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Alterations in *TP53*, *cyclin D2, c-Myc, p21$^{WAF1/CIP1}$* and *p27^{KIP1}* expression associated with progression in B-CLL

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Abstract: B-cell chronic lymphocytic leukaemia (B-CLL) originates from B lymphocytes that may differ in the activation level, maturation state or cellular subgroups in peripheral blood. Tumour progression in CLL B cells seems to result in gradual accumulation of the clone of resting B lymphocytes in the early phases (G0/G1) of the cell cycle. The G1 phase is impaired in B-CLL. We investigated the gene expression of five key cell cycle regulators: *TP 53, c-Myc, cyclin D2, p21$^{WAF1/CIP1}$* and *p27^{KIP1}*, which primarily regulate the G1 phase of the cell cycle, or S-phase entry and ultimately control the proliferation and cell growth as well as their role in B-CLL progression. The study was conducted in peripheral blood CLL lymphocytes of 40 previously untreated patients. Statistical analysis of correlations of *TP53, cyclin D2, c-Myc, p21$^{WAF1/CIP1}$* and *p27^{KIP1}* expressions in B-CLL patients with different Rai stages demonstrated that the progression of disease was accompanied by increases in *p53, cyclin D2* and *c-Myc* mRNA expression. The expression of *p27^{KIP1}* was nearly statistically significant whereas that of *p21 $^{WAF1/CIP1}$* showed no such correlation. Moreover, high expression levels of *TP53* and *c-Myc* genes were found to be closely associated with more aggressive forms of the disease requiring earlier therapy.

Key words: B-CLL, *c-Myc, TP-53, cyclin D2, p27^{KIP1}*

Introduction

B-cell chronic lymphocytic leukaemia (B-CLL) is a disease characterized by great clinical, phenotypic and prognostic heterogeneity. Its heterogeneity reflects in diverse genome gene expression profiles, including *ZAP-70* (*zeta-chain-associated protein kinase 70*) and *CD38 (cluster of differentiation 38)* genes, different *IgV (immunoglobulin variable region)* gene mutations or their absence. B-CLL originates from B lymphocytes that may differ in the activation level, maturation state or cellular subgroups in peripheral blood. The essence of the disease is continuous complementing replacement of dying B-CLL cells with daughter leukaemic lymphocytes. About 0.1% – 1% of total tumour cells arise every day [1]. It was proved that B-CLL is an accumulative disease with higher levels of proliferation than it was earlier thought [2]. The accumulation of neoplastic cells impairs the equilibrium between cell death [inhibited by accumulation of the anti-apoptotic protein BCL2 (*B-cell lymphoma protein 2*)] and cell proliferation [3]. Tumour progression in B-CLL seems to result in gradual accumulation of the clone of resting B lymphocytes in the early phases (G0/G1) of the cell cycle.

In B-CLL, like in other mammalian tumours, the G1 cell cycle phase is deregulated. The crucial genetic factors responsible for that deregulation remain undetermined.

The cell cycle progression in normal mammalian cells is governed by interactions between cyclins, cyclin-dependent kinases (cdks) and cyclin-dependent kinase inhibitors. Cyclin D2 seems to play a special role in the regulation of B lymphocytes passing through the G1 phase restriction point [4]. The inhibitory effect on the cdks or cdk-cyclin complexes is exerted by cdk inhibitors, *e.g.* p21$^{WAF1/CIP1}$ (*cyclin-*

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dependent kinase inhibitor 1A) and p27Kip1 (*cyclin-dependent kinase inhibitor 1B*) proteins [5]. p21WAF1/CIP1 inhibits the cell cycle in the G1/S phase whereas p27Kip1 acts both as a cyclin-dependent kinase activator and inhibitor [6,7]. The commonest anomalies in B-CLL occur in the G1 phase and G1/S restriction point. Most of the proto-oncogenes and suppressor genes take control at that level, *e.g. c-Myc (v-myc myelocytomatosis viral oncogene homolog)* proto-oncogene and *TP-53 (tumor protein 53 gene)* suppressor gene. *c-Myc* is required for cell proliferation.

Deregulation of *c-Myc* gene expression in many human tumours is associated with poor prognosis and indicates the key role of this oncogene in tumour progression [8]. However, p53 (*tumor protein 53*) is important in multicellular organisms, where it regulates the cell cycle and functions as a tumour suppressor preventing cancer. Moreover, it can arrest the cell cycle at the G_1/S restriction point upon recognition of DNA damage. p53 is essential to prevent the transmission of genetic disorders to daughter cells.

