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Current Knowledge of Microarray Analysis for Gene Expression Profiling in Chronic Lymphocytic Leukemia

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1. Introduction

Chronic lymphocytic leukemia (CLL) is characterised by the accumulation of mature CD5+/CD19+ B-lymphocytes in the blood, bone marrow, lymph nodes and spleen (Caligaris-Cappio & Hamblin, 1999). Although the role of cellular proliferation disorders in CLL may originally have been underestimated, the typical characteristic of the disease is still regarded as a failure of malignant cells to undergo apoptosis (Munk Pedersen & Reed, 2004). CLL is a heterogeneous disease and although it is relatively stable in some patients, it progresses rapidly in others (Caligaris-Cappio & Hamblin, 1999). The mutational status of immunoglobulin heavy chain variable gene segment (*IGHV*) and the expression of CD38 and/or ZAP70 are important prognostic factors of disease so their detection is very useful for stratification of patients into indolent or aggressive subgroups (Hamblin et al., 1999; Krober et al., 2002; Orchard et al., 2004).

However, a more robust approach to subclassifying CLL is to identify the genomic changes in the malignant clone. The heterogeneity of the disease may result from different genetic abnormalities in distinct subclasses of patients. Furthermore, there is a strong relationship between specific genetic aberrations and the clinical course of the disease. On the basis of the mutational status of the variable region of the IGH, CLL can be divided into two subtypes. Somatic hypermutation of *IGHV* occurs in more than half of the patients and is associated with a more indolent clinical course. Additionally, deletions of the long arm of chromosome 13 or 11 and the short arm of chromosome 17, as well as trisomy of chromosome 12, are prognostically most important for the CLL patients. The most common abnormality in CLL, observed in more than 50% patients, is del(13)(q14) and, along with hypermutation of *IGHV*, this is linked with a good prognosis (Damle et al., 1999; Schroeder & Dighiero, 1994).

2. Gene expression profiling

Recent advances in genomics have transformed research on hematologic malignancies by improving molecular approaches to gene networks. New technologies have been designed to meet the need for methods to address the functional significances of nucleotides sequences. Microarrays have emerged as powerful tools for increasing the potential of

standard methods through genome-wide biological studies. They have been focused mainly on gene expression profiling (GEP), but also on mutational screening, genotyping of polymorphisms and copy number analyses.

2.1 Contribution of microarray study to comprehension of CLL pathophysiology

DNA microarrays can be used to detect either DNA, as in comparative genomic hybridization, or to detect RNA, usually as complementary DNA (cDNA) after reverse transcription. The process of measuring gene expression via cDNA is called *expression analysis* or *expression profiling* (Schena et al., 1995). Alizadeh et al., (2000) investigated the construction of a commercial cDNA microarray (Lymphochip) for studies of normal and malignant human cells. They examined each stage of lymphocyte differentiation that can be defined by a characteristic gene expression signature. Genes that are coregulated by over hundreds of experimental conditions often encode functionally related proteins. GEP also provide an unprecedented ability to define the molecular and functional relationships between normal and malignant lymphocyte cell populations (Alizadeh & Staudt, 2000).

Using different microarray platforms such as oligonucleotide arrays, cDNA arrays printed on glass slides and on nylon membranes, Wang et al., (2004) found that several genes were consistently differently expressed between CLL and normal B-cell samples. The following 10 genes were shown to be expressed differently in CLL compared with tonsillar B-lymphocytes and plasma cells: *FCER2* (*CD23*), *FGR*, *TNFRSF1B*, *CCR7*, *IL4R*, *PTPN12, FMOD, TMEM1, CHS1* and *ZNF266* (Zent et al., 2003).

The results of GEP tests on CLL cells indicated that their profile was more closely related to non-proliferating B cells, or memory B cells, than to cells from a naïve germinal centre (GC), mitogenically activated blood cells or CD5+ B cells (Klein et al., 2001). Over the last few years, global GEP has been revised and defined CLL as a tumor of antigen-experienced B cells. These could be marginal zone or memory B cells. Now we know, that CLL results not only from an accumulation of transformed B cells, due to an imbalance between cell generation and cell death rate, but also from a proliferation of B cells in particular microenvironments in the lymphoid tissues and bone marrow (Klein & Dalla-Favera, 2010). Based on these findings, it can be suggest, that the leukemic B cells are more complex mixture than we have hitherto expected. Other genes have been dubbed *CLL signature genes* because they are selectively expressed in CLL and not in normal cells or other types of B-cell malignancy (Rosenwald et al., 2001). The CLL signature includes genes already known to be characteristic for CLL, such as *CD5*, *IL2Rα (CD25)* and *BCL2*, and genes not previously known to be expressed in CLL, such as *WNT3*, *TITIN*, *ROR1* and *MRC-OX2. ROR1* and *MRC-OX2* encode membrane proteins, so they might be useful for decisions concerning treatment with humanised monoclonal antibodies. *WNT3* probably regulates B lymphocyte proliferation (Zent et al., 2003; Reya et al., 2000). A study by Zent et al., (2003) showed that the GEP of CLL lymphocyte is different from multiple myeloma (MM) cells. CLL expressed higher levels of tumour necrosis factor (TNF) and TNF receptor pathway genes (*LTB, TRAF5, TNFRSF9, TNFSF7* and *LITAF).* The *IAP* family gene (*BIRC1*) and the *XIAP* antagonist (*HSXIAPAF1*) were expressed at higher levels only in CLL to MM, similar to *BCL-2* expression.

2.2 Contribution to the identification of new genes that might be considered as prognostic factors

In the last few years, the development of cytogenetics and molecular biology has led to the release of new genetic prognostic markers such as *IGHV* mutational status, genomic aberrations and individual gene mutations.

To determine possible genetic and molecular abnormalities related to early clinical progression in CLL, Fernandez et al., (2008) investigated alterations in genomic and gene expression profiles in a series of samples sequentially obtained at diagnosis in early stage of the disease and at the time of clinical progression before treatment. A group of 58 genes was identified by supervised analysis comparing the initial and progressed samples: 37 were over-expressed while 21 were down-regulated. No significant differences were observed in the expression of these genes in samples from the three CLL cases with stable clinical decease. Functional analysis of the over-expressed genes showed that they are involved in different pathways, including cell cycle and cell growth (*MCM4, RAPGEF2, OGG1, ESCO1, ESR1, ACTL6A, CENPJ, ATG5*) and ion regulation (*MYLC2PL, ADRB1, TRPV5, TMCO3*). Interestingly, 6 of the 21 down-regulated genes were considered negative regulators of integrin-mediated cell adhesion and motility (*PRAM1, CDC42EP4, COL4A2, PLCB2, RAPGEF1, FLNA*). These findings suggest that in early stage CLL, clinical progression is associated with inactivation of tumour suppressor genes and modulation of the expression of a small number of genes that are inhibitors of cell adhesion and motility.

Ferrer et al., (2004) performed gene expression profiling on 31 CLL cases and investigated the *HV* gene mutation status by nucleotide sequencing. The array data showed that the greatest differences between the unmutated (20 cases) and the mutated (11 cases) groups were observed in the expression of such genes as: *ZAP70, RAF1, PAX5, TCF1, CD44, SF1, S100A12, NUP214, DAF, GLVR1, MKK6, AF4, CX3CR1, NAFTC1* and *HEX*. *ZAP70* was significantly more highly expressed in the *IGHV*-unmutated CLL group, whereas all the other genes were more highly expressed in the *IGHV*-mutated cases. This study confirmed that *ZAP70* expression can predict the *HV* mutation status and suggested that *RAF1, PAX5* and other differentially expressed genes may be good markers for differentiating between these two groups and can serve as prognostic markers.

