prognosis, disease progression, miR-155 could be a key target for future cancer therapy. The first transgenic mouse model (E μ -mmu-miR-155) confirms that miR-155 acts on development of B-cell leukemia/lymphoma¹⁸². MiR-155 expressing mice initially displayed pre-leukemic B-cell proliferation in spleen and bone marrow followed by

splenomegaly and progression in B-cell lymphoma within seven months¹⁸². Recent work on miR-155 knock-in mouse model (tTA-miR-155 ROSA26) with elevated levels of miR-155 proved an anti-miR-155 therapy as possible treatment of B-cell lymphoma¹⁸⁴. The majority (~60%) of knock-in mice with induced expression of miR-155 developed in five months lymphoma. In contrast, animals without induction of miR-155 expression did not develop aggressive lymphoma. After one week of treatment miR-155 knock-in mice with lymphoma by anti-miR-155 oligonucleotides encapsulated in nanoparticles led to the visible improvement e.g. delay in tumor growth. Efficacy of anti-miR-155 treatment was dose dependent and coated nanoparticles may improve tumor cells uptake that led to increased therapy efficacy¹⁸⁴. These data underline the anti-cancer potential of anti-miR-155 nanoparticles in therapy of B-cell malignancies. It has been noted that “antagomirs” (the single-stranded RNA analogues complementary to the specific miRNA) or other chemically modified oligonucleotides need an appropriate vehicle to achieve efficient inhibition²⁸⁸. Up to now, such experiments with delivery of anti-miR-155 were done only in mice models¹⁸⁴ or *in vitro* on the primary B-CLL cells (Figure 4.36; and²⁶⁹). All these data collectively suggest that approaches based on interruption of miR-155 function may have therapeutic ability in treatment of B-cell malignancies.

The miR-155 acts cooperatively with other proto-oncogene or tumor suppressor gene in driving the leukemogenesis; here we found that one such cooperating gene is proto-oncogene MYB in B-CLL¹⁴⁴. Our data support the importance of MYB proto-oncogene in enhancing the miR-155 production in primary B-CLL cells (Figure 4.30B). In normal hematopoiesis, MYB regulates cell proliferation and differentiation²¹². Higher levels of MYB were observed in acute myeloid²³⁵ and lymphoid leukemia²¹⁷ but not in B-CLL. Recently become rediscovered the role of MYB during leukemogenesis. While the mRNA of MYB is often aberrantly produced or duplicated in leukemia – the targeted inhibition of MYB can have wide clinical uses. The importance of the MYB transcription factor can be confirmed by the fact that homozygous null mice die at the embryonic stage due to inability to switch from fetal to adult erythropoiesis; hence, the mice became anemic²²⁴. Authors Lieu and Reddy demonstrated reduced proliferative capacity, an aberrant, accelerated differentiation of hematopoietic stem cells (HSCs) due to disruption of *MYB* gene in mouse model. Hematopoiesis was also impaired it resulted in decreased production of neutrophils, monocytes, B-cells, erythrocytes and megakaryocytes in mice. These data indicate that MYB

is a key regulator of self-renewal and differentiation of HSCs ²⁸⁹. Expression of MYB is required for leukemia maintenance while MYB suppression in mice resulted in delayed disease progression, induced by clearance of leukemia cells from all infiltrated organs and led to the complete remission ²⁹⁰. This sensitivity to MYB inhibition could be applied also to human leukemia cells because the inhibition of MYB in mouse model was much more efficient than mimicking the conventional AML therapy used in humans. The authors found that MYB suppression leads to eradication of aggressive AML without any impact on the normal myelopoiesis ²⁹¹. The mRNA and protein half-life should be considered in the target gene selection. The MYB mRNA, as well as its encoded protein, has an estimated half-life of approximately 30 to 50 minutes ²⁹² that is an important factor for efficiency of mRNA targeting. Luger SM *et al.*, showed that mRNA and protein levels correlate. Further, it is clear that in the absence of mRNA to translate, the protein levels of MYB decline rapidly ²⁹³. In 2008, a trial study with official title “Infusional MYB Antisense Oligodeoxynucleotide in Advanced Hematological Malignancies” was started. The aim of the study was evaluation of MYB antisense oligonucleotides in treatment of advanced hematologic malignancies as B-CLL, chronic myeloid leukemia and multiple myeloma (<http://clinicaltrialsfeeds.org/clinical-trials/show/NCT00780052>). Results of this study have not been published yet, but results seem to be very promising for leukemia patients. Based on all the evidence presented above, anti-microRNA or small interfering mRNA based synthetic molecules seem to be very promising therapy tools in leukemia treatment.

Aim of the Thesis was to explore and understand the molecular mechanism of miR-155 and its target genes: MYB, PU.1 in B-CLL and seek for association between expressions of studied molecules and prognosis/progression of B-CLL. Importance of miR-155 in prognosis/progression confirms its increased level in advanced Rai stage and in patient group with unfavorable prognosis. We shown that aberrant expression of miR-155 in B-CLL inhibits both mRNA and protein levels of its target gene PU.1. Here we answer the question what causes an aberrant expression of miR-155 in B-CLL and if miR-155 can be used as biomarker of B-CLL progression. We provide evidence that MYB binds and stimulates *MIR155HG* in B-CLL. Moreover, the MYB recruited loci at *MIR155HG* promoter region overlapped with pattern of active epigenetic marks as H3K9Ac and H4K4Me3. It should be noted that the MYB elevation in B-CLL is possibly important not only for the up-regulation of

miR-155 but also for the regulation of additional target genes, including *BCL2* and others known as deregulated in B-CLL. As well as to look on the global gene expression arrays to find out if the miR-155 and MYB programs are deregulated in B-CLL. Finally, we created the working model of B-CLL based on the *in vitro* functional assays on the primary B-CLL cells. The criteria of this model fulfilled 20% of B-CLL patients samples in our cohort.

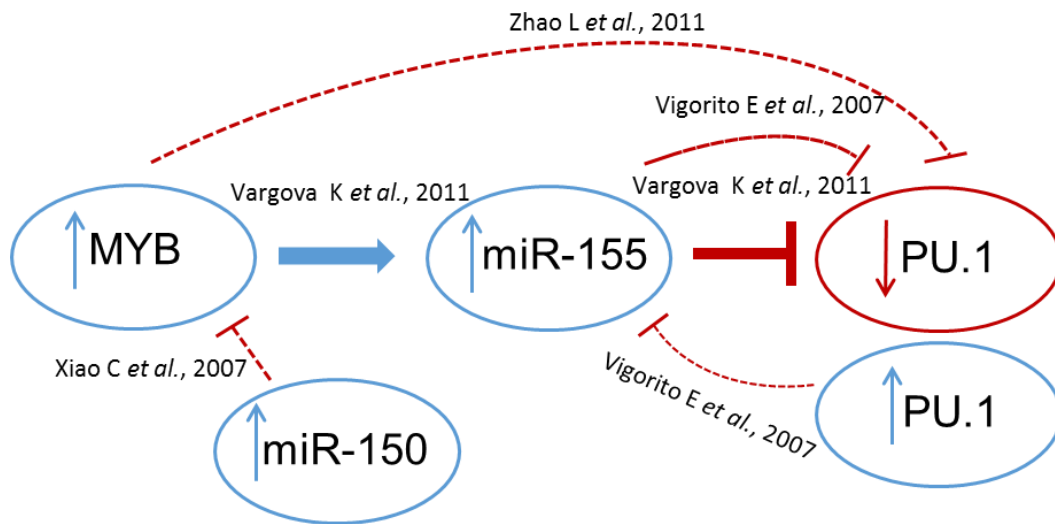


Figure 5.1: Working model of MYB-miR-155-PU.1 in B-CLL. This model describes the relationship between aforementioned molecules based on our results and published data.