Unfortunately, *TP-53* suppressor gene dysfunction is observed in 50% of all tumours. The *cyclin D2* gene is one of the targets of both *p53* and *c-Myc* proteins [9]. The wild-type p53 protein induces *p21WAF1/CIP1* expression. The expression level of the *p21* cell cycle inhibitor is considered a marker of proper function of p53 as a transcription activator. Increased levels of p21 expression reflect *p53* overexpression caused by DNA damage [10]. Most of the peripheral blood CLL lymphocytes are arrested in the G0/G1 phase of the cell cycle, which is mainly attributed to high p27 protein expression [11,12].

B-CLL, like other tumours, is characterized by growth abnormalities. The aim of our study was to determine c-Myc, TP-53, cyclin D2, p21WAF1/CIP1, p27KIP1 mRNA expression in B-CLL lymphocytes and its connection with disease progression.

Materials and methods

Patients. The study was conducted in peripheral blood lymphocytes of 40 patients diagnosed with chronic lymhocytic B-cell leukaemia (B-CLL). The study cohort was recruited from the previously untreated, B-CLL patients of the Haematooncology and Bone Marrow Transplantation Department, Medical University of Lublin, Poland. The follow up period was four years. The control group included 16 healthy volunteers, 5 females and 11 males of comparables ages (median 62, range 38±79). The disease stages were determined according to the Rai classification [13]. Clinical data are presented in Table 1.

Flow cytometry analysis. The study was conducted in peripheral blood lymphocytes obtained using the standard Böyum method [14]. The immunophenotype was detected by flow cytometry with monoclonal antibodies selectively binding the following antigens: CD5 PE /CD19 FITC (Serotec/Serotec), CXCR4 PE/ CD19 FITC (Becton Dickinson/ Becton Dickinson), CD23 PE/ CD19 PE-Cy5 (DacoCytomation/ Becton Dickinson), CD21 FITC/ CD19 PE-Cy (DacoCytomation/ Becton Dickinson).

RNase Protection Assay (RPA). Total RNA was extracted from peripheral blood lymphocytes using TRI Reagent and was subjected to ribonuclease protection assay (RPA). Detection of studied mRNA genes was performed with RPA system (RiboQuant, Pharmingen, San Diego, CA). Multiprobe custom template sets (p53, CXCR4, p27, p21, cycD2, c-myc) and two housekeeping genes, L32 and glyceraldehydes-3-phosphate dehydrogenase (GAPDH), were used for *in vitro* transcription using T7 RNA polymerase to direct synthesis of highly specific [^{32}P]-labeled antisense RNA mixtures. Each template set was transcribed using Riboprobe system (Promega, Madison, WI) in the presence of [^{32}P] uridine triphosphate (UTP) (3000 Ci/mmol, NEN, Boston, MA). Total RNA (20 µg) was hybridized with [^{32}P]-labeled antisense RNAs at 56°C overnight and was subjected to ribonuclease treatment. Protected fragments were precipitated and separated on a 5% acrylamide gel. Electrophoresis was conducted in Consort EV233 under the following conditions: 45W, 1500V, 2h. Signal detection was performed by autoradiography with the use of X-ray film cassette placed in -20°C. The X-ray was scanned and optical density of each band was analysed using the Gel Scanv1.13 (Kucharczyk).

Ethical issues. The study was approved by the ethics committee of Medical University in Lublin and written informed consent was obtained from patients for the use of the clinical specimens for research.

Statistical analysis. Experimental data was subjected to statistical analysis using Statistica 6.0 software (Kaplan-Meier's survival analysis, log-rank and Spearman rank correlation). The differences were considered significant at $p<0.05$.

Results

The gene expression was defined as a quotient of optical density of the gene band studied and optical density of *L32 (60S ribosomal protein L32 gene)* housekeeping gene band (Figs 1 and 2).

Another issue analysed was a link between *p53* expression and disease progression. For this purpose, patients were divided into two groups with high and low *p53* expression levels. The highest value of normal *p53* expression in peripheral blood lymphocytes of healthy volunteers was considered a discriminating value. According to the normal distribution theory, the upper limit of the parameter range is the sum of mean (X) and two standard deviations (2SD): X+ 2SD= 0.095+ 2 x 0.074=0.24.