2.2.1 Deregulated apoptosis in poor-prognosis CLL

CLL is a heterogeneous disease with marked variability in its clinical course. With the aim of identifying genes potentially related to disease progression, Fält et al., (2005) performed gene expression profiling on CLL patients with non-aggressive disease or with progressive disease requiring therapy. The Affymetrix GeneChip U95Av2 technique was used in 11 samples obtained from CLL patients with stable and 10 patients with clinically progressive disease. To discriminate samples from progressive and stable disease, a group of genes was chosen as markers; two genes in particular, *PPP2R5C* and *RBL2*, were included among the best discriminators as both were expressed at lower levels in progressive than in stable CLL. These genes are known to be key regulators of both the cell cycle and the mitochondria/cytochrome c apoptotic pathway. This procedure allowed samples with progressive and stable disease to be identified with 70-90% accuracy.

Stratowa et al., (2001) studied 54 peripheral blood lymphocyte samples obtained from patients with CLL to determine the expression levels of 1024 genes on a cDNA microarray and to correlate them with patient survival. Overall survival (OS) of CLL patients displaying low expression of genes coding for IL-1β, IL-8 and L-selectin was shorter than for patients with high expression of these genes. However, high expression of *TCL1* was connected with decreased patient survival. These findings suggest that CLL prognosis may be connected with a defect in lymphocyte trafficking, causing accumulation of leukemic B cells in the blood.

Edelmann et al., (2008) used a microarray-based GEP (Affymetrix U95A) to study how the stroma modulates the survival of CLL cells in *in vitro* co-culture model employing the murine fibroblast cell line M2-10B4. CLL cells cultured in direct contact with the stromal layer (STR) showed significantly better survival than cells cultured in transwell (TW) inserts above the M2-10B4 cells. STR induced a more marked up-regulation of the PI3K/NF-κB/Akt signaling pathway genes (*INPP4A, NFKB2, REL* and *MAPKAPK2*) than TW conditions and mediated a pro-angiogenetic switch in the CLL cells by up-regulating *VEGF* and *OPN* and down-regulating the anti-angiogenetic molecule *TSP-1*. The findings also suggest that *TSP-1* expression in CLL cells may be related to both disease stage and CLL subtype as defined by *ZAP70* and *CD38* expression. OPN protein secretion may be correlated to disease progression in CLL.

GEP used to predict the prognosis in CLL is presented in Table 1 and Table 2.

2.3 Contribution of GEP microarray study to pharmacogenomics

Drug resistance remains a major problem of CLL treatment. Owing to their high adaptability to therapeutic conditions, malignant tumour cells frequently develop escape mechanisms in response to cytostatic drugs. It is very difficult to predict a tumour's reaction to drugs because it can deploy multiple cellular mechanisms such as enhanced DNA repair, elevated levels of drug transporters, over-expression of detoxifying enzymes or apoptosis inhibition, which are often involved in the development of drug resistance. To monitor the multiple alterations by which CLL may become drug-insensitive, highly parallel analyses such as the DNA microarray technique are required. This technique affords new ways of predicting resistance and sensitivity to therapy (Dietel & Sers, 2006).

2.3.1 *In vitro* experiments

There is now well documented that some genes induce apoptosis, whereas the others can inhibit this phenomenon (Table 3). It is also known that drugs used for therapy regimens can change GEP and modify apoptosis. However, the knowledge concerning the drug influence on GEP is still insufficient and demands further studies.

The study by Vallat et al., (2003) combined two series of microarray analyses (Hu-FL GeneChips, Affymetrix, 7,070 genes) with four sensitive and three resistant CLL samples and compared their gene expression patterns before and after *in vitro* irradiation-induced apoptosis. Sixteen differentially expressed genes (≥2-fold, specifically in resistant cells) were disclosed by data analysis. After the validation of the selected genes by quantitative RT-PCR on seven microarray samples, their altered expression level was confirmed on a further 15 CLL samples not previously included in the microarray analysis. Eleven patients with

	Alterations in gene expression	Gene description
Good-prognosis CLL	***Cell cycle and transcription genes***	
	RAF1 ⇑	V-raf-1 murine leukemia viral oncogene homolog 1
	PAX5 ⇑	Paired box gene 5
	TCF1 ⇑	Transcription factor 1
	CD44 ⇑	CD44 antigen
	SF1 (ZNF162) ⇑	Splicing factor 1 (zinc finger protein 162)
	S100A12 ⇑	S100 calcium binding protein A12
	NUP214 ⇑	Nucleoporin 214 kD
	DAF ⇑	CD55 molecule, decay accelerating factor for complement
	GLVR1 ⇑	Solute carrier family 20 (phosphate transporter) member 1 (Glv-r)
	MKK6 ⇑	Mitogen-activated protein kinase kinase 6 (MKK6, MAPKK6, MEK6)
	AF4 ⇑	Pre-B-cell monocytic leukemia partner 1; (AF4, AFF1, MLLT2)
	CX3CR1 ⇑	Chemokine (C-X3-C motif) receptor 1 (CCRL1, GPR13)
	NAF ⇑	T cell chemotactic factor (NAF, IL-8)
	HEX ⇑	Hematopoietically expressed homeobox (HEX, HHEX)
	Cell cycle and cell growth	
	MCM4 ⇑	Minichromosome maintenance complex component 4
	RAPGEF2 ⇑	Rap guanine nucleotide exchange factor (GEF) 2 (RAPGEF2)
	OGG1 ⇑	8-oxoguanine DNA glycosylase (OGG1, HMMH, HOGG1)
	ESCO1 ⇑	Establishment of cohesion 1 homolog 1 (ESCO1, CTF, ECO1)
	ESR1 ⇑	Estrogen receptor 1 (ESR1, ER α)
	ACTL6A ⇑	Actin-like 6A (ACTL6A, Arp4, BAF53A, INO80K, MGC5382)
	CENPJ ⇑	Centromere protein J (CENPJ, BM032, CPAP, LAP, LIP1)
	ATG5 ⇑	ATG5 autophagy related 5 homolog (APG5-LIKE, APG5L, ASP)
	Ions regulation	
	MYLC2PL ⇑	Myosin, light chain 10, regulatory (MYL10, MYLC2PL, PLRLC)
	ADRB1 ⇑	Adrenergic, beta-1-, receptor (ADRB1, B1AR, BETA1AR, RHR)
	TRPV5 ⇑	Transient receptor potential cation channel, subfamily V, member 5
	TMCO3 ⇑	Transmembrane and coiled-coil domains 3 (TMCO3, C13orf11)
	Cell signalling	
	INPP4A ⇑	Inositol polyphosphate-4-phosphatase, type I (TVAS1, INPP4A)
	NFKβ2 ⇑	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2
	REL ⇑	V-rel reticuloendotheliosis viral oncogene homolog
	MAPKAPK2 ⇑	Mitogen-activated protein kinase-activated protein kinase 2 (MK2)

