Simultaneous assessment of p53 and MDM2 expression in leukemic cells in response to initial prednisone therapy in children with acute lymphoblastic leukemia

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Ineffective apoptosis is one of main causes of a treatment failure in childhood acute lymphoblastic leukemia (ALL). p53 plays a crucial role in triggering apoptosis of ALL in response to prednisone treatment. MDM2 is the endogenous inhibitor of apoptosis that downregulates the functional activity of p53 protein. This study is aimed to evaluate changes in MDM2 and p53 expression in peripheral blood mononuclear cells collected from children with ALL prior to and after 6 and 12 h of prednisone administration in relation to early treatment response.
The study comprised 35 children with newly diagnosed ALL, subdivided into good (n = 24) and poor (n = 11) early treatment responders. MDM2 – associated APC fluorescence and p53 – associated FITC fluorescence were measured by the laser scanning cytometer.
In the group of poor responders, p53 and MDM2 fluorescence were significantly higher than in the group of good responders. In the group of good early treatment responders, a statistically significant rise of p53 fluorescence measured in the nucleus and in the cytoplasm 12 h after prednisone administration as well as increase in MDM2 fluorescence measured in the cytoplasm 6 and 12 h after prednisone administration were seen. These data suggest that pretreatment overexpression of MDM2 protein may contribute to poor early treatment response.

Key words: acute lymphoblastic leukemia, children, p53, MDM2, early treatment response.

Introduction

Acute lymphoblastic leukemia (ALL) accounts for approximately 80% of all leukemias in children [1-3]. One of the most important prognostic factors in ALL is an early response to treatment including a response to a preliminary therapy with glucocorticoids (GC) which is an independent and strong prognostic factor in ALL in children. Patients that respond well to preliminary GC treatment have a statistically better prognosis as compared to those who demonstrate a poor response to initial GC therapy [2-4]. GC act through binding to GC receptors (GR). In the absence of hormone, GR resides in the cytosol combined with a variety of proteins including the heat shock protein 90, the heat shock protein 70 and the protein FKBP52. A direct mechanism of action involves homo-dimerization of the receptor, translocation via active transport into the nucleus, and binding to specific DNA responsive elements activating gene transcription [5-7]. The mechanism of GC acting upon leukemic cells has not been fully elucidated to date, yet it is assumed that the said mechanism arrests the cells in G1 phase to induce

apoptosis and modulate the expression of apoptosis-regulating proteins, e.g. through exerting a direct inhibitory effect on Bcl-2 protein or via direct activation of the caspase cascade (including caspase-3) [5, 7-9].

The key gene that controls apoptosis is the *TP53* gene. Its protein product is a 53-kilodalton phosphoprotein composed of 393 amino acids, which participates in regulation of proliferation and apoptosis [10-12]. p53 protein initiates the process of apoptosis – on the one hand, via activation of proapoptotic proteins from the Bcl-2 family, e.g. Bax, Bak, and Bcl-xS, which subsequently activate the anion channels of the external mitochondrial membrane or directly damage the mitochondrial membrane, thus releasing cytochrome c, or, on the other hand, via inhibition of expression of antiapoptotic Bcl-2 family proteins, e.g. Bcl-2, Bcl-xL, Mcl-1, Bcl-W, Bfl-1, Bcl-B [11, 13, 14]. No direct association has been demonstrated between p53 protein and GC, apart from the fact that p53 regulates the *BCL-2*, *BAX* genes that participate in GC-induced apoptosis [5].

The murine double minute 2 protein (MDM2) encoded by the *MDM2* gene is a nuclear 90-kilodalton phosphoprotein, the synthesis of which is induced by p53 protein in consequence of DNA damage. As experimental studies show, MDM2 protein, through binding to the N-terminal domain, triggers p53 inactivation through three mechanisms:

- MDM2 binding to p53 directly inhibits p53-dependent transcription,
- MDM2 facilitates the ubiquitinization of p53 and its subsequent degradation in proteasomes,
- MDM2 binding to p53 facilitates the translocation of p53 protein from the nucleus to the cytoplasm [15-18].

p53 and MDM2 shuttle constantly through the nuclear membrane and are assumed to interact in a negative feedback mechanism [19-21]. MDM2 has also several p53-independent functions, which have been extensively discussed by Ganguli and Wasylyk [22].