The Kaplan-Meier's survival analysis was used for two groups of B-CLL patients with the *p53* expression level higher and lower than 0.24 (higher and lower than the upper limit of the normal *p53* expression range). The period between the diagnosis and onset of treatment or latest follow up was analysed.

Patients with the *p53* mRNA expression levels higher than 0.24 had shorter treatment-free survival than the other group ($p=0.03$) (Fig. 3). As seen in Fig. 3, three years after the diagnosis, the number of untreated patients with high *p53* expression levels declined to zero, *i.e.* each patient in this group received cytotoxic treatment.

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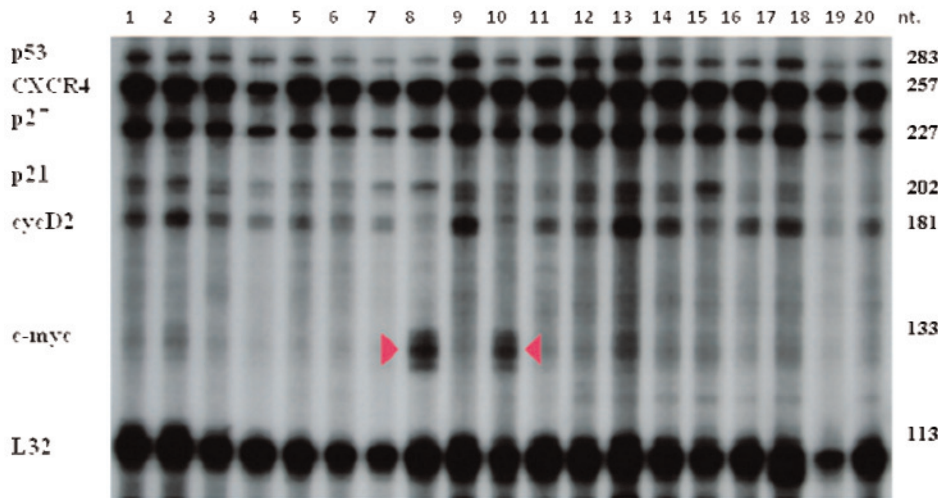

Fig. 1. Electrophoresis of hybrids of the probe and gene transcripts studied. The identification of the consecutive bands was performed based on the calibration curve traced according to the manufacturer's instructions. Arrowheads mark the bands of highly expressed c-Myc mRNA in patients No. 8 and 10. Bands molecular weight in nucleotides – nt (protected fragments) is marked on the right side of the figure.

Table 1. Patients clinical data.

Number of patients	n=40
Female/male	22/18
Age (median, range)	68 (39 - 83)
Rai stage	Stage 0 - 5 Stage I - 10 Stage II - 13 Stage III - 9 Stage IV - 3
White blood cell count (WBC) (G/L)	47.05 (16.5 - 638)
Lymphocyte count (G/L)	40.7 (11.4 - 630)
Hemoglobin level (g/dl)	13.35 (6.82 - 17.2)
Platelet count (PLT) (G/L)	174.5 (76.9 - 345)

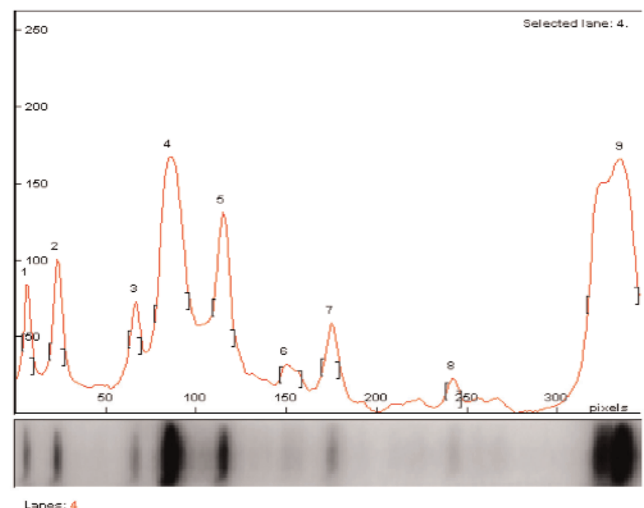

Fig. 2. An example of expression level analysis of the studied genes in Gel Scan v1.13 computer programme. Numbered peaks correspond to 3- *p53,* 5- *p27,* 6- *p21,* 7- *cyclin D2,* 8- *c-Myc* and 9-*L32.* The curve deflection depended on the magnitude and intensity of the band and was correlated with the amount of the radioactive isotope bound. The relative optical density was defined as a quotient of the optical density of the gene band studied and optical density of *L32* housekeeping gene.