6. CONCLUSIONS

- We detected significant overproduction of both primary transcript of miR-155 and its mature form in B-CLL patient cells as compared with the normal healthy B-cells. Expression of miR-155 in the peripheral blood cells and in plasma was similar. The B-CLL patient samples with advanced Rai stage (II-IV) and with unfavorable prognosis (ZAP-70+/CD38+/IgVH unmutated) showed significantly higher expression of miR-155 in comparison with the patient group with favorable prognosis. Further, expression of miR-155 was significantly higher in B-CLL patient group with deletions of chromosome 11q22-23 and 13q14. Expression changes of miR-155 in patient groups with trisomy of chromosome 12 or with deletion of 17p13 chromosome weren't statistically significant.
- In our B-CLL patient samples we detected low levels of PU.1 at both mRNA and protein level. In the group of patient samples with advanced Rai stage (II-IV) and after several therapy regimens, the PU.1 mRNA was significantly increased. Expression of PU.1 mRNA in both (favorable and unfavorable) prognostic patient group remains overall low. Expression of PU.1 mRNA in the B-CLL patient cells with deletion of 17p13 chromosome was significantly decreased, while expression changes of PU.1 in another cytogenetic subgroups weren't statistically significant.
- In subset of ~40% of B-CLL patient samples, we detected elevated level of MYB mRNA. The protein level of MYB was in selected B-CLL patient lysates overproduced as compared to lysates from normal healthy B-cells. The ChIP assay data showed that MYB binds at the three specific loci at the promoter region of *MIR155HG*. Our data from luciferase assay showed that MYB enhances the transcription of miR-155 in B-CLL cells. In the B-CLL patient samples with unfavorable clinical outcome and with advanced Rai stages (II-IV) we detected increased expression of MYB mRNA. The expression changes of MYB mRNA in our B-CLL patient samples based on cytogenetics weren't statistically significant.

- Another B-CLL specific microRNA is miR-150, that is also involved in the maturation of B-cells. In subset of ~30 % of B-CLL patient samples we detected elevated expression of miR-150. We observed significantly decreased expression of miR-150 in B-CLL patient samples with advanced Rai stage (II-IV) and with unfavorable prognosis. Expression of miR-150 was significantly decreased in B-CLL patient samples with deletions of 11q22-23 and 17p13.
- The *in vitro* functional assays on Raji cell line and primary B-CLL patient B-cells (where expression level of miR-155 was manipulated by anti-miR-155 or mimic miR-155 and MYB by siRNA MYB or pcDNA MYB), confirmed dependence of miR-155 expression to MYB and expression of PU.1 to levels of miR-155. Furthermore, we were able to push down the expression level of miR-155 by increasing the level of PU.1 in PU.1 rescue experiment. By microarray approach we have shown that miR-155 and MYB programs are deregulated in B-CLL.
- Generally, our data indicate relationship between miR-155, MYB and PU.1 in B-CLL. We created working model of this relationship in B-CLL patient cells. About 20% of B-CLL patients from our cohort match this model. Finally, it should be concluded that the read out of miR-155, MYB and PU.1 ratio by RT-qPCR could be used as an additional and reliable prognostic marker of B-CLL disorder progression.

7. SUMMARY

This PhD Thesis is focused on the detection of the expression changes of two microRNAs related to B-CLL (miR-155 and miR-150) and two transcription factors (PU.1 and MYB) involved in B-cell maturation process.

Our expression data confirmed elevated levels of miR-155 in the B-CLL patients' peripheral blood mononuclear cells as well as in the blood plasma. We detected increased expression of miR-155 in patient samples with advanced Rai stage (II-IV) and with unfavorable B-CLL clinical outcome. From cytogenetic point of view, the B-CLL cells with deletion of chromosome 17p13 expressed more miR-155.

The expression level of PU.1 fluctuates during the maturation of blood cells; however, when the level of PU.1 expression declines under 20%, the leukemia develops. We detected significant decrease of PU.1 mRNA expression and protein level in B-CLL cells. In B-CLL cells of patients with advanced Rai stage (II-IV), the level of PU.1 mRNA was decreased; however, in cells of patients with therapy regimens, the level of PU.1 mRNA was increased. The difference between expression of PU.1 mRNA in the B-CLL patient samples with favorable and unfavorable prognosis was not statistically significant. Interestingly, B-CLL patient samples with deletion of 17p13 chromosome displayed significantly lower expression of PU.1 mRNA.

Transcription factor MYB plays a crucial role during the development of B-cells. Aberrant expression of MYB leads to lymphoproliferative disorders. Up to now, the role of MYB has been described only in acute and chronic myeloid leukemia but not in B-CLL. We observed increased expression of MYB mRNA and protein in a subset of B-CLL patient samples (~40%). By CHIP assay, we further detected that MYB specifically binds at the promoter region of *MIR155HG* and thereby stimulates the transcription of miR-155 in B-CLL cells. In B-CLL patient samples with advanced Rai stage (II-IV) the expression of MYB was higher as in patients with Rai stage 0-I. The group of B-CLL patient samples with unfavorable prognosis displayed higher expression of MYB in comparison to favorable prognosis group. The expression of MYB mRNA in B-CLL patient samples did not correlate with cytogenetic aberrations.

Another B-CLL specific microRNA is miR-150, that is also involved in the maturation of B-cells. The miR-150 plays a role in B-CLL progression. Expression of miR-150 decreased with the disease progression: lower miR-150 expression was detected in cells of patients with advanced Rai stage (II-IV) and with unfavorable prognosis. Similarly, expression of miR-150 was significantly lower in the B-CLL patient samples with deletions of 11q22-23 and 17p13.

Our data, generated from *in vitro* functional assays, indicated the existence of a tight relationship between miR-155, PU.1 and MYB molecules in B-CLL cells. We created the working model of such tight relationship. Briefly, the expression of MYB molecule stimulates the expression of miR-155 molecule. The aberrantly expressed miR-155 inhibits the PU.1 expression. This leads to B-CLL progression.

The read out of miR-155, MYB and PU.1 ratio by RT-qPCR could be used as a reliable prognostic marker of B-CLL progression. Furthermore, the restoration of the normal balance between studied molecules in leukemic B-cells could be used as a future therapy of B-CLL.

8. AUTHOR CONTRIBUTION

The author wrote the Thesis and, under the kind supervision, performed following experimental procedures: routine cell culture work, patient/control samples preparation (Ficoll-Paque and Rosette separation), mRNA/microRNA extraction, qRT-PCR and delta-delta analysis, transfections (Amaxa, DMRIE-c, JetPEI). Other members of the laboratory helped me with following methods: Pavel Burda with chromatin immunoprecipitations, Nikola Čuřík with western blotting and luciferase assay and Filipp Savvulidi with the flow cytometry analysis and FACS cell sorting. FISH analysis (cytogenetics) was performed by Adela Berková. The patient clinical data was collected by Petra Obtrlíková. The bioinformatical analyses were performed by Vojtech Kulvait and Jiří Zavadil (microarrays and GSEA). My mentor, Tomáš Stopka helped me with the experimental design, reviewed the results and revised the PhD Thesis and research publications.