Gene expression: upregulation ⇑

Table 1. GEP in CLL, which may predict a good prognosis. (Edelmann et al., 2008; Fält et al., 2005; Fernandez et al., 2008; Ferrer et al., 2004; Stratowa et al., 2001)

	Alterations in gene expression	Gene description
Genes underexpressed	***Cell adhesion and motility***	
	RAPGEF1 ⇓	Rap guanine nucleotide exchange factor (GEF) 2
	FLNA ⇓	Alpha-filamin; endothelial actin-binding protein
	PRAM1 ⇓	PML-RARA regulated adaptor molecule 1
	CDC42EP4 ⇓	CDC42 effector protein (Rho GTPase binding) 4
	COL4A2 ⇓	Collagen alpha-2(IV) chain
	PLCB2 ⇓	Phospholipase C, beta 2
	IL1β ⇓	Interleukin 1 beta
	IL-8 ⇓	Interleukin 8 (T cell chemotactic factor)
	L-selectin ⇓	Leukocyte-endothelial cell adhesion molecule 1 (LECAM-1)
	Cell cycle and signal transduction	
	PPP2R5C ⇓	Protein phosphatase 2, regulatory subunit B', gamma
	RBL2 ⇓	Retinoblastoma-like 2 (p130)
	Cell growth	
	TSP-1 ⇓	Thrombospondin-1
Genes overexpressed	***Cell adhesion and motility***	
	TCL-1 ⇑	T-cell leukemia/lymphoma 1A
	Cell cycle and signal transduction	
	VH 3.21 ⇑	Immunoglobulin heavy variable 3-21
	ZAP70 ⇑	Tyrosine-protein kinase ZAP-70
	Cell growth	
	OPN ⇑	Osteopontin
	VEGF ⇑	Vascular Endothelial Growth Factor

Gene expression: up ⇑ - and downregulation ⇓

Table 2. GEP in CLL, which may predict the poor prognosis. (Edelmann et al., 2008; Fält et al., 2005; Stratowa et al., 2001; Thorselius et al., 2006)

malignant B cells that were sensitive to *in vitro* radiation-induced apoptosis had never been treated, whereas eight of the 11 patients with resistant disease had previously been treated with fludarabine (FA), cyclophosphamide (C) or chlorambucil (CHB). In the 11 sensitive and 11 resistant CLL samples tested, genes were found to be specific for all the resistant samples; *TR3, HLA-DQA1, MTMR6, C-MYC, C-REL, C-IAP1, MAT2A* and *FMOD* were up-regulated, whereas *MIP1A/GOS19-1* homolog, *STAT1, BLK, HSP27* and *ECH1* were down-regulated. The result of this study was defining clinically relevant new molecular markers specific to resistant CLL subtypes.

Morales et al., (2005) investigated the regulation of apoptosis in B-CLL cells using cDNA microarrays (Human Apoptosis GEArray Q Series, Superarray) with 96 known genes. Data were obtained from and compared between two groups of CLL patients with either non-progressive, non-aggressive, previously untreated disease in which the leukemic cells were sensitive to *in vitro* FA-induced apoptosis, referred to as sensitive B-CLL (sB-CLL), or progressive, chemotherapy-refractory disease in which the leukemic cells were resistant to

in vitro FA-induced apoptosis, referred to as resistant B-CLL (rB-CLL). By performing a supervised clustering of genes that most clearly discriminated rB-CLL from sB-CLL, a small group of genes was identified. *BFL1* was the most strongly discriminating gene, with higher expression in rB-CLL. This finding suggests that *BFL1* may be an important regulator of CLL apoptosis, which could contribute to disease progression and resistance to chemotherapy, and could be a potential future therapeutic target.

Direct physical interaction of stromal cells with CLL cells and overexpression of *RAD51* and *LIG4* (DNA ligase IV) in the leukemic cells have been found. These genes code for DNA repair enzymes in mammalian cells (Edelmann et al., 2008). Given that *RAD51* expression in CLL was previously reported to correlate with resistance to CHB. These findings may provide a molecular-level explanation of the capacity of stromal cells to protect CLL cells from drug-induced apoptosis (Christodoulopoulos et al., 1999).

Segel et al., (2003) have used a cDNA microarray containing approximately 40,000 human gene sequences to obtain GEP for untreated and tetradecanoyl phorbol acetate (TPA)-treated B-CLL cells. Three genes, *EGR1*, *DUSP2* and *CD69*, showed a 2-fold or greater increase in mRNA transcription in two studies. Several genes (*PKC, N-MYC, JUN D* and *BCL2*), previously reported to be overexpressed in CLL lymphocytes, were also overexpressed in these studies but were not altered by TPA treatment. These findings suggest that the products of these three genes may be central to early steps in the TPA-induced evolution of B-CLL cells to a plasma-cell phenotype. A variety of stimulators such as TPA, bryostatin, IL-2 and others can induce CLL lymphocytes to mature *in vitro* to an immunoglobulin-producing and -secreting phenotype. Such treatment corrects some metabolic defects such as impairment of the L-system amino acid transport, but not others such as diminished membrane gamma-glutamyl transpeptidase (GGTP) activity.

GEP allows the study of a large number of genes and analysis of global pathways rather than single targets. Stamatopoulos et al., (2009) revealed the influence of valproic acid (VPA) on molecular changes in two key pathways in cancer: apoptosis and proliferation. The study was conducted on purified B cells obtained from 14 CLL patients. Microarray analysis was performed with an Affymetrix GeneChip Human Genome U133 Plus 2.0 array. Several genes (i.e. *CD5, BCL2, CD23, LCK, PIM1*) described as overexpressed in CLL by Wang et al., (2004) were downregulated by VPA in this study, whereas genes described by Wang et al., (2004) as underexpressed in CLL (i.e. *BCLA1, C-MYC, DUSP2* and *PEA15*) were upregulated by VPA. The authors suppose that these results indicate that VPA could restore a more 'normal' epigenetic code and, in this way, could allow normal cellular processes that were silenced after malignant transformation. No differences among the GEP of ZAP^+ and ZAP^- patients (poor and good prognosis, respectively) were found, indicating that VPA was acting independently of disease aggressiveness. It had also been observed that VPA acted on an important number of genes involved in apoptosis: *BCL2*, *XIAP*, *FLIP*, *BCL-xL, AVEN* and *cIAP*, which as a result, were significantly downregulated, whereas *CASP 2, 3, 6, 8, 9,* and *BAX, BAK, APAF1* and *P53* were all significantly upregulated. The ratio of anti- and proapoptotic genes determines the tendency towards cell death or cell survival. Moreover, a large number of cell-cycle genes were upregulated, not only *CDK1, 2, 4,* and *6, cyclin B1, B2, D1, D2, E1* and *E2*, but also inhibitors of cell cycle, such as *P15, 16, 18, 19* and *21*. The deregulated and simultaneous expression of all these genes is probably one of the reasons for proliferation inhibition (Stamatopoulos et al., 2009).