In two patients (No. 8 and 10), the c-Myc mRNA expression level was surprisingly high (Fig. 4). To analyse this phenomenon, the Kaplan-Meier method was used. In unstimulated B-CLL cells, the *c-Myc* expression level is significantly lower in comparison with normal lymphocytes [15]. With regard to this, the median c-Myc mRNA expression in B-CLL patients was chosen as the discriminating value ($M_{c\text{-}Myc}$=0.0604).

Kaplan-Meier's analysis implies that patients with higher c-Myc mRNA expression levels had much shorter treatment-free survival than those with lower *c-Myc* expression levels.

Moreover, Kaplan-Meier's analysis was carried out in groups with different cyclin D2 mRNA expression. The median cyclin D2 mRNA expression level in B-CLL patients was considered the discriminating value ($M_{Cyclin\ D2}$=0.19). Cyclin D2 mRNA expression levels defined by this value were not related to treatment-free survival (p=0.14) (Fig. 5).

In four randomly selected patients, gene expression analysis was repeated one year after the diagnosis. During this period, the patients did not receive any cytotoxic treatment. The repeated analysis indicated a gradual increase in expression of each gene studied. The highest increase was noted in the patient with progressive disease (LDT<6 months, treatment-free survival – 21 months).

To explain the above observation further, the correlation analysis between the parameters studied and Rai stages was conducted (Table 2, Fig. 6).

The results suggest that the expression level of p53, cyclin D2 and c-Myc was correlated with the Rai stage of the disease; the p27 and Rai stage correlation was near statistical significance (p=0.066). The p21 expression level did not correlate with the Rai stage.

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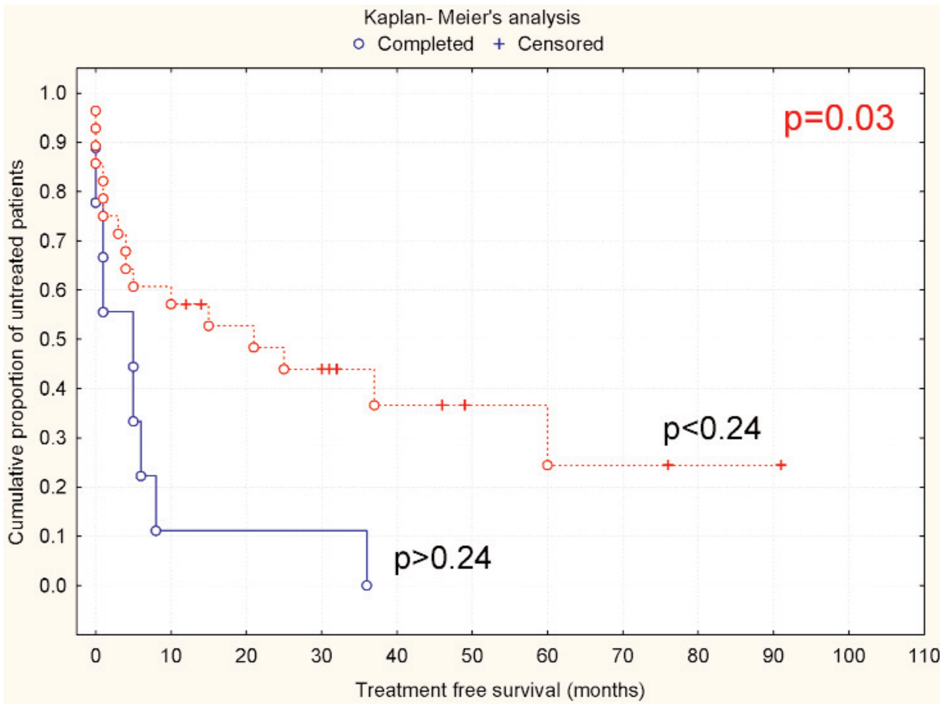

Fig. 3. A high *p53* expression level as a negative prognostic factor indicating shorter treatment-free survival. The period between diagnosis and initiation of treatment was analysed. Patients with *p53* expression levels, estimated as the relative optical density of the proper band, higher than the border value of 0.24, required the institution of treatment earlier compared to patients with lower *p53* expression levels. After approximately 36 months, the number of untreated patients in the *p53*>0.24 group declined to zero (blue curve), *i.e.* each patient in this group underwent treatment.