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Other sources of literature (internet links):

- [1] <http://moon.ouhsc.edu/kfung/jty1/HemeLearn/HemeCase/PB-001-Ans.htm> (B-CLL cells blood smear picture)
- [2] <http://www.ncbi.nlm.nih.gov/gene/114614> (*MIR155HG* gene)
- [3] <http://www.ebi.ac.uk/ebisearch/search.ebi?query=MYB&db=allebi&requestFrom=searchBox&submit=Search> (*MYB* gene)
- [4] <http://ncicll.com/>(WHO B-CLL classification)
- [5] <http://www.stemcell.com/en/Products/All-Products/RosetteSep-Human-B-Cell-Enrichment-Cocktail.aspx> (Rosette separation scheme)
- [6] http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_041461.pdf (TaqMan qRT-PCR)
- [7] <http://bioinfogp.cnb.csic.es/tools/venny/index.html> (Wenn diagram)
- [8] <http://clinicaltrialsfeeds.org/clinical-trials/show/NCT00780052> (MYB trial study, 2008)

miR-155 target gene databases:

PicTar: <http://pictar.mdc-berlin.de/>

Targetscan: <http://www.targetscan.org/>

Microna: <http://www.microna.org/>

Microcosm: <http://www.ebi.ac.uk/enright-srv/microcosm/>

TargetsDiana: <http://diana.cslab.ece.ntua.gr/tarbase/>

GSEAsset: <http://www.broadinstitute.org/gsea/msigdb/cards/AGCATT,MIR-155.html>

MYB target gene databases:

Set MYB TARGETS GENEGO: <http://www.genego.com/>

Set MYB TARGETS RULAI.CSHL.EDU: <http://rulai.cshl.edu/cgi-bin/TRED/>

Our GSEA data are available at: <http://www1.lf1.cuni.cz/~vkulv/gsea>

10. SUPPLEMENT

Supplemental table 10.1: Clinical and expression data of patient cohort from General Faculty Hospital in Prague (at the last page of the Thesis).

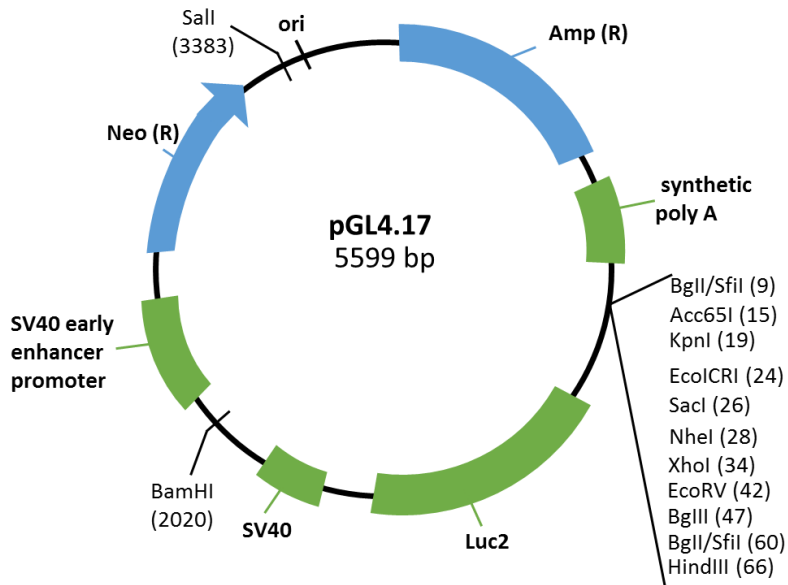
Supplemental table 10.2: Clinical and expression data of patient cohort from University Hospital and Medical Faculty Brno (at the last page of the Thesis).

Supplemental table 10.3: Clinical and expression data of healthy donors (controls) from Institute of Hematology and Blood Transfusion in Prague.

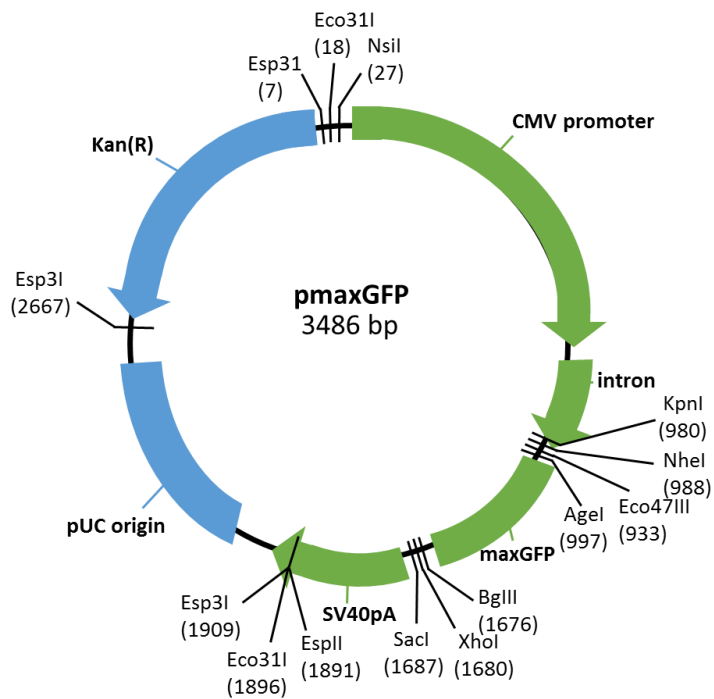
Sample info					Expression (fold change)						
Code of sample	Date of sample	Gender	Donors age at the date of sample	Sample origin (PBMC or CD19+)	miR-155	PU.1	MYB	miR-150	pri-miR-155	FOS	MYC
C63	24.8.2009	M	36	PBMC	NA	NA	0,51	NA	ND	ND	ND
C76	10.9.2010	M	22	PBMC	0,61	NA	1,68	NA	ND	ND	ND
C77	10.9.2010	M	20	PBMC	1,17	1,17	1,78	NA	ND	ND	ND
C83	27.10.2010	M	22	PBMC	NA	NA	NA	NA	ND	ND	ND
C81	26.10.2010	M	31	PBMC	NA	NA	0,21	NA	ND	ND	ND
C84	27.10.2010	M	22	PBMC	NA	NA	0,61	NA	ND	ND	ND
C93	6.2.2010	M	31	PBMC	0,44	0,83	0,72	0,99	ND	ND	ND
C94	6.2.2010	M	29	PBMC	0,34	1,58	0,73	0,98	ND	ND	ND
C95	6.9.2010	M	38	PBMC	0,46	0,77	0,60	1,01	ND	ND	ND
C96	7.9.2010	M	38	PBMC	0,42	0,65	0,47	1,02	ND	ND	ND
C51	14.7.2010	M	38	CD19+	1,11	0,44	NA	1,53	0,04	0,61	0,74
C52	20.7.2009	M	28	CD19+	0,87	0,33	NA	NA	NA	0,58	1,05
C53	20.7.2009	M	35	CD19+	0,98	0,66	0,00	0,76	0,01	0,77	0,72
C55	27.7.2009	M	30	CD19+	0,62	2,78	0,00	0,59	0,02	3,23	2,07
C56	8.3.2009	M	35	CD19+	2,40	0,93	0,06	2,30	0,06	0,31	1,29
C57	8.3.2009	M	29	CD19+	1,30	0,56	NA	1,69	0,03	0,13	1,71
C58	8.10.2009	M	31	CD19+	0,44	0,47	0,01	1,07	0,00	1,16	1,88
C60	17.8.2009	F	29	CD19+	NA	0,70	NA	NA	0,38	0,25	0,35
C62	24.8.2009	M	43	CD19+	0,77	0,67	0,01	0,89	0,03	1,34	0,51
C75	10.6.2009	F	25	CD19+	0,93	0,55	0,01	NA	0,70	0,57	1,01
C46	29.6.2009	M	35	CD19+	0,46	2,95	0,01	NA	1,45	NA	0,57
C47	29.6.2009	M	28	CD19+	0,78	0,97	0,00	NA	0,13	1,18	0,09