In our department, we identified differentially expressed genes in lymphocytes obtained from CLL patients and incubated with FA or cladribine (2-chlorodeoxyadenosine; 2-CdA) (Table 4). Among 93 studied apoptotic genes by means of 384 TaqMan Low Density Array (Applied Biosystems) most of them were downregulated, whereas such a few of them were upregulated: *BAD, TNFRSF21, DAPK1* – in 2-CdA cultured group and *CARD6* and *CARD9* in FA cultured group. We have also noticed 4 genes (*BAK1, BAX, FAS* and *PUMA*) with about a 20- or more –fold decrease in gene expression with respect to control samples. Interestingly, in the above-mentioned genes we have found great differences in fold change value between FA and 2-CdA. The expression of two of them, *BAX* and *PUMA,* were considerably decreased when lymphocytes were incubated with FA. It may be hypothesized that the high ratio between anti- and proapoptotic gene expression might account for the failure to achieve complete response after purine nucleoside analogues (PNAs) therapy. Additionally, 2-CdA has inhibited to a lower extent the expression of *PUMA* and *BID* as compared to FA (Franiak-Pietryga I, Korycka-Wolowiec A, unpublished data), which might confirm the results reported by Robak et al., (2009) that 2-CdA, but not FA, is the most effective drug against *P53*-defective cells. At this stage of our knowledge, probably it is too far-fetched to make a suggestion that FA mostly triggers apoptosis in intrinsic pathways to caspase activation, while 2-CdA induces apoptosis via death receptor activation (extrinsic pathway) and by stress-inducing stimuli (intrinsic pathway). To confirm this hypothesis, further experiments are to be conducted in our department. Besides the *in vitro* experiments also *in vivo* studies play an important role in the increase of our knowledge on gene expression profiling.

2.3.2 *In vivo* studies

The study of CLL by Plate et al., (2000) was directed at understanding the signals that maintain viability *in vivo* and are lost when the leukemic cells are removed from the body, such that they immediately begin to undergo apoptosis *ex vivo*. Differences in gene expression between freshly isolated B-CLL cells and those maintained *in vitro* with and without FA were measured using the ATLAS apoptosis cDNA microarray (Clontech, Palo Alto, CA). Many genes, especially *cyclin D1*, were under-expressed after culturing. The anti-apoptotic genes *BAG1* and *AKT2* were over-expressed. The greatest positive effect of FA was the up-regulation of *JNK1*.

Rosenwald et al., (2004) profiled gene expression in CLL leukemic samples obtained before and during FA administration using Lymphochip DNA arrays prepared from 17,856 cDNA clones. The procedure selected 27 microarray elements, 18 of which represented named genes while the other 9 represented novel genes of unknown function. In seven CLL samples, a consistent gene expression (GE) signature of *in vivo* FA exposure was identified. Many of the FA signature genes were known *P53* target genes and genes involved in DNA repair (*P21, MDM2, DDB2, TNFRSF10B, PCNA* and *PPMID*). Because *in vivo* treatment with FA induces a *P53*-dependent GE response, it has the potential to select *P53* mutant CLL cells, which are more drug-resistant and are associated with an aggressive clinical course. Therefore, treatment of CLL patients with FA has the potential to select for outgrowth of *P53* mutant subclones that would be cross-resistant to several other chemotherapeutic agents. Moreover, the gene expression response to γ radiation was highly similar to the response to FA.

The purine metabolism of B-CLL lymphocytes was studied by Marinello et al., (2006). Gene expression analysis was performed on samples obtained from 2 B-CLL patients. Data analysis revealed 17 genes whose expression varied at least 2-fold. Some purine metabolism genes

expressed differently from controls were identified. Among the de novo enzymes, the *Gars-Airs-Gart* complex was over-expressed and *IMPDH1* and *APRT* seemed under-expressed. An imbalance in the expression of the adenosine-related protein gene was also observed, with over-expression of *CD26*, *CD38* and *mtAK3*, while *ADORA 1* and *cAK1* were under-expressed (Table 5). Simultaneous gene profiling of apoptosis-related factors and purine metabolism enzymes is of particular interest for drugs such as FA and 2-CdA, which are commonly used in CLL treatment. Three years later the above-mentioned data was confirmed on samples obtained from 5 B-CLL patients on a chip prepared with 57 genes. To the group of genes described previously some of new ones were added, including apoptosis-related proteins. *CASP6*, *CASP8* and *BCL2L1* (*BCL-xL*) were under-expressed, whereas *IL-4*, *IL-18* were over-expressed. In contrast, less significant changes were observed in the expression of some other anti- or proapoptotic factors like *BAX* and *BCL10*, respectively.

To identify novel genes involved in the molecular pathogenesis of CLL, Proto-Siqueira et al., (2008) performed a serial analysis of gene expression (SAGE) in CLL cells and compared it with healthy B cells (nCD19+). A gene ontology analysis revealed that *TOSO*, which plays a functional role upstream of the *FAS* extrinsic apoptosis pathway, was over-expressed in CLL cells. A positive correlation was observed between *TOSO* and *BCL2,* but not between *TOSO* and *FLIP*. The over-expression of *TOSO* and *BCL2* might be responsible for *BAX* inhibition, which leads to the suppression of apoptosis and might be associated with poor prognosis in CLL. It is also known that bortezomib blocks *BAX* degradation in malignant B cells. *TOSO* might therefore be considered a possible target for small molecule therapy in combination with newer pro-apoptotic drugs such as bortezomib and lumiliximab.

Giannopoulos et al., (2009) provided novel biological insights into the molecular effects of thalidomide and suggested the existence of a signature predictive of thalidomide response in CLL. GEP data on day 0 and 7, based on a paired supervised analysis, revealed a thalidomide-induced signature comprising 123 differentially expressed genes. Upon thalidomide monotherapy, an upregulation of genes, known to be involved in mediating thalidomide response, was observed. Such genes as *FAS* and *CDKN1A*, as well as novel candidate genes, such as *STAT1* and *IKZF1* were reported. Gene expression differences in responders as compared to nonresponders after thalidomide monotherapy on day 7 were determined. Responders showed lower expression of gene coding pro-survival cytokine such as *IL-8* and lower level of *TGFB1*, whereas genes involved in apoptosis, i.e. *CASP1*, were more highly expressed than in nonresponders. Higher expression of *ZAP70*, as well as anti-apoptotic genes such as *TRAF1*, and genes involved in angiogenesis, (eg. *ECGF1)* was observed in nonresponders group. Thalidomide responders showed also lower *JUN* and *CASP9* expression levels associated with deregulated insulin and *RAS* signalling pathways. In CLL being induced by *NFKB* activation, *IL-8* may function as an autocrine growth and apoptosis resistance factor promoting cell survival.

Our data depicts changes in apoptotic GEP in CLL patients treated with cladribine, cyclophosphamide and rituximab (CCR). The measurements were conducted by means of 384 TaqMan Low Density Arrays (Applied Biosystems). Data analysis pointed 20 out of 93 examined apoptotic genes, whose expression has significantly changed. Changes in GEP are mostly related to the intrinsic apoptotic pathway. The most significant differences in gene expression before, as opposed to after, treatment are demonstrated by antiapoptotic genes such as *BCL2, BCL2L1, BIRC1, BIRC5* and *BIRC8,* whose expression is considerably

decreased. Of the proapoptotic genes, *NOXA, CASP10, ESRRBL1* and *NFKBIZ* are particularly distinguished, because they are significantly overexpressed (Table 4). Additionally, genes specifically clustered in terms of GEP, which was different in particular genes depending *IGHV* mutational status (Franiak-Pietryga et al., 2010).