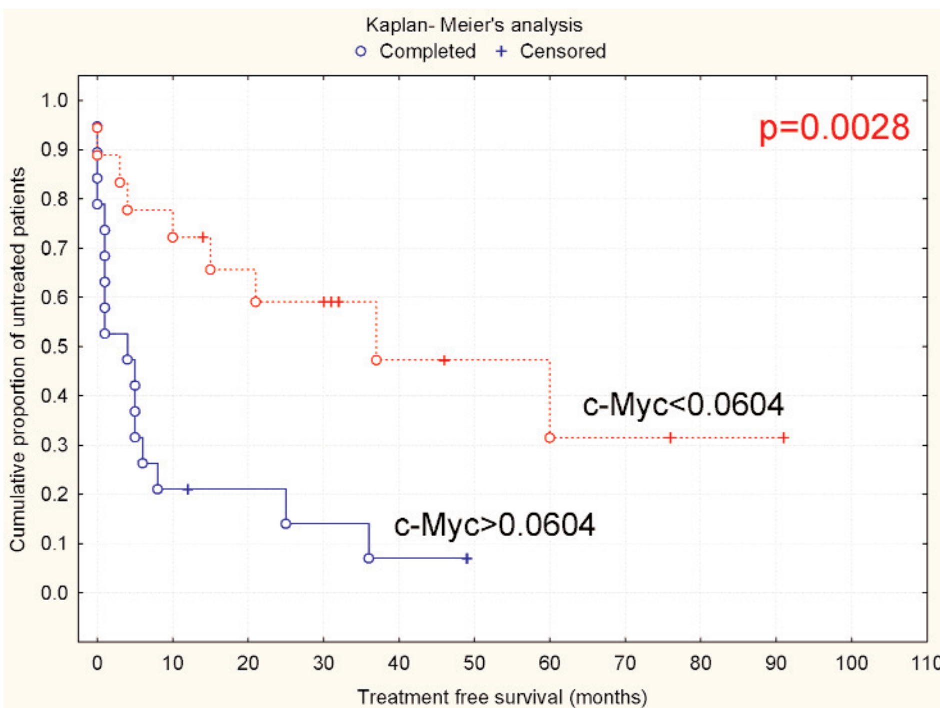

Fig. 4. A high *c-Myc* expression level as a negative prognostic factor indicating shorter treatment-free survival. The period between diagnosis and initiation of treatment was analysed. The number of untreated patients decreased over time. The discriminating value- 0.0604 was the median *c-Myc* expression level in B-CLL patients. Treatment-free survival in groups with high and low c-Myc mRNA expression levels varied in a statistically significant way (p=0.0028).

Discussion

Cell cycle progression is regulated by both intracellular and extracellular control mechanisms. Intracellular control guarantees that cell cycle progression is stopped in response to DNA damage, whereas extracellular factors may determine the cell proliferation, differentiation or apoptosis. New evidence suggests that antigenic stimulation, along with interactions with accessory cells and cytokines, is a promoting factor in CLL that stimulates the proliferation of CLL cells and allows them to avoid apoptosis. *In vivo* studies using nonradioactive methods suggest that CLL cells are more dynamic than is usually appreciated [1]. This dynamic process is also composed of cells that proliferate and die, often at appreciable levels.

In our study, we specifically investigated the gene expression of five key cell cycle regulators*: TP 53, c-Myc, cyclin D2, p21* $^{WAF1/CIP1}$ and *p27*^{KIP1}, which primarily regulate the G1 phase of the cell cycle, or S