Supplemental material 10.1: Restriction maps of plasmids

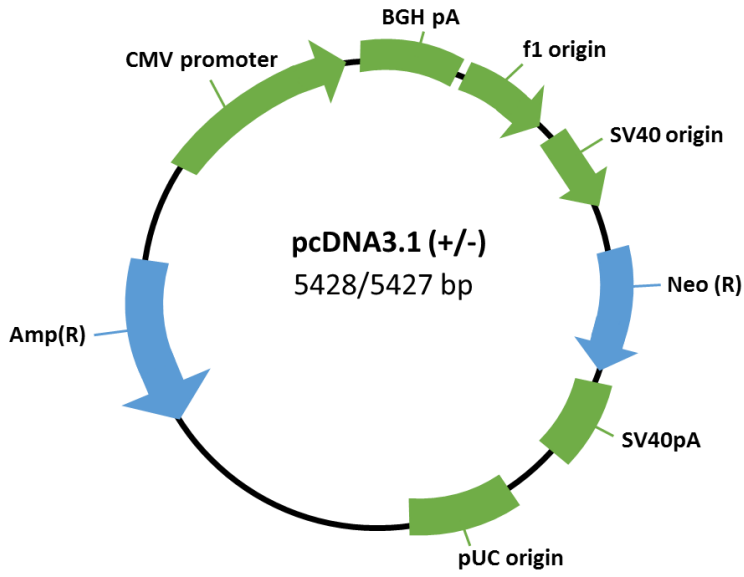
Generic pGL4.17 vector map



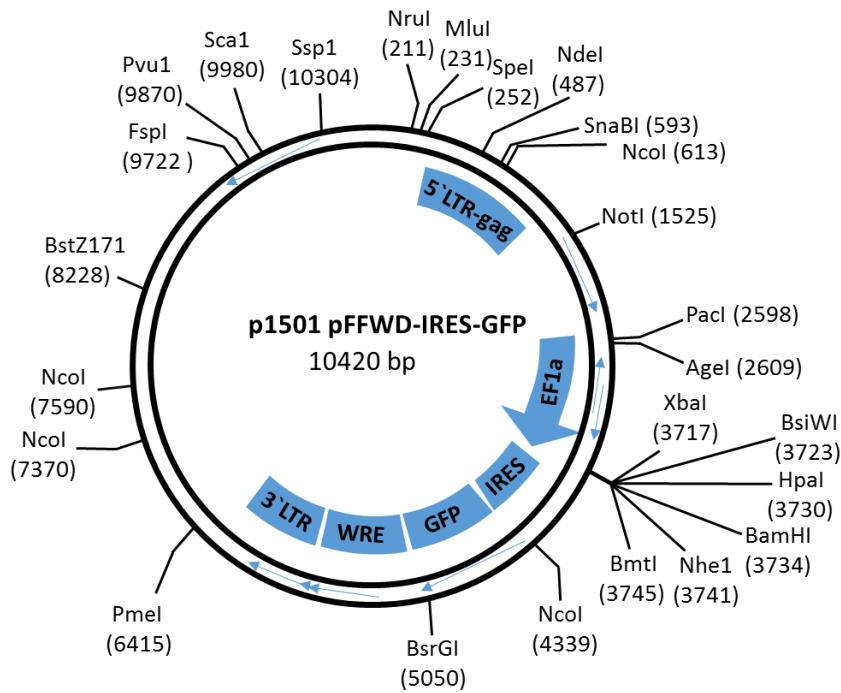
Generic pmaxGFP vector map



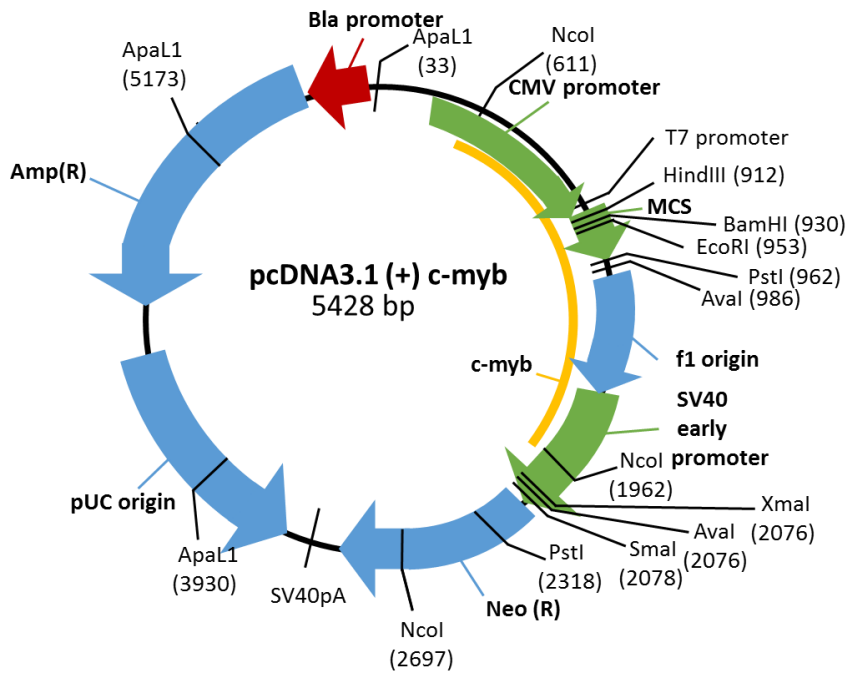
Generic pcDNA3.1 (+/-) vector map



Generic PU.1-IRES-GFP vector map



Generic pcDNA3.1 (+) c-myb vector map



Supplemental material 10.2: Detailed protocols.

Isolation of peripheral blood mononuclear cells (PBMCs) by Ficoll – Paque:

- 1) Dilute the peripheral anti-coagulated blood with sterile 1xPBS in ratio 1:1.
- 2) Load carefully diluted blood sample on the Ficoll-Paque PREMIUM layer in ratio 1:1.
- 3) Centrifuge at 2000rpm for 25min at 18-20°C; with brake off and use swing out rotor.
- 4) Harvest and wash the PBMCs (“foggy ring”) with sterile 1xPBS (3x volume) at 1500rpm for 6min at 4°C.
- 5) The separated PBMCs can be now used for culturing or RNA/miRNA or DNA isolating or can be frozen for future experiments.

Isolation of CD19+ B-cells by RosetteSep kit:

- 1) To the 1mL of the whole anti-coagulated blood add 50µL of RosetteSep® Human B Cell Enrichment Cocktail and incubate for 20min at room temperature. (NOTE: If you have frozen blood cells, thaw the cells and wash with sterile 1xPBS supplemented with 2% of FBS; then follow the protocol).
- 2) Dilute the sample with sterile 1xPBS with 2% of FBS in ratio 1:1 and mix gently.
- 3) Load carefully the diluted blood sample on the Ficoll-Paque PREMIUM layer in ratio 1:1.
- 4) Centrifuge at 2000rpm for 20min at 18-20°C; with brake off; with swing out rotor.
- 5) The desired CD19+ B-cells will be at the interlayer between Ficoll-Paque and plasma.
- 6) Wash desired CD19+ B-cells with sterile 1xPBS (3x volume) at 1500rpm for 6min at 4°C.
- 6) The desired CD19+ B-cells (untouched) can be used for culturing or RNA/miRNA/DNA isolation or can be frozen for future experiments.