An increasing number of experimental as well as still scarce clinical data indicate that one of the most important causes of a poor response to GC treatment in childhood ALL is insufficient apoptosis, which may be caused by overexpression of proteins belonging to the group of endogenous apoptosis inhibitors, of which MDM2 seems to be the most interesting [13, 18, 22, 23]. Therefore, the purpose of this study was simultaneous evaluation of changes in the expression of p53 protein and its inhibitor, MDM2, in peripheral blood mononuclear cells of children with ALL within the first 12 hours of GC therapy. To the best of our knowledge, such study has not been reported yet, although, the better insight into MDM2 role in response to initial treatment is of a clinical importance from the point of view of specific targeted therapy with the use of MDM2 inhibitors e.g. nutlin that in experimental conditions enhance apoptosis of leukemic cells in children with ALL [24, 25].

Materials and methods

Patients

The study comprised 35 children (14 girls, 21 boys) aged 6-192 months (mean 89.4 months; median 95.0 months) with *de novo* ALL diagnosed between June 2002 and October 2004. The diagnosis was based on morphologic examination of bone marrow smears stained with MGG and confirmed by results of flow cytometric immunophenotyping of bone marrow cells. Characteristics of patients are given in Table I. All children were treated according to BFM ALL 90 Protocol. The initial phase of therapy consisted of oral administration of prednisone 60 mg/m^2/day in three divided doses plus one age-related dose of intrathecal methotrexate on the first day of treatment (6 mg $<$ 1 year, 8 mg 1-2 years, 10 mg 2-3 years, 12 mg $>$ 3 years.). During the first day of treatment three consecutive doses of prednisone were given at 6 hours' intervals. Based on two criteria: prednisone response (as defined by the BFM Group) and bone marrow day 15 leukemic cells rate, patients were classified as good early treatment responders (*i.e.* patients with peripheral blood leukemic cell count no more than 1×10^3/µl on day 8 and with no more than 5% of leukemic cells in their bone marrow on day 15; n = 24) and poor early treatment responders (*i.e.* patients with peripheral blood leukemic cell count exceeding 1×10^3/µl on day 8 and/or with more than 5% leukemic cells in their bone marrow on day 15; n = 11). The follow-up time was 0-42 months (mean 22.5 months, median 21 months). Causes of treatment failure were defined as lack of remission, relapse and death. The study was approved by the Ethical Committee of the Pomeranian Medical University.

Preparation of peripheral blood cells for analysis

Peripheral blood was sampled prior to prednisone administration and 6 and 12 h after first dose of prednisone (prior to 2nd and 3rd dose of oral prednisone) into polystyrene tubes containing 50 µl of Heparin (Polfa Warszawa, Poland). 2-5 ml of peripheral blood was mixed with equal volume of PBS (phosphate buffered saline), pH 7.4 without Ca and Mg. The mixture was then laid on onto ficoll (Gradisol, Polfa Kutno, Poland) in ratio 2 volumes of diluted blood to 1 volume of ficoll followed by centrifugation at 800 g for 30 minutes at room temper-

ature (Eppendorf Centrifuge 5403, Germany). Mononuclear cells containing layer was collected and rinsed twice in PBS each time at 200 g for 5 min (Eppendorf Centrifuge 5403, Germany). Cell pellet was resuspended in appropriate volume of PBS to obtain final cell concentration of 5×10^6/ml. Aliquots of 200 µl were centrifuged at 300 g for 5 min (Shandon Cytospin 3, USA). Cytospin preparations were fixed in cold (0-4°C) 1% buffered paraformaldehyde for 15 min followed by cold (–20°C) 70% ethanol for up to 24 hours. Slides were subsequently air dried then stored at –20°C.