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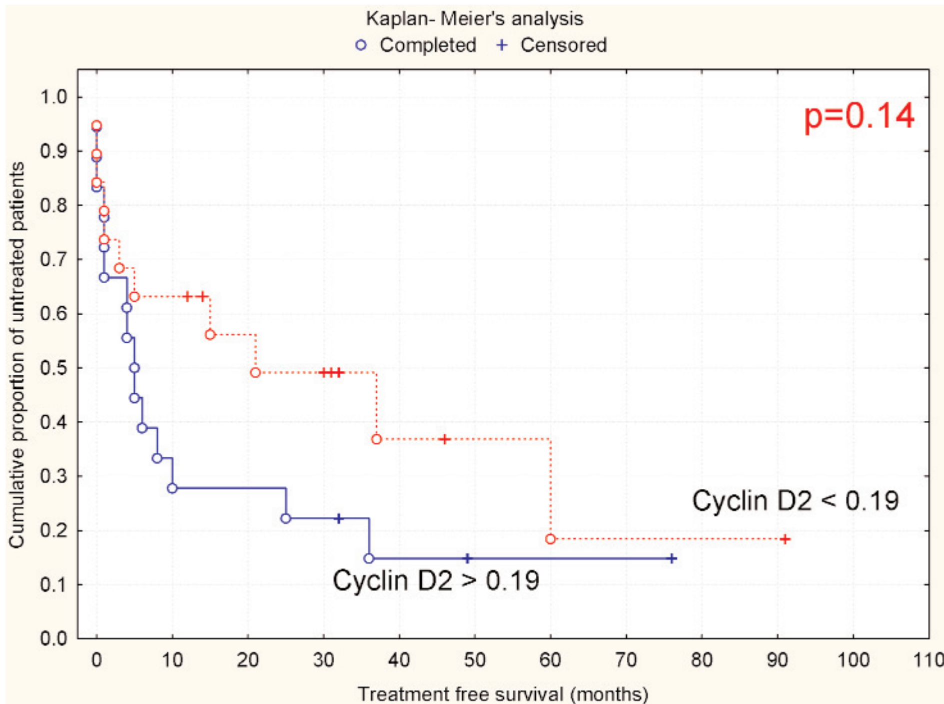

Fig. 5. A *cyclin D2* expression level as a negative prognostic factor of treatment-free survival. The period between diagnosis and initiation of treatment was analysed. The number of untreated patients decreased over time. The discriminating value 0.19 was the median cyclin D2 expression level in B-CLL patients. Treatment-free survival in groups with high and low *cyclin D2* mRNA expression levels showed no statistical variation (p=0.14).

Table 2. Correlation between gene expression levels and Rai stages according to the Spearman R rank correlation coefficient and p value.

Rai stage correlation with	Spearman R correlation coefficient	p value
p53	0.41	0.011
p27	0.30	0.066
p21	0.20	0.23
cyclin D2	0.35	0.033
c-Myc	0.41	0.011

phase entry and ultimately control the proliferation and cell growth. We analyzed mRNA expression levels of those genes and their role in B-CLL progression.

Statistical correlation analysis of *TP53, cyclin D2, c-Myc, p21* $^{WAF1/CIP1}$ and *p27*^{KIP1} expression in B-CLL patients with different Rai stages demonstrated that progression of disease was accompanied by increases in p53, cyclin D2 and c-Myc mRNA expression. *p27*^{KIP1} expression was nearly statistically significant whereas *p21* $^{WAF1/CIP1}$ expression showed no such correlation. Moreover, we found that high expression of *TP53* and *c-Myc* genes was closely associated with more aggressive disease course requiring earlier therapy.

Patients with high *p53* expression were characterized by significantly increased β_2- *microglobulin* levels, reflecting high disease activity and correlating with advanced Rai stages. Similar observations were published by Cordone *et al*. [16] and Ghia *et al*. [17].

Such findings suggest that the disease progression may lead to the selection of the damaged genome clone, which is visible in increased p53 mRNA expression observed in our study. According to Pettitt *et al.* [10], increased p53 expression always results in increased p21$^{WAF1/CIP1}$ levels. This feedback relation seems to be much more complex. Kienle *et al.* [18] found that the level of p21 mRNA expression was abnormally high in patients with 17p13.1 deletion (*p53* locus). It may be two fold higher than the one observed in CLL patients without that structural chromosomal aberration. Generally, the level of p21 mRNA does not correlate with p21 protein expression. Abnormal, high mRNA p21 $^{WAF1/CIP1}$ levels, frequently without increased protein expression levels, are observed in B-CLL with deletions of chromosome 17p13.1 locus. In such cases, p21 $^{WAF1/CIP1}$ levels may be twice higher than those of normal B-CLL karyotypes. Koníková and Kusenda [19] suggest that decreased p21 $^{WAF1/CIP1}$ protein expression in CLL patients may result from high MDM2 (*the murine double minute 2*) protein "activity", which is responsible for the p21 transport to proteasomes. On the other hand, abnormally high enzymatic activity of a proteasome itself [20] is likely to decrease significantly the p21 protein level. Cell cycle abnormalities may be the consequence of that phenomenon, since the cells of low or undetectable p21 $^{WAF1/CIP1}$ protein expression are able to enter the cell cycle much more easily, or even reach and pass the S phase despite the accompanying DNA damage [21].