Lysis of erythrocytes by ammonium chloride (NH₄Cl):

- 1) Warm the solution of NH₄Cl up to 37°C.
- 2) To the 1mL of peripheral blood add 49mL (50x) of warmed up NH₄Cl and invert the tube several times, incubate at 37°C for 15-20min (in shaking incubator).
- 3) Centrifuge the cells after lysis at 1500rpm for 10min at 4°C.
- 4) Aspirate the supernatant and the cell pellet resuspend in 10mL of 1xPBS and centrifuge as in the step 3.
- 5) Resuspend the cell pellet in the appropriate culture medium or start the isolation of RNA, DNA or just freeze the cell pellet for later analysis. (NOTE: freezing medium contains 90% of FBS and 10% of DMSO).

Magnetic separation of CD19+ B-cells by MACS magnetic beads:

- 1) Determine the cell number and wash cells with MACS buffer (5mL).
- 2) Pellet the cells by centrifuging at 1500rpm for 10min at 4°C.
- 3) To the cell pellet add 7µL of anti-CD19 FITC antibody and incubate on ice for 20min.
- 4) Wash out the unbound antibody by MACS buffer (3mL), centrifuge at 1500rpm for 10min at 4°C.
- 5) Add 80µL of MACS buffer to the cell pellet and 20µL of FITC beads per 10⁷ cells.
- 6) Mix well and incubate for 15min in the refrigerator by occasional vortexing.
- 7) Wash with 3mL of MACS buffer 1500rpm for 10min at 4°C.
- 8) Aspirate the supernatant completely and resuspend the cell pellet in 500µL of MACS buffer.
- 9) Prepare appropriate magnetic separator and magnetic column.
- 10) Rinse the column with appropriate volume of MACS buffer (LS column with 3mL of MACS buffer) and apply the cell suspension onto the empty column reservoir.
- 11) The fraction of unlabeled cells will pass through the column; collect this fraction into appropriate tube.
- 12) Wash the column with MACS buffer 3 times (LS column with 3x3mL of MACS buffer).
- 13) Apply 5mL of MACS buffer onto empty column reservoir and flush out the labeled CD19+ B cell fraction.
- 14) Centrifuge both fractions (labeled, unlabeled) at 1500rpm for 10min at 4°C and prepare aliquots (10x10³ cells) for flow cytometry measurement.

Transfection of primary B-CLL cells by lipofection reagent DMRIE-c:

- 1) Grow the cells to ~80% of confluency and determine the cell number.
- 2) Prior the transfection with DMRIE-c wash the cells (0.8x10⁶ cells/mL/6 well) with OPTI-MEM medium (2mL).
- 3) Centrifuge the cells at 1500rpm for 6min at 18-20°C and resuspend in 400µL of OPTI-MEM medium/well.
- 4) Prepare transfection reactions with the final volume 100µL/well. Transfection reaction contains: DMRIE-c (2µL/reaction) + oligonucleotides (MYB siRNA (30nM) or anti-miR-155 (40nM) or negative control oligonucleotides) + pmaxGFP plasmid (2µg/reaction). Fill up the reaction with OPTI-MEM medium to get the final volume 100µL/well.

- 5) Apply the transfection reactions on the cells and incubate for 5hrs in humidified atmosphere with 5% CO₂ at 37°C. (NOTE: the transfection reactions should be applied very slowly – by drop-wise).
- 6) After 5hrs of incubation add the equal volume of cultivation IMDM medium supplemented with 20% FBS and 1% of MEM non-essential amino acids (500µl).
- 7) After 48-96hrs harvest the cells, isolate RNA and perform RT-qPCR.

Transfection of HeLa cells by lipofection reagent JetPEI:

- 1) Grow the cells in confluence ~60% and seed 80×10^3 cells per 24 well tissue plate in 1mL of culture medium (RPMI-1640 supplemented with 10% of FBS).
- 2) Prepare transfection reactions in the final volume of 100µL.
- 3) To the 50µL of 150mM NaCl solution add 1.5µg of plasmid DNA. Vortex gently and spin down briefly.
- 4) To the 50µL of 150mM NaCl solution add 2µL of jetPEI solution. Vortex gently and spin down briefly.
- 5) Mix the prepared solutions, vortex and spin down. Incubate for 25min at room temperature.
- 6) Apply 100µL of the prepared solution drop-wise onto the cells and culture cells in humidified atmosphere with 5% CO₂ at 37°C.
- 7) After 48hrs harvest the cells, prepare cell lysates and perform the reporter gene assay and western blot.

Transfection of Raji cells by Amaxa nucleofector:

- 1) 24hrs prior to transfection determine the cell number, seed in concentration 2×10^6 cells per 1/6 well plate and culture in IMDM medium supplemented with 10% of FBS, 1% of MEM non-essential amino acids, but without antibiotics!
- 2) On the next day, wash the cells with 1xPBS (2mL).
- 3) Centrifuge the cells at 400g for 6min at 18-20°C and resuspend in 2mL/well of IMDM medium supplemented with 10% of FBS and 1% of MEM non-essential amino acids.
- 4) Prepare the working nucleofection solution by mixing the nucleofection solution and supplement in ratio 4.5:1.
- 5) Combine the 100µl of nucleofection solution with: oligonucleotides [MYB siRNA (30nM, 100nM)/anti-miR-155 (40nM, 100nM)/mimic-miR-155 (40nM, 100nM) and appropriate

negative control oligonucleotides] + pmaxGFP plasmid (2µg/reaction) or MYB and control pcDNA plasmids (0.2µg, 2µg).

6) Transfer the cell/RNA/DNA suspension into certified cuvette (sample must cover the bottom of the cuvette without air bubbles), close the cuvette with the cap, insert into nucleofector and select the appropriate nucleofector program M-013 for Raji cell line.

7) Take the cuvette out of the holder once the program is finished and transfer cells immediately into prepared warmed up IMDM medium.

8) Incubate cells in humidified atmosphere with 5% CO₂ at 37°C until analysis.

9) Harvest the cells after 24hrs for mimic hsa-miR-155 transfection and 48hrs for siRNA MYB, anti-miR-155 and plasmid MYB transfections. Isolate RNA and perform RT-qPCR.

Transduction of primary B-CLL cells with lentivirus:

1) Plate 1x10⁶ B-CLL cells* in 24 well plates.

2) For sensitizing of cells add polybrane in the final concentration 8µg/ml.

3) Add the lentivirus with 3 MOI (MOI in range 3-10).

4) Spin the plate for 30min at 1000g 32°C, incubate overnight in the 37°C incubator.

5) Change the culture medium for cells on the next day.

6) After 72hrs harvest cells, isolate RNA and perform RT-qPCR.

* Cell concentration depends on the cell type, lower is better, because lower cell concentration could increase the MOI.

Calculation of MOI: If 60µL of virus (virus titer: 5x10⁷ TU/mL (transduction unit/mL)) was added to 1x10⁶ cells, the MOI will be 3.

MOI = virus amount (mL) x virus titer (TU/mL)/cell number

MOI = 0.06 mL x 5x10⁷TU/mL /1x10⁶=3 (e.g.).

Luciferase reporter assay:

- 1) Prepare the luciferase reagent by combining the Dual-Glo[®] Luciferase buffer with Dual-Glo Luciferase substrate in the ratio 1:100. Mix by inversion until the substrate is thoroughly dissolved.
- 2) Resuspend the cells in the prepared reagent (75 μ L/well). (NOTE: Reagents should be added 10min before quantifying the luminescence. For the maximal light intensity, samples should be measured within 2hrs of reagent addition.)
- 3) In 10min measure the luminiscence on the luminometer.