Immunocytochemistry

For simultaneous immunocytochemical detection of MDM2 and p53, slides were rinsed in PBS twice, each time for 5 min, then 100 µl of 1 : 20 diluted monoclonal mouse anti-human MDM2 protein (Clone SMP-14; DakoCytomation, # M 7146) was added and the slides were incubated for 1 h at 37°C. The slides were rinsed in PBS for 5 min and 100 µl of 1 : 40 diluted APC-conjugated goat anti-mouse IgG1 (Molecular Probes, # A 865) was added, and the slides were incubated for 1 h at room temperature. After rinsing in PBS, the cells were treated with 100 µl mouse serum – normal (DakoCytomation, # X 0910) at room temperature for 10 minutes to suppress nonspecific staining. The slides were rinsed in PBS for 5 min and 100 µl of 1 : 10 diluted FITC-conjugated monoclonal mouse anti-human p53 protein (Clone DO-7; DakoCytomation, # F 7054) was placed onto cells. The slides were incubated for 30 min at room temperature, rinsed once again in PBS and then incubated for 30 min at room temperature with 200 µl of propidium iodide (PI, Sigma Aldrich) in concentration of 10 µl/ml in the presence of ribonuclease A (Ribonuclease A type I-AF from bovine pancreas, Sigma Aldrich) in concentration of 100 µl/ml. During all incubations cells were deposited in a humid chamber and protected from light. Slides prepared from the same blood sample and stained according to the described procedure but with respective isotype antibodies (for MDM2 – mouse serum – normal; for p53 – mouse IgG2b DakoCytomation, # X 0944) served as negative controls.

Fluorescence measurements

Slides were mounted with coverslips and MDM2 – associated APC long red fluorescence (emission maximum around 660 nm), p53 – associated FITC green fluorescence (emission maximum 520 nm) and DNA-associated PI red fluorescence (emission maximum 580-nm) excited by 633-nm helium-neon laser and 488-nm argon laser, respectively were measured simultaneously by laser scanning cytometer (LSC, CompuCyte, USA). DNA-associated PI red fluorescence was used as a contouring parameter (threshold contour). Values of integrated MDM2 – associated APC long red fluorescence and integrated p53-associated FITC green fluorescence were measured separately over the cell nucleus and over the rim of cytoplasm, and recorded as FCS 3.0 files by WinCyte 3.4 software. The integrated fluorescence is the sum of fluorescence of all the pixels within the integrated fluorescence contour. Its value ranges between 0 and 8 000 000 of relative units for a single fluorescence measurement.

Principles of evaluating MDM2-associated APC long red fluorescence and p53-associated FITC green fluorescence are presented in Fig. 1. Based on two parameters, DNA-associated PI red fluorescence maximal pixel and cell area cells were selected for further analysis (dot plot A). To measure integrated MDM2-associated APC long red fluorescence (Long Red Fluorescence Integral) and integrated p53-associated FITC green fluorescence (Green Fluorescence Integral) over the nucleus, integration contour was set at +4 pixels from threshold contour (dot-plots B and D), whereas peripheral inner and outer contours were introduced at +6 and +8 pixels from threshold contour in order to measure long red (Long Red Fluorescence Priph Int) and green fluorescence (Green Fluorescence Priph Int) intensity over a rim of cytoplasm (dot-plots C and E). Background fluorescence was subtracted automatically by the software. In each slide, at least 5000 cells were measured. The ratio of the p53/MDM2 that represents the relative proportion of the studied protein to its inhibitor was calculated separately in the nucleus and the cytoplasm by dividing the values of relative units of p53-associated FITC green integrated fluorescence by MDM2-associated APC long red integrated fluorescence.