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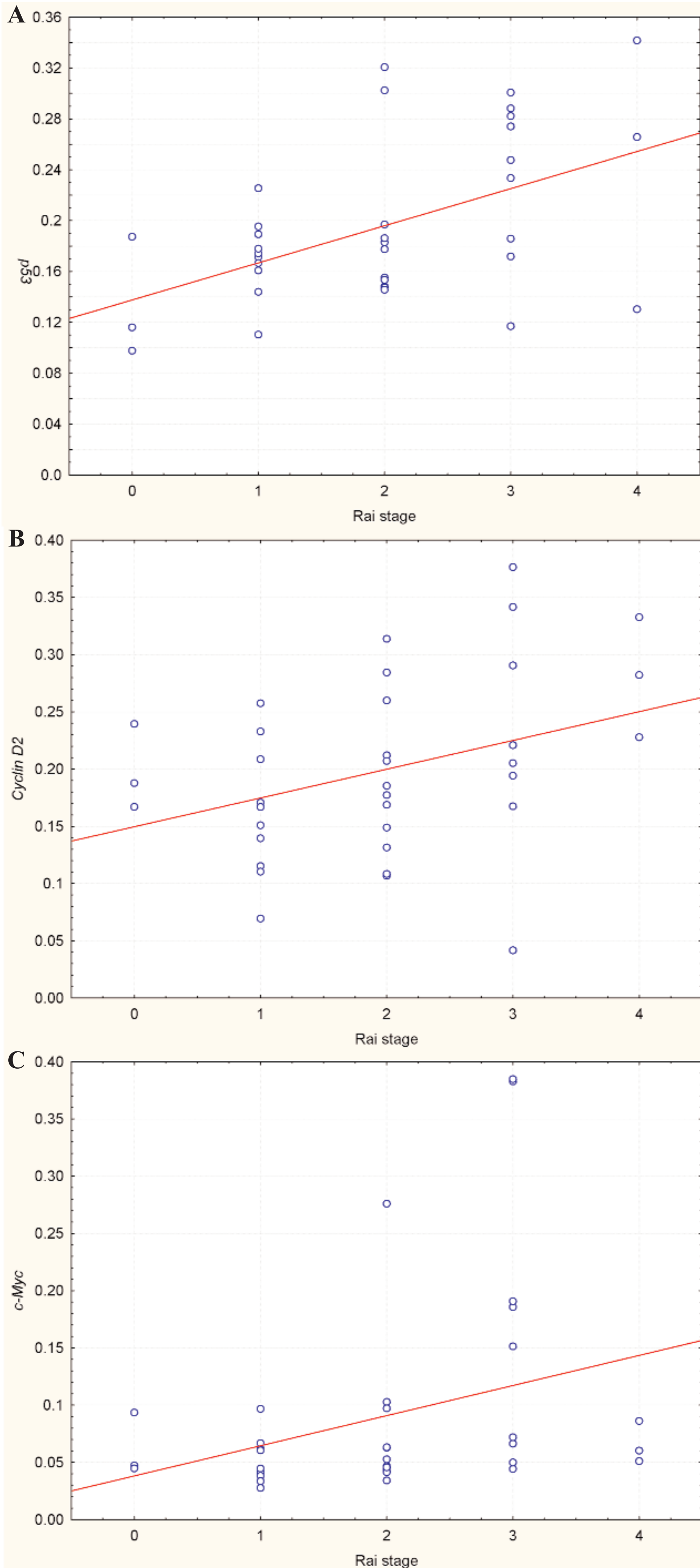

Fig. 6. Rai stage and *p53* (**A** – R=0.41; p=0.011), cyclinD2 (**B** – R=0.35; p=0.033) and *c-Myc* (**C** – R=0.41; p=0.011) correlation graphs.