Control measurements for CD19 purity after MACS separation:

- 1) Wash the cells (10x10³ cells) with 1xPBS/1% BSA buffer; centrifuge at 400g for 6min at 4°C.
- 2) Add 7 μ L/tube of CD19 FITC antibody to the cell pellet and incubate on ice for 20min.
- 3) Wash the cells with 3mL of 1xPBS/1% BSA buffer; centrifuge at 400g for 6min at 4°C.
- 4) Resuspend the cell pellet in 300 μ L of 1xPBS/1% BSA buffer.
- 5) Add 1 μ L of PI and immediately measure on FACS.

AnnexinV staining:

- 1) Harvest cells (~1x10⁶ cells) and wash in 1xPBS; centrifuge at 400g for 6min at 4°C.
- 2) Resuspend the cell pellet in 3mL of 10x diluted binding buffer; centrifuge at 400g for 6min at 4°C.
- 3) Resuspend cell pellet in 100 μ L of 1x diluted binding buffer and add 5 μ L of FITC conjugated AnnexinV, incubate at room temperature for 10-15min in dark.
- 4) Wash cells in 1x binding buffer and centrifuge at 400g for 6min at 4°C.
- 5) Resuspend cells in 200 μ L of 1x binding buffer and proceed FACS analysis.

Western blot:

- 1) Harvest cells (3x10⁶); centrifuge at 2000rpm for 5min at RT.
- 2) Wash the cells with 1xPBS (500 μ l); centrifuge at 2000rpm for 5min at RT.
- 3) Lyse the cells in RIPA buffer (200 μ l) and incubate on ice for 12min. In time intervals 3, 6, 9 and 12min during the incubation time vortex the samples for 15sec.
- 4) Sonicate the protein lysates on the Sonic Dismembrator with a micro tip (50% amplitude, 3 cycles for 1sec, pause 5sec). Immediately after sonication procedure place the samples on ice for 10min.

- 5) Determine the protein concentration on Biophotometer. Use Bradford reagent for protein measurement and construct the calibration curve.
- 6) To the 20ng protein lysate add 6 μ L of NuPAGE LDS sample buffer (4x) and fill up with the NuPAGE MOPS SDS buffer to the final volume 24 μ L.
- 7) Denature proteins at 70°C for 10min and immediately load (20ng per lane) on the NuPAGE 10% Bis-Tris gel in NuPAGE MOPS SDS (1x) running buffer. The running conditions are as follows: 80V (15min) - 120V (1hr), 125mA, for 90min.
- 8) After running the gel, transfer gel into prepared iBlot gel transfer stacks and blot the gel on the PVDF membrane (program: P2, blotting time 7min).
- 9) To block the unspecific binding, incubate the membrane with 7.5% non-fat milk in PBS/0.1% Tween 20 for 45min on the shaking platform.
- 10) Incubate with primary antibody (anti-PU.1, anti-MYB, anti-actin) in concentration 1:600 (PU.1, c-MYB) and 1:100 (β -actin) diluted in 7.5% non-fat milk in PBS/0.1% Tween 20 at 4°C overnight.
- 11) Wash the membrane 3x with 7.5% non-fat milk in PBS/0.1% Tween 20 for 20min on the shaking platform.
- 12) Incubate with secondary antibody in concentration 1:4000 diluted in 7.5% non-fat milk in PBS/0.1% Tween 20 at room temperature for 1hour on the shaking platform.
- 13) Wash the membrane 3x with 7.5% non-fat milk in PBS/0.1% Tween 20 for 20min on the shaking platform.
- 14) For detection of signal use ECLPlus Western blot system; mix an equal volume of detection solution 1 with detection solution 2 allowing sufficient total volume to cover the membrane.
- 15) Dry the excess of wash buffer from the membrane and place with protein side up on wrap; pipette the mixed detection reagent on to the membrane and incubate for 1min at room temperature.
- 16) Dry the excess of detection buffer from membrane and place with protein side down on to a fresh piece of wrap; wrap up the blots and gently smooth out any air bubbles. Place the wrapped blots with the protein side up in an X-ray film cassette.
- 17) Place a sheet of autoradiography film (Hyperfilm ECL) on top of the membrane, close the cassette and expose for e.g. 15 sec. Develop the first piece of film immediately, and on the

basis of its appearance estimate how long to continue the exposure of the second piece of film.

18) After developing of film store the membrane wet and wrapped in a refrigerator at 4°C.

Chromatin immunoprecipitation (ChIP):

1) Harvest the cells ($\sim 1 \times 10^6$) and crosslink with formaldehyde in the final concentration 1% (3mL into 30mL of medium) for 15min at room temperature.

2) Stop the crosslink reaction by adding 2.5M glycine to a final concentration of 0.15M (2.6ml to 50ml mixture); incubate on ice for 5min.

3) Centrifuge the cells at 1900g for 4min at 4°C, remove supernatant and leave on ice for 5 min. Resuspend and rinse the pellets in pre-cold 1xPBS (app. 7.5ml) by up and down pipetting several times and leave on ice for about 30min.

4) Centrifuge at 4000g for 3min to collect pellets. Prepare fresh lysis buffer I, II, III and keep them on ice. (NOTE: during all following steps the cell pellets should be done on ice!)

5) Add to the pellet 10mL of lysis buffer I; mix and centrifuge at 400g for 10min at 4°C.

6) Add to the pellet 10mL of lysis buffer II; mix and leave on ice for 10min.

7) Centrifuge at 400g for 10min at 4°C; add 2mL of lysis buffer III to the pellet, mix by short vortex and transfer into 15mL polypropylene tube and follow to sonication step or freeze at -80°C.

8) Sonicate the samples in a dry ice-ethanol cooling bath with 40% intensity (amplitude) in 500 cycles of 2sec of pulses to yield the 200-600bp DNA fragments. Centrifuge at 400g for 10min at 4°C.

9) Add proteinase K buffer (170 μ L to 20 μ L of sonicated aliquot), 10 μ g/tube of RNase, 10mg/mL of proteinase K and incubate overnight at 37°C.

10) Add 20 μ L of 3M sodium acetate (pH=7), 2 μ L of glycogen, 500 μ L of 96% ethanol and incubate at -20°C for 15min.

11) Centrifuge at max. speed for 15min at 4°C, carefully remove supernatant, wash the pellets with 500 μ L of 70% ethanol; Centrifuge at max. speed for 10min at 4°C and dry the DNA pellets on air.