	Gene expression	Gene description	Response to drugs and other chemical substances
Genes overexpressed	***BAG1***	BCL2-associated athanogene	Positive effect to FA
	AKT2	Protein kinase Akt-2; promoter of cell survival	Positive effect to FA
	BCL2	B cell lymphoma 2 associated oncogene	Positive effect to TPA
	JNK1	Mitogen-activated protein kinase 8; stress-activated protein kinase; *MAPK8*	Positive effect to FA
	TR3	TR3 orphan receptor; early response protein NAK1	Resistance to FA, C, CHB
	MTMR6	Myotubularin related protein 6	Resistance to FA, C, CHB
	C-MYC	Transcription factor, puf, and kinase	Resistance to FA, C, CHB; Positive effect to VPA
	C-REL	Proto-oncogene c-Rel	Resistance to FA, C, CHB
	C-IAP1	Apoptosis inhibitor 1; *BIRC2*	Resistance to FA, C, CHB
	N-MYC	V-myc myelocytomatosis viral related oncogene	Positive effect to TPA
	JUND	Transcription factor jun-D	Positive effect to TPA
	P21	Cyclin-dependent kinase inhibitor 1A, *CDKN1A*	FA signature genes involved in DNA repair
	MDM2	P53 binding protein homolog	FA signature genes involved in DNA repair
	TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b, apoptosis inducing protein	FA signature genes involved in DNA repair
	BFL1	BCL2-related protein A1, *BCL2A1*	Resistance to F, Positive effect to VPA
	BAX	BCL2 associated protein, apoptotic death-initiating protein	FA, 2-CdA
	BCL10	CARD-containing apoptotic signaling protein	FA, 2-CdA
	TOSO	Fas apoptotic inhibitory molecule 3; *FAIM3*	Bortezomib, Lumiliximab
	DUSP2	Dual specificity phosphatase 2	Positive effect to TPA, VPA

Genes underexpressed	***FAS***	TNF receptor superfamily, member 6; *TNFRSF6*	Positive effect to Thalidomide Resistance to FA, C, CHB
	CASP6	Caspase 6, enzyme of apoptotic pathway	FA, 2-CdA
	CASP8	Caspase 8, enzyme of apoptotic pathway	FA, 2-CdA
	BAX	BCL2 associated protein, apoptotic death-initiating protein	Bortezomib, Lumiliximab
	BCL-xL	Anti-apoptotic BCL2-like 1; *BCL2L1*	Positive effect to FA
	BCL2	B cell lymphoma 2 associated oncogene	Positive effect to TPA

C – cyclophosphamide, 2-CdA – cladribine, CHB – chlorambucil, FA – fludarabine,
TPA – tetradecanoyl phorbol acetate, VPA – valproic acid

Table 3. Expression of apoptotic genes under the influence of drugs and other chemical substances. (Giannopoulos et al., 2009; Marinello et al., 2006; Morales et al., 2005; Plate et al., 2000; Proto-Siqueira et al., 2008; Rosenwald et al., 2004; Segel et al., 2003; Stamatopoulos et al., 2009; Vallat et al., 2003).

	Gen expression	**Gen description**	**Response to drug**
Proapoptotic genes	***BAD*** ⇑	BCL2-associated agonist of cell death	2-CdA
	TNFRSF21 ⇑	Tumor necrosis factor receptor superfamily, member 21	2-CdA
	DAPK1 ⇑	Death associated protein kinase 1	2-CdA
	CARD6 ⇑	Caspase recruitment domain family member 6	FA
	CARD9 ⇑	Caspase recruitment domain family member 9	FA
	BAK1 ⇓	BCL2-antagonist/killer1; *BAK1*	2-CdA, FA
	BAX ⇓	BCL2-associated X protein, isoform delta	2-CdA, FA
	PUMA ⇓	BCL2 binding component 3; p53 up-regulated modulator of apoptosis; *BBC3*	2-CdA, FA
	FAS ⇓	TNF receptor superfamily, member 6; *TNFRSF6*	2-CdA, FA, CCR
	NOXA ⇑	Phorbol-12-myristate-13-acetate-induced protein 1; *PMAIP1*	CCR
	CASP10 ⇑	Caspase 10, apoptosis-related cysteine peptidase	CCR
	ESRRBL1 ⇑	Intraflagellar transport 57 homolog-IFT57; *HIP1*	CCR
	NFKBIZ ⇑	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta; *IKBZ*	CCR
Antiapoptotic genes	***BCL2*** ⇓	B-cell leukemia/lymphoma 2	CCR
	BCL2L1 ⇓	BCL2 like isoform 1	CCR
	BIRC1 ⇓	Baculoviral IAP repeat-containing 1	CCR
	BIRC5 ⇓	Baculoviral IAP repeat-containing 5	CCR
	BIRC8 ⇓	Baculoviral IAP repeat-containing 8	CCR

Gene expression: up ⇑ - and downregulation ⇓

2-CdA – cladribine, CCR – cladribine, cyclophosphamide and rituximab; FA – fludarabine

Table 4. Genes involved in apoptosis (Franiak-Pietryga et al., 2010; Franiak-Pietryga I, Korycka-Wolowiec A, unpublished data)

GEP may have a predictive value for the effectiveness of anti-cancer therapy. Although numerous experiments remain to be performed, it might become possible to predict chemoresistance and to avoid ineffective drugs. The possibility of pretherapeutic discrimination between responders and non-responders will further stimulate the development of an individualised therapeutic strategy using a personalised combination of drugs (Dietel & Sers, 2006). A list of the genes and their response to therapy and drug resistance is presented in Table 5.

	Gene	Description	Response to drugs
Genes overexpressed	***HLA-DQA1***	HLA class II histocompatibility antigen, DQ alpha 1 chain	Resistance to FA, C, CHB
	MAT2A	Methionine adenosyltransferase 2	Resistance to FA, C, CHB
	FMOD	Fibromodulin	Resistance to FA, C, CHB
	EGR1	Early growth response protein 1	Positive effect to TPA
	CD69	Early lymphocyte activation antigen	Positive effect to TPA
	PKC	Protein kinase C	Positive effect to TPA
	DPB2	DNA polymerase epsilon	FA signature genes involved in DNA repair
	PCNA	Proliferating cell nuclear antigen	FA signature genes involved in DNA repair
	ADORA3	Adenosine A3 receptor	FA, 2-CdA
	Gars-Airs-Gar complex	Phosphoribosylglycinamide synthetasephosphoribosylaminoimidazole synthetase-phosphoribosylglycinamide formyltransferase	FA, 2-CdA
	mtAK3	Adenylate kinase 3 (mitochondrial)	FA, 2-CdA
	NMN	Myodenylate deaminase	FA, 2-CdA
	CD26	Adenosine deaminase complexing protein	FA, 2-CdA
	CD38	Cyclic ADP-ribose hydrolase	FA, 2-CdA
	IL-18	Interleukin 18; interferon-gamma-inducing factor	FA, 2-CdA
	IL-4	Interleukin-4; lymphocyte stimulatory factor 1	FA, 2-CdA
	RAD51	DNA repair protein	Resistance to CHB
	BFL1	BCL2-related protein A1, *BCL2A1*	Positive effect to VPA
	C-MYC	Transcription factor, puf, and kinase	Positive effect to VPA
	DUSP2	Serine/threonine specific protein phosphatase	Positive effect to VPA
	PEA15	Homolog of mouse MAT-1 oncogene	Positive effect to VPA