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An overwhelming majority of B-CLL lymphocytes remains in the G0/G1 phase of cell cycle and this anergic state largely depends on the p27 protein expression. The $p27^{Kip1}$ protein expression is found to be even twofold higher in 80% of CLL compared to normal B lymphocytes [15]. In our study a nearly statistically significant correlation between p27 protein expression and lymphocyte-doubling time was observed in CLL patients. Our results are consistent with the ones published by Vrhovac *et al*. [22]. They found that high $p27^{Kip1}$ protein expression in B-CLL correlated with disease progression, faster lymphocyte-doubling time and poor prognosis. More stable disease progression is not accompanied by increased p27 protein expression [22,23].

The family of cyclins is one of the most important factors involved in the regulation of the cell cycle of both normal and malignant cells. As far as *cyclin D2* is concerned, the gene for *cyclin D2 (CCND2)* is activated in the early G1 phase and may play a role in carcinogenesis, as its amplification has been found in several malignancies including B-CLL [24]. *Cyclin D2* expression depends on the degree of lymphocyte B activation, which seems to increase in proliferating B-CLL cells [25]. *Cyclin D2* expression was demonstrated [26] to increase threefold upon induction of proliferation of lymphocytes through the BCR (*B cell receptor*) in "unmutated B-CLL." It proves a significant role of cyclin D2 in B-CLL lymphocyte proliferation. We demonstrated that *cyclin D2* increased with disease progression, which supports the hypothesis of considerable amounts of B-CLL lymphocytes exit the quiescence state and enter the cell cycle.

Furthermore, some inducing factor(s) are involved, which stimulate *cyclin D2* expression in B-CLL cells. The hypothesis is confirmed by the fact that D-type cyclins are synthesized as long as growth factors are present, and therefore, *cyclin D* has been suggested to act as a growth factor sensor [27]. The presence of cells with higher *cyclin D2* expression levels in peripheral blood may reflect their increased proliferative index. Similarly, with the disease progression, cells with *ZAP-70* and CD38 expression may be observed, which are normally absent [28]. Our results are different from those reported by Paul *et al*. [29], who did not find any correlation between cyclin D2 mRNA expression and disease progression according to the Rai stage.

However, our study was carried out on lymphocytes of untreated B-CLL patients; Paul *et al*. analysed lymphocytes of patients in the 3rd and 4th Rai stage after chemotherapy, 4 weeks before the initiation of treatment. It is well known that at least one of the chemotherapeutics administered in B-CLL therapy, fludarabine, strongly impedes the *cyclin D2* expression [30].

Moreover, we demonstrated a correlation between increased mRNA c-Myc expression and B-CLL progression, which is an unfavourable prognostic factor. B-CLL patients with increased c-Myc mRNA expression required shorter time to treatment (TTT), which may indicate the selection of cells, which transmit the proliferation- and not apoptosis-stimulating signal. High *c-Myc* expression reflects aberrant function of the DNA repair pathway, dependant on *p53* and/or ATM (ataxia telangiectasia mutated) [31,32], which may be one of the characteristic factors of B-CLL.

It seems that mobilization of cell-cycle factors and their overexpression in B-CLL is triggered by abnormal BCR-mediated signals due to either as-yet-undiscovered lesions of the signal transducing pathway or still-elusive cytogenetic lesions responsible for the neoplastic transformation. The reason why the signal from BCR causes either cell cycle initiation or cell death remains to be determined. Cross-linking of surface IgM (*Immunoglobulin M*) in CLL cells may trigger signal transduction that can cause or protect from apoptosis [33,34], whereas cross-linking of surface IgD invariably prevents apoptosis [33]. The two surface isotypes express the same clone-specific antigen-binding site and provide concordant signals in mature B cells. The possibility that the B-CLL cell precursors could be stimulated through receptors other than the BCR should be taken under consideration. Bacterial products such as LPS (*lipopolysaccharide*), CpG, or peptidoglycans that bind to different Toll-like receptors can directly stimulate B-cells [35,36].

Increased expression of the genes observed with the disease progression may imply permanent existence of unknown growth factors in CLL, which are capable of binding with cell receptors and transmitting stimulatory signals to the cell nucleus leading to cell cycle progression. Identification of stimulating antigens, their receptors, signal transduction pathways and still-elusive cytogenetic lesions responsible for neoplastic transformation in B-CLL is necessary to explain the pathogenesis of the disease.

Periodic yet regular gene expression profiling may be useful for monitoring the disease progression and instituting proper, appropriately timed therapy.

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