- 12) Dissolve DNA pellets in 25 μ L of TE buffer by incubating at 37 $^{\circ}$ C for 30min at shaking platform. Run an aliquot of sample on 1% agarose gel to check for presence of 200-300bp DNA bands.
- 13) Add 5 μ L of protein A and G beads to the 90 μ L of immunoprecipitation buffer (IP); centrifuge at 5000g for 5min at room temperature. Resuspend pellet with beads in 150 μ L of IP buffer and add 30 μ L of BSA (10mg/mL). Incubate for 2hrs at 4 $^{\circ}$ C on shaking platform.
- 14) Clear the chromatin by adding 105 μ L of IP buffer and 10 μ L of protein A and G beads, gently shake and centrifuge at 400g for 5min at 4 $^{\circ}$ C.
- 15) To the pellet add 30 μ L of IP buffer and incubate for 4 hrs (or overnight) at 4 $^{\circ}$ C on shaking platform.
- 16) Add 1-2 μ g of antibody per tube (MYB, H3K9acetyl, H3K4methyl and control antibody (IgG)) and incubate for 2hrs at 4 $^{\circ}$ C on shaking platform.
- 17) Centrifuge at 14000g for 10min at 4 $^{\circ}$ C. Take out 100 μ L of supernatant as "DNA input" control.
- 18) Add 100 μ L of chromatin per 100 μ L of antibody-beads and add 800 μ L of IP buffer, incubate overnight at 4 $^{\circ}$ C on shaking platform.
- 19) On the next day continue the incubation at room temperature for 1hour on the shaking platform.
- 20) Centrifuge at 5000g for 5min at 4 $^{\circ}$ C, remove supernatant. Add 900 μ L of IP buffer and centrifuge again.
- 21) Remove 950 μ L of supernatant and add 1mL of wash buffer.
- 22) Centrifuge at 6000g for 6min at 4 $^{\circ}$ C, remove supernatant. Add 900 μ L of IP buffer and centrifuge again. Repeat step 21 and 22.
- 23) Add 1mL of wash buffer II and centrifuge at 6000g for 6 at 4 $^{\circ}$ C, remove supernatant.
- 24) Resuspend pellet in 100 μ L of proteinase K buffer and add 1 μ L of proteinase K, 10 μ L of RNase per 100 μ L of buffer. Incubate at 37 $^{\circ}$ C for 30min, overnight at 55 $^{\circ}$ C and 2hrs at 65 $^{\circ}$ C to reverse crosslink.
- 25) Dilute 10% of input into 2%, 1%, 0.5%, 0.25%, 0.125% and 0.0625% for the preparation of standard curve. Perform the qPCR.

Isolation of mRNA/miRNA from PBMCs, B-cells, Raji cells by TRIzol reagent:

- 1) Harvest the cells ($\sim 5 \times 10^6$) and centrifuge at 1500rpm for 6min at room temperature.
- 2) Decant the supernatant completely and resuspend (by up and down pipetting) the cell pellet in 1mL of TRIzol reagent until it's clear (use syringe with needle), leave for 5 min on the bench to complete the cell lysis. In this step you can stop and store the sample at -80°C or continue RNA isolation.
- 3) To the TRIzol cell suspension add 200 μL of chloroform (CH_3Cl) (20% of the total volume), cap the tube securely and vortex for 20sec. Centrifuge at 11000rpm for 15min at 4°C .
- 4) Aspirate the clear upper phase ($\sim 500\mu\text{L}$) into a new eppendorf tube and mix with the same volume of CHCl_3 , vortex and centrifuge at 11000rpm for 10min at 4°C .
- 5) Aspirate the upper aqueous clear phase and transfer into new eppendorf tube and add 1 μL of linear acrylamid, 500 μL of isopropanol, vortex and spin shortly, and precipitate at -20°C overnight.
- 6) On the next day vortex the tube/s shortly and centrifuge at max. speed (14000rpm) for 30min at 4°C .
- 7) Decant the supernatant, add 1mL of 75% ethanol, vortex gently and centrifuge at max speed (14000rpm) for 5min at 4°C .
- 8) Decant the supernatant, spin down shortly and decant the rest of liquid completely, leave the pellet to dry on the air for 3-5 min covered with a paper towel. (NOTE: do not over dry the RNA pellet!).
- 9) Resuspend the RNA pellet in 10-20 μL of nuclease free water with RNase inhibitors (1%) and analyze the RNA quantity by Nanodrop and quality by Bioanalysator Agilent. Store the RNA at -80°C . The good quality RNA should have following parameters: OD A260/A280 as well as the OD A260/A230 ratio=2.0 (or close to 2.0), the RIN (RNA Integrity Number) should be more >7 .

Isolation of miRNAs from B-CLL patient plasma samples by miRNAeasy kit:

- 1) Thaw the plasma on ice. (NOTE: The plasma samples should be kept on ice until step 6!).
- 2) Cool down the centrifuge for 4°C. Replace 250µl of plasma into 1.5 ml sterile tube. Spin down at 3000g (5500rpm) for 5min at 4°C (keep the supernatant).
- 3) Transfer 200µl of plasma to a new sterile 1.5 ml tube and add 1mL of QIAzol (5x volume) and vortex for 30sec.
- 4) Incubate for 5min at room temperature (RT) (20-25°C).
- 5) Add 200µl of chloroform (in the equal volume to the starting material), vortex and incubate for 2min at RT.
- 6) Centrifuge at 12000g (10500rpm) for 15min at 4°C.
- 7) Heat the centrifuge to RT.
- 8) Transfer the upper aqueous phase (~600µL) into 2ml tube.
- 9) Add 900µL of ethanol (1.5 volume of obtained aqueous phase) and 25µl of glycogen, mix by pipetting. Incubate the samples for 10min at 4°C.
- 10) Transfer the 750µl to the RNAeasy mini spin column, centrifuge at 13000g (11500rpm) for 30sec at RT, discard the flow-through. Repeat this step until whole sample is used – remix the suspension by pipetting before loading it again.
- 11) Discard the flow-through. Wash the column with 700µl of RWT, centrifuge at 13000g (11500rpm) for 1min at RT.
- 12) Discard the flow-through. Wash the column 3x with 500µl of RPE, centrifuge at 13000g (11500rpm) for 1min at RT. (NOTE: repeat this step 3x).
- 13) Transfer the column into a new collection tube and spin down at 16000g (14000rpm) for 2min at RT. Leave the tubes open for 2min.
- 14) Transfer column to the new 1.5 mL eppendorf tube and carefully add 50µl of RNase-free water, the liquid should be centered on the membrane!
- 15) Incubate for 2min at RT, then centrifuge at 16000g (14000rpm) for 2min at RT.
- 16) Store the isolated miRNA at -80°C.

The isolated miRNA can be directly used for reverse transcription reaction and followed by TaqMan RT-qPCR.

Supplemental material 10.3: Data from sequencing of B-CLL patient samples and control sample.

MYB F in E-box 2 (from +128bp to +607bp): ACCAAGGAGACGCTCTGGCACTG

C41 (+128) GTCTCGCGCT-CCGCCCGCCTTTCCTCTCTTGAACGTGGCAGGGACGCCGGGGGACTTCS
P89 GTCTCGCGCTCCCGCCCGCCTTTCCTCTCTTGAACGTGGCAGGGACGCCGGGGGACTTCS
P143 -TCTCGCGCT-CCGCCCGCCTTTCCTCTCTTGAACGTGGCAGGGACGCCGGGGGACTTCS

C41 GTGCGAGGGTCACCGCCGGGTTAACTGGCGAGGCAAGGCGGGGGCAGCGCGCACGTGGCC
P89 GTGCKAGGGTCACCGCCGGGTTAACTGGCGAGGCAAGGCGGGGGCAGCGCGCACGTGGCC
P143 YTGCGAGGGTCACCGCCGGGTTAACTGGCGAGGCAAGGCGGGGGCAGCGCGCACGTGGCC

C41 GTGRAGCCCGCCTGGTCCCGCGCGCGCCTGCGGGTGCCCCCTGGGGACTCAGTGGTGTG
P89 GTGGAGCCCGCCTGGTCCCGCGCGCGCCTGCGGGTGCCCCCTGGGGACTCAGTGGTGTG
P143 GTGGAGCCCGCCTGGTCCCGCGCGCGCCTGCGGGTGCCCCCTGGGGACTCAGTGGTGYC

C41 SCCTCGCCCGGGACCAGAGATTGCGCTGGATGGATTCCCGCGGGCAGAGGCAGGGGGAAG
P89 GCCTCGCCCGGGACCAGAGATTGCGCTGGATGGATTCCCGCGGGCAGAGGCAGGGGGAAG
P143 GCCTCGCCCGGGACCAGAGATTGCGCTGGATGGATTCCCGCGGGCAGAGGCAGGGGGAAG