Genes underexpressed	***STAT1***	Signal transducer and activator of transcription 1	Positive effect to Thalidomide Resistance to FA, C, CHB
	BLK	B lymphoid tyrosine kinase	Positive effect to Thalidomide Resistance to FA, C, CHB
	HSP27	Heat shock protein beta-2	Positive effect to Thalidomide Resistance to FA, C, CHB
	ECH1	Enoyl CoA hydratase 1, peroxisomal	Positive effect to Thalidomide Resistance to FA, C, CHB
	P21	CDKN1A, cyclin-dependent kinase inhibitor 1A	Positive effect to Thalidomide Resistance to FA, C, CHB
	APRT	Adenine phosphoribosyltransferase	FA, 2-CdA
	IMPDH1	IMP dehydrogenase 1	FA, 2-CdA
	ADORA1	Adenosine A1 receptor	FA, 2-CdA
	cAK1	Cytosolic adenylate kinase 1	FA, 2-CdA
	GRK6	G-prot-coupled receptor kinase 6	FA, 2-CdA
	CD73	5'-nucleotidase, ecto	Bortezomib, Lumiliximab Positive effect to FA
	CD5	Lymphocyte antigen T1/Leu-1	Positive effect to VPA
	BCL2	B cell lymphoma 2 associated oncogene	Positive effect to VPA
	CD23	FCER2, Fc fragment of IgE, low affinity II, receptor for CD23	Positive effect to VPA
	PIM1	Proto-oncogene serine/threonine-protein kinase PIM-1	Positive effect to VPA

C – cyclophosphamide, 2-CdA – cladribine, CHB – chlorambucil, FA – fludarabine,
TPA – tetradecanoyl phorbol acetate, VPA – valproic acid

Table 5. The influence of GEP on response to therapy or drug resistance (Edelmann et al., 2008; Giannopoulos et al., 2009; Marinello et al., 2006; Plate et al., 2000; Proto-Siqueira et al., 2008; Rosenwald et al., 2004; Segel et al., 2003; Stamatopoulos et al., 2009; Vallat et al., 2003).

3. Genotyping

3.1 Introduction

Owing to a greater availability of the human genome sequence, the focus of research has now been shifted to identifying sequence polymorphisms. It is of utmost importance to understand how biological functions may be affected by these variations and be associated with heritable phenotypes.

A single nucleotide polymorphism (SNP) array is a type of DNA microarray that is used to detect polymorphisms within a population. SNPs are the most frequent type of variation in the genome. It is estimated that about 10 million SNPs have been identified in humans, an average of one SNP every 400–1000 base pairs (Botstein & Risch, 2003). Currently, about 5.6 million have been typed (dbSNP Build ID: 126), about half of which are estimated to have a minor allele frequency over 10% (Kruglyak & Nickerson, 2001). As SNPs are highly conserved throughout evolution and within a population, a map of SNPs serves as an excellent genotypic marker for research. SNPs from the whole genome form a *genetic fingerprint.* Although SNPs are spaced randomly throughout the genome and could therefore lie in coding sequences, only a small fraction has functional significance (i.e. are non-silent), such as those found in the transcribed or regulatory regions of genes (Mohr et al., 2002). SNPs on a small chromosomal segment tend to be transmitted as a block, forming a haplotype. This correlation between alleles at nearby sites is known as linkage disequilibrium (LD) and enables genotypes at a large number of SNP loci to be predicted from known genotypes at a smaller number of representative SNPs, called 'tag SNPs' or 'haplotype tag SNPs' (Gabriel et al., 2002; Dutt & Beroukhim, 2007). This reduction in the complexity of genetic variation among individuals enables an overall genotype to be determined much more efficiently and economically; roughly 500,000 tag SNPs are sufficient to genotype an individual with European ancestry (Dutt & Beroukhim, 2007; Nicolas et al., 2006).

The mechanisms of an SNP array and the DNA microarray are identical; the convergence of DNA hybridization, fluorescence microscopy and solid surface DNA capture. In order to study the genetic vulnerability of a germline to complex diseases, oligonucleotide arrays have been developed to interrogate such large numbers of SNP markers in multiple databases (Dutt & Beroukhim, 2007; Gunderson et al., 2005).

3.2 Genome-wide association studies

CLL and other B-cell lymphoproliferative disorders (LPDs) show clear evidence of familial aggregation, but the inherited basis is still largely unknown. To identify a susceptibility gene for CLL, Sellick et al., (2005) conducted a genome-wide linkage analysis of 115 families, using a high-density SNP array (GeneChip Mapping 10Kv1 Xba, Affymetrix) containing 11,560 markers. Multipoint linkage analyses were undertaken using both nonparametric (model-free) and parametric (model-based) methods. It confirmed that high LD between SNP markers could lead to inflated nonparametric linkage (NLP) and LOD scores (Dawn Tare & Barrett, 2005). After the high-LD SNPs were removed, a maximum NPL of 3.14 ($p<0.0008$) on chromosome (11)(p11) was obtained. The highest multipoint heterogeneity LOD (HLOD) score under both dominant (HLOD 1.95) and recessive (HLOD 2.78) models was yielded by the same genomic position. Moreover, four other chromosomal positions (5)(q22-23), (6)(p22),

(10)(q25) and (14)(q32) displayed HLOD scores >1.15 ($p<0.01$). None of those regions coincided with areas of common chromosomal abnormalities frequently observed for CLL. These results support an inherited predisposition to CLL and related B-cell LPDs.

Pfeifer at al., (2007) explored high-density 10k and 50k Affymetrix SNP arrays to assess genetic aberrations in the tumour B-cells of patients with CLL. Among the prognostically important aberrations, del(13)(q14) was present in 51%, trisomy 12 (+12) in 13%, del(11)(q22) in 13% and del(17)(p13) in about 6% of cases. A prominent clustering of breakpoints on both sides of the genes *MIRN15A/MIRN16-1* indicated the presence of recombination hot spots in the 13q14 region. Patients with a mono-allelic del(13)(q14) had slower lymphocyte growth kinetics than patients with bi-allelic deletions. In four CLL cases with unmutated *HV* genes, a common minimal 3.5-Mb gain of 2p16 spanning the *REL* and *BCL11A* oncogenes was identified, implicating these genes in the pathogenesis of CLL.

New risk variants for CLL were identified by Crowther-Swanepoel et al., (2010). A genome-wide association (GWA) study of 299,983 tagging SNPs (by means of HumanCNV370-Duo BeadChips, Illumina) was conducted with validation in four additional series totalling 2,503 cases and 5,789 controls. In 2008, the authors reported the results of a GWA study of CLL based on an analysis of 299,983 tagging SNPs in 505 cases and 1, 438 controls and through fast track analysis of SNPs, identified risk loci at 2q13, 2q37.1, 6p25.3, 11q24.1, 15q23 and 19q13.32 (Di Bernardo et al., 2008). The authors identified 4 new risk loci for CLL at 8q24.21 (rs2456449, *TCF4*), 2q37.3 (rs757978, *FARP2*), 15q21.3 (rs7169431, *NEDD4, RFX7*) and 16q24.1 (rs305061, *IRF8*). The evidence for risk was found for two more loci: 15q25.2 (rs783540, *CPEB1*) and 18q21.1 (rs1036935, *CXXC1, MBD1*). *TCF4* binds to an enhancer for *MYC*, providing a mechanistic basis for this 8q24.21 association. It had also been shown that variation in *IRF4* influences CLL risk. There is a possibility that the effect of the other 8q24.21 cancer risk loci is by *MYC,* which is a direct target of *IRF4* in activated B-cells and this observation needs further study.

FARP2 is a gene connected with signalling downstream of G protein-coupled receptors. rs757978 is involved in the substitution of threonine for isoleucine at amino acid 260 (T260I), whereas rs305061 maps within a 30-kb region of LD at 16q24.1 locus and localises 19kb telomeric to *IRF8*, which regulates α and β-interferon response. There is still no evidence for a direct role of *NEDD4* in CLL, but it is a credible candidate gene because it has a role in regulating viral latency and pathogenesis of EBV. Particularly, *NEDD4* regulates EBV-LMP2A, which mimics signalling induced by the B-cell receptor, altering B-cell development. *CPEB1* plays a role in regulating cyclin B1 during embryonic cell division and differentiation. *CXXC1* and *MBD1* are involved in gene regulation. *MBD1* expression in EBV-transformed lymphocytes correlated with risk genotype. Although *MBD1* has no documented role in CLL, it can affect CLL development through translational control of *MYC*. No connection between 17p deletion status and genotype was observed. Although there was evidence that the rs305061 risk genotype was associated with worse overall survival, *IGHV*-mutation status was highly correlated with rs305061, but risk genotype correlating with unmutated-CLL (Crowther-Swanepoel et al., 2010; Di Bernardo et al., 2008).

To identify genetic variants associated with outcome of CLL, Sellick et al., (2008) genotyped 977 non-synonymous SNPs (nsSNPs) in 755 genes relevant to cancer biology in 425 patients participating in a trial comparing the efficacies of FA and CHB ± C in first-line treatment. A

total of 78 SNPs (51 dominantly acting and 27 recessively acting) were associated with progression-free survival (PFS), nine of them also affecting overall survival (OS) at the 5% level. These included SNPs mapping to the immunoregulatory genes *IL16* P434S, *IL19* S213F, *LILRA4* P27L, *KLRC4* S29I and *CD5* V471A, as well as the DNA response genes *POLB* P242R and *TOPBP1* S730L, which were all independently prognostic of *IGHV* mutational status. A total of five SNPs associated with PFS were common to patients treated with CHB or FA (*DST* L22S, *LILRA4* P27L, *SEC23B* H489Q, *XRCC2* R188H and *ZAK* S531L); three SNPs were common to patients treated with either CHB or FA with C (*APBB3* C236R, *ENPPS* I171V, and *C21orf57* S2L); and four were common to patients treated with either FA alone or FA with C (*DDX27* G206V, *DPYD* S534N, *WNT16* G72R and *DHX16* D566G). The variants have proved to be invaluable prognostic markers of patient outcome (Table 6).

Gene	Description	Chemotherapy	Response to treatment
DST L22S	Dystonin	CHB or FA	PFS
LILRA4 P27L	Leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 4		PFS
SEC23B H489Q	SEC23-related protein B		PFS, OS
XRCC2 R188H	DNA repair protein XRCC2; RAD51-like		PFS
ZAK S531L	MLK-like mitogen-activated protein triple kinase		PFS
APBB3 C236R	Amyloid beta A4 precursor protein-binding family B member 3	CHB or FC	PFS
ENPPS I171V	Ectonucleotide pyrophosphatase/phosphodiesterase 1		PFS
C21orf57 S2L	Chromosome 21 open reading frame 57		PFS
DDX27 G206V	DEAD box protein 27	FA or FC	PFS
DPYD S534N	Dihydropyrimidine dehydrogenase		PFS
WNT16 G72R	Wingless-type MMTV integration site family, member 16		PFS
DHX16 D566G	DEAH (Asp-Glu-Ala-His) box polypeptide 16		PFS

FA – fludarabine, FC – fludarabine with cyclophosphamide, CHB – chlorambucil;
PFS – progression-free survival, OS – overall survival

Table 6. Relationship between SNPs and drug response (Sellic et al., 2008)

3.3 Copy number variation analyses

Gunnarsson et al., (2008) compared platform dynamics, an in-depth analysis of copy-number alterations (CNAs) using four high-resolution microarray platforms: BAC arrays (32K), oligonucleotide arrays (185K, Agilent) and two SNP arrays (250K, Affymetrix and 317K, Illumina). Ten CLL samples were analysed. The evaluation of baseline variation and copy-number ratio response showed that the Agilent platform performed best and confirmed the robustness of BAC arrays. These platforms demonstrated more platform-specific CNAs. The SNP arrays showed more technical diversity, although the high density of elements compensated for this. Affymetrix detected more CNAs than Illumina, but the latter showed a lower noise level and a higher detection rate in the LOH analysis. Application of high-resolution microarrays will enhance the possibility of detecting new recurrent microevents in CLL leading to identification of new important subgroups, refining the prognostic hierarchy established by FISH. The whole-genome screening with SNP arrays (Affymetrix GeneChip Mapping 250K Nsp1) was conducted and a high frequency of known recurrent alterations in 203 newly diagnosed CLL patients was revealed (Gunnarsson et al., 2010). Moreover, the genome-wide analysis allowed detection of a novel combination of gain of 2p and del(11q), and additional large and small CNAs, which are important for the evaluation of overall complexity in CLL patients. The authors identified genomic complexity as a poor prognostic marker in the survival analysis. However, they noted that this characteristic was strongly linked to established poor-risk molecular markers. The small alterations were mostly non-overlapping. It seems unlikely that there are unknown recurrent CNAs > 200 kbp involved in the CLL pathophysiology detectable in this setting (Gunnarsson et al., 2010). Similar results have been presented by Kujawski et al., (2008), who reported a correlation between genomic complexity and a significantly shorter time to first and second treatment and presented the number of CNAs as an independent prognosis factor.

The discovery of microRNA and its biological functions is a significant step towards the understanding of the molecular bases of human physiology and pathology. MicroRNAs constitute a class of short, non-coding RNA molecules involved in the regulation of a number of important biological process including cell proliferation, differentiation and apoptosis by down-regulation of gene expression during the translation phase. On the basis of these findings, CLL is a genetic disease in which the main alterations occur in microRNAs (miRNAs). Down-regulation of *MIR15A* and *MIR16* as a part of del(13)(q14) has been suggested as good prognostic factors. Both miRNAs negatively regulate *BCL2* at a post-transcriptional level. In CLL cases with unmutated *IGHV* or high level of expression *ZAP70* the overexpression of *TCL1* was observed. This is due to low-level expression of *MIR29* and *MIR181*, which directly targets this oncogene. The overexpression of *TCL1* is correlated with del(11)(q22) and with the aggressive CLL. These miRNAs might be used to target *BCL2* or *TCL1* for therapy of the disease (Calin et al., 2007; Cimmino et al., 2005).