C41 GAGGGGTGTTCGAAACCTAATACTTGAGCTTCTTTGCAAAGTTTCCTTGGATGGTTGGGG
P89 GAGGGGTGTTCAAACCTAATACTTGAGCTTCTTTGCAAAGTTTCCTTGGAKGGTTGGGG
P143 GAGGGGTGTTCGAAACCTAATACTTGAGCTTCTTTGCAAAGTTTCCTTGGATGGTTGGGG

C41 ACGTACCTGTATAATGGCCCTGGACCAGCTTCCCTGTTGGAGTGGCCAGAGAAGTGTGTA
P89 ACGTACCTGTATAATGGCCCTGGACCASCTTCCCTGTTGGAGTGGCCAGAGAAGTGTGTA
P143 ACGTACCTGTATAATGGCCCTGGACCAGCTTCCCTGTTGGAGTGGCCAGAGAAGTGTGTA

C41 AAACACACTAGAGGGGCAGGGTGGAAAAAGAGACTGCCTTCAAACCTTGTATCTTTTCGA
P89 AAACACACTAGAGGGGCAGGGTGGAAAAAGAGACTGCCTTCAAACCTTGTATCTTTTCWA
P143 AAACACACTAGAGGGGCAGGGTGGAAAAAGAGACTGCCTTCAAACCTTGTATCTTTTCGA

C41 (+548) TTTTCATTTTGAAAAATAACTACAAATCTATTTTAATTTTACAAAGTTAGACTCATAGCAT
P89 TTTTCATTTTGAAAAW-AACTA-AA-TCTATTTTAATTTTACAAAGTTR-ACTC-TAGY-T
P143 TTTTCATTTTGAAAAATAACTACAAATCTATTTTAATTTTACAAAGTTAGACTCATAGCAT

Mutations:
+188 (P143)
+546 (P89)

MYB R in E-box 2 (from +119 to -361bp): CGTCCCTGCCACGTTCAAGAGAGG

C41 (+119) GCGTACCTGCAGTGCCAGGAGCGTCTCCTTGGTTCCCCGCGCTTGCTCTGCGGGCGGCTC
P89 G-G-AC-TGCAG-G-CA-GA-CGTCTCCTTGGTTCCCCGCGCTTGCTCTGCGGGCGGCTC
P143 GCGTACCTGCAGTGCCAGGAGCGTCTCCTTGGTTCCCCGCGCTTGCTCTGCGGGCGGCTC

C41 GGGGCTCCGCTATCCGCTCCCTTCCCAGGCTGCAGGCGGCGCTGGGCCCGGGCTCGGCC
P89 GGGGCTCCGCTATCCGCTCCCTTCCCAGGCTGCAGGCGGCGCTGGGCCCGGGCTCGGCC
P143 GGGGCTCCGCTATCCGCTCCCTTCCCAGGCTGCAGGCGGCGCTGGGCCCGGGCTCGGCC

C41 GCGCACAGGAAGCCCGCGCCTGCGAACGTGCGACCCTTTTATAACTCACTTGTGACTCAT
P89 GCGCACAGGAAGCCCGCGCCTGCGAACGTGCGACCCTTTTATAACTCACTTGTGACTCAT
P143 GCGCACAGGAAGCCCGCGCCTGCGAACGTGCGACCCTTTTATAACTCACTTGTGACTCAT

C41 AACCGACCAGGCGCCTTTTCTGCAACCCCCCCCCCTTCTCCCCCYCKGCKGTTYTK
P89 AACCGACCAGGGGCCTTTTCTGCAACCCCCCCCCCTTCTCTCCCCCCTC-----
P143 AACCGACCAGGCGCCTTTTCTGCAACCCCCCCCCCTTYCCCCCYCKGCKGTTYTK

C41 GGAACCWAMAGYTKGAAAAAYTTTTTYTTTTWAAAGRAAAARYCAAAAAASCCMSCSC
P89 -----
P143 GGAACCWAMAGYTTGAAAAAWTTTTTCTTTTAAAGRAAAAGYCRAAAAAGCCASSSC

C41 CSGGGWAGKKGGGGTTTTYCCCTTTCCCTTTCCCGGGGGYCWTTGGGGGGGGTTTTTTT
P89 -----
P143 CCGGGRAGKKGGGGTTTTYCCCTTTCCCTTTCCCGGGGKCATGSGKGGGTGRTTTTTT

C41 TTTCTCCAACMCMSCCGGGTACTTTCTGCCCCGCGCCSCAGGGYTTGGGCTCCAACC
P89 -----
P143 TTTCTCCAACMCMSCCGGGTACTTTCTGCCCCGCGCCSCARGGSTTGGGCTCCAACC

C41 (-311) TTTGTTCTTAAAAAAAAGGAAACSCCTTCAAAGKASGGCCCAAASGTCSCCCGAACTTTC
P89 -----
P143 TTTGTTCTTAAAAAAAAGGAAACSCCTTCAAAGKACGGCCCAAAMGTCSCCCGAACTTTC

Mutations:

- 234 (P143)
- 242 (P143)
- 244 (P143)
- 307 (P143)

11. LIST OF PUBLICATIONS

Related to the Thesis:

M Pesta, **K Vargova**, J Vargova, N Dusilkova, V Kulvait, P Obrtlíkova, A Berkova, K Michalova, M Trněny and T Stopka. Model to understand the clinical relevance of prognostics of CLL in the real time. *Blood*. 2013. (*in prep.*).

Petra Basova, Vit Pospisil, Filipp Savvulidi, Pavel Burda, **Karina Vargova**, Libor Stanek, Martina Dluhosova, Erika Kuzmova, Anna Jonasova, Ulrich Steidl, Peter Laslo and Tomas Stopka. Aggressive acute myeloid leukaemia in PU.1/p53 double mutant mice. *Oncogene*. 2013 (*accepted*).

Vargova K, Curik N, Burda P, Basova P, Kulvait V, Pospisil V, Savvulidi F, Kokavec J, Necas E, Berkova A, Obrtlíkova P, Karban J, Mraz M, Pospisilova S, Mayer J, Trněny M, Zavadil J, Stopka T. MYB transcriptionally regulates the miR-155 host gene in chronic lymphocytic leukemia. *Blood*. 2011 Apr 7; 117(14):3816-25. IF=10.5

Unrelated to the Thesis:

N Curik, P Burda, **K Vargova**, V Pospisil, M Belickova, P Vlckova, F Savvulidi, E Necas, H Hajkova, C Haskovec, J Cermak, M Krivjanska, M Trněny, P Laslo, A Jonasova and T Stopka. 5-Azacitidine in aggressive myelodysplastic syndromes regulates chromatin structure at *PU.1* gene and cell differentiation capacity. *Leukemia*, 2012 Aug; 26(8):1804-11. IF=9.51

Pospisil V, **Vargova K**, Kokavec J, Rybarova J, Savvulidi F, Jonasova A, Necas E, Zavadil J, Laslo P, and Stopka T. Epigenetic silencing of the oncogenic miR-17-92 cluster during PU.1-directed macrophage differentiation. *EMBO J*. 2011 Sep 6. IF=10.1

Vargova J, **Vargova K**, and Stopka T. Nuclear Localization of Chromatin Remodeling ISWI ATPase Smarca5 (Snf2h) in Mouse. *Front Biosci*. E1, 553-559, June 1, 2009. IF=3.308

Kokavec J, Vargova J, Burda P, **Vargova K**, Necas E, Zavadil J, and Stopka T. SWI/SNF2 proteins in germinal and early embryonic development. *Folia Zool.* – 58 (Suppl. 1), 2009. IF=0.522