Ouillette et al., (2008) analysed 171 CLL cases for LOH and subchromosomal copy loss on chromosome 13 in DNA from FACS-sorted $CD19^+$ cells by means of the Affymetrix *Xba*I 50k SNP array platform. Detailed analysis suggests the existence of distinct subtypes. Categorisation is based on del(13)(q14) lesions with Rb loss as type II [40% of del(13)(q14) cases] and consequently without such a loss as type I [60% of del(13)(q14) cases]. Rb is a decisive regulator of cell cycle progression and genomic stability. The loss of one or two alleles

could differentially affect the biology of CLL cases Hernando et al., 2004. In the type I 198 genes were analysed. In this group reduced expression of *FLJ11712, KCNRG, RFP2, RFP2OS* and *DLEU1* was identified. Many other genes have emerged as candidate differentially expressed genes by means of qPCR: *LATS2, DFNA5, PHLPP, LPIN1, SERPINE2, ARHGAP20, CYTB5, SLA2*, and *AQP3*. *LATS2* RNA levels were lower in CLL cases with del(13)(q14) type I as opposed to type II cases or all other CLL cases without del(13)(q14). *LATS2* is involved in cell cycle progression control. It is possible that Rb and *LATS2* may be regulators [in non-del(13)(q14) cases] in different processes of CLL subsets (Ouillette et al., 2008). Further subdivision of del(13)(q14) type I cases into type Ia and type Ib is suggested by the occurrence of deletions that appear of relatively uniform length [del(13)(q14) type Ia] and that displays centromeric breaks within the vicinity of the *MIR15A/MIR16* cluster. Bi-allelic del(13)(q14) type Ia lesions were associated with significant reductions in *MIR15A/MIR16* expression levels. As opposed to Calin et al. (2007), this observation reveals that *BCL2* levels were not correlated with *MIR15A/MIR16* levels. In important recent discovery is that about 50% of all CLL cases with del(13)(q14) do not express the *PHLPP* gene. *PHLPP* dephosphorylates activated *AKT* and low or absent *PHLPP* expression may allow for sustained *AKT* signalling after proper cell surface stimuli (Ouilette et al., 2008).

Multiple, discrete, genomic alterations in the 13q region, including *MIR15A/MIR16, Rb* and others were also observed by Grubor et al., (2009). It might suggest greater complexity of lesions in the 13q region than already known. Moreover, they focused on intraclonal heterogeneity within CLL patients and they searched for genomic differences between $CD38^+$ and $CD38^-$ populations in the same patient. The study was conducted by means of a high-resolution CGH technique called representational oligonucleotide microarray analysis (ROMA). This method is very sensitive to examining the clonal heterogeneity of CLL within the same patient from mixed subpopulations. Copy number differences, in separated $CD38^+$ and $CD38^-$ fractions, were detected in 3 of 4 samples at various loci throughout the genome, some of clinical relevance (ie. *ATM* and *TP53*). With the exception of the del(6)(q21), reported major cytogenetic imbalances have been observed previously. The majority of lesions (315/419) were deletions and not amplifications, which is typical of CLL. Two novel regions were observed: del(8)(p21.2-p12) and del(2)(q37.1), including genes *TRIM35* and *SP100/110/140*, respectively. The apparent on-going evolution of CLL clones in a patient may improve the understanding of the disease and the ability to identify patients at risk. The above-demonstrated capabilities offer opportunities for patient treatment individualisation and the identification of new therapeutic agents.

Lehmann et al., (2008) performed molecular allelokaryotyping on 56 samples of early stage CLL using the 50k XbaI GeneChip from Affymetrix (50,000 SNP probes). Excluding the four common abnormalities [+12, del(17)(p13), del(11)(q22) and del(13)(q14)], SNP-chip analysis identified a total of 45 copy number changes in 25 CLL samples (45%). Four samples had del(6)(q21) that involved *AIM1*. UPD was detected in four samples, two of them involved the whole of chromosome 13, resulting in homozygous deletion of *MIR15A/MIR16-1*. The data suggests that genetic abnormalities including gain, loss and UPD of genetic materials frequently occur at an early stage of CLL. In addition to well-documented common genetic abnormalities, deletions of 5q, 6q and Xp were observed to be frequent in early-stage CLL. *AIM1* was examined as a target of this deletion. In the study, expression levels of *ZAP70* and

the mutational status of *IGHV* were analysed. It was demonstrated for the first time that *ZAP70* expression was correlated with del(11)(q22) in early-stage CLL. It was also observed that non-hypermutation of *IGHV* was correlated with +12, del(11)(q22) and del(13)(q14) in early-stage CLL.

4. Conclusion

Microarray technology provides comprehensive data on the expression patterns of thousands of genes in parallel, which positions this method in the centre of optimisation of diagnosis and the classification of leukemias. GEP may lead to the detection of new biologically defined and clinically relevant subtypes of chronic lymphocytic leukemia as a basis for specific therapeutic decision. If such testing is to be used as a routine method for diagnostic purposes in parallel with current standard methods, it is crucial to include GEP in future routine diagnostic applications and in clinical trials. With promising initial results, genome-wide association studies using SNPs are becoming increasingly well established as tools for discovering disease genes. SNP array is an important application in determining disease susceptibility, and consequently in pharmacogenomics, by measuring the specific effectiveness of a form of drug therapy for the patient. As each individual has many SNPs that together create a unique DNA sequence. SNPs may be performed to map disease loci, and hence determine individual-specific disease susceptibility genes. As a result, drugs can be personally designed to act efficiently on a group of individuals who share a common allele, or even a single individual.

5. Acknowledgements

This work was supported by grant No. PBZ/MNiSW/07/2006/28 from the Ministry of Science and Higher Education, Poland to I. F-P and partially by statutory means No. 503/3-015-02/503-01 of the Department of Pharmaceutical Biochemistry, Medical University of Lodz, Poland.

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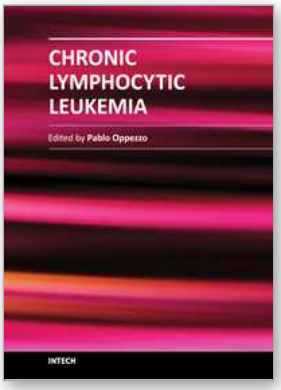

Chronic Lymphocytic Leukemia
Edited by Dr. Pablo Oppezzo

ISBN 978-953-307-881-6
Hard cover, 448 pages
Publisher InTech
Published online 10, February, 2012
Published in print edition February, 2012

B-cell chronic lymphocytic leukemia (CLL) is considered a single disease with extremely variable course, and survival rates ranging from months to decades. It is clear that clinical heterogeneity reflects biologic diversity with at least two major subtypes in terms of cellular proliferation, clinical aggressiveness and prognosis. As CLL progresses, abnormal hematopoiesis results in pancitopenia and decreased immunoglobulin production, followed by nonspecific symptoms such as fatigue or malaise. A cure is usually not possible, and delayed treatment (until symptoms develop) is aimed at lengthening life and decreasing symptoms. Researchers are playing a lead role in investigating CLL's cause and the role of genetics in the pathogenesis of this disorder. Research programs are dedicated towards understanding the basic mechanisms underlying CLL with the hope of improving treatment options.

How to reference
In order to correctly reference this scholarly work, feel free to copy and paste the following:

Ida Franiak-Pietryga and Marek Mirowski (2012). Current Knowledge of Microarray Analysis for Gene Expression Profiling in Chronic Lymphocytic Leukemia, Chronic Lymphocytic Leukemia, Dr. Pablo Oppezzo (Ed.), ISBN: 978-953-307-881-6, InTech, Available from: http://www.intechopen.com/books/chronic-lymphocytic-leukemia/current-knowledge-of-microarray-analysis-for-gene-expression-profiling-in-chronic-lymphocytic-leukem

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