Statistical analysis

Censoring events were defined as lack of remission, death in remission and relapse. Event-free survival was defined as the time from diagnosis to any event defined above, whereas disease-free survival – as the time from the point of achieved remission to a relapse. Probabilities of event-free survival (p-EFS) and disease-free survival (p-DFS) were calculated with the use of life table analysis and compared in respective groups of patients with logrank test. Cytometric data were expressed as mean ± SD. W Shapiro-Wilk test for normality and the U Manna-Whitney test, t-test for two samples assuming unequal variances, paired t-test and analysis of variance were used for statistical analysis. P-values equal or less than 0.05 were considered statistically significant.

A

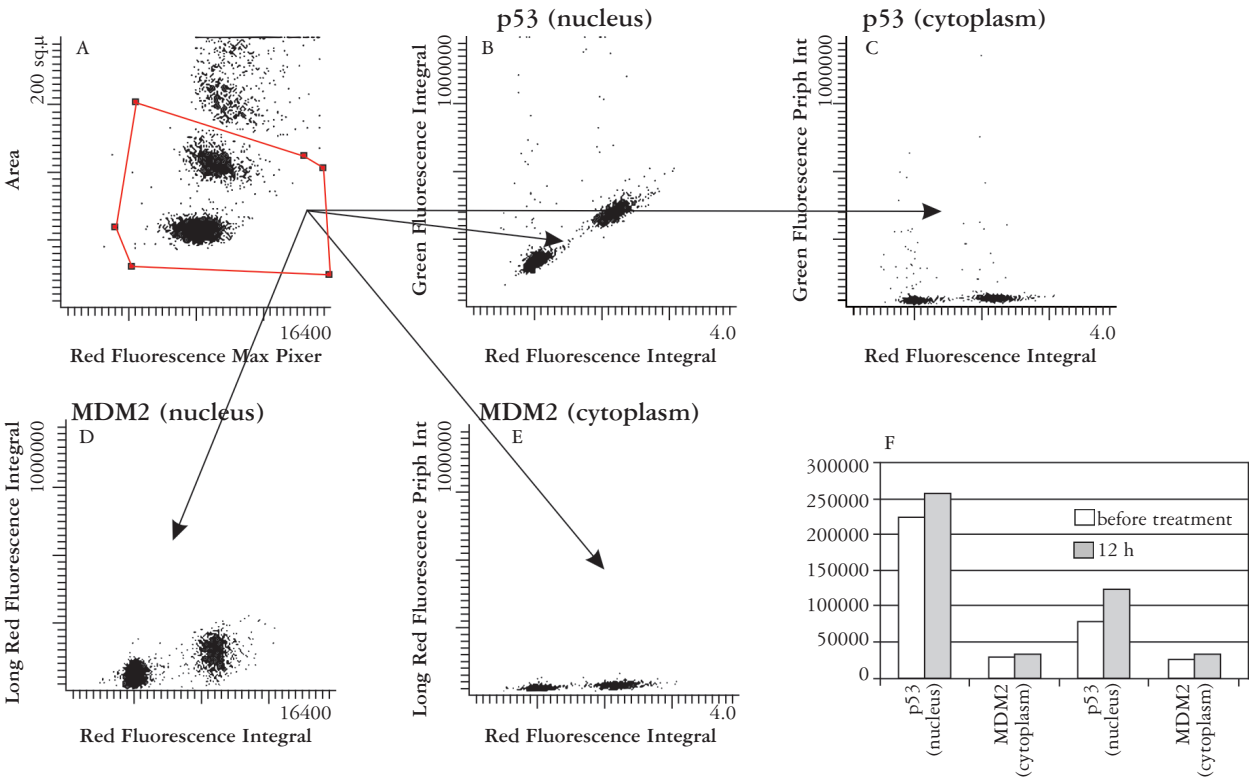

Fig. 1. Principles of MDM2-associated APC long red fluorescence and p53-associated FITC green fluorescence measurements. Based on two parameters DNA-associated PI red fluorescence maximal pixel and cell area cells were selected for further analysis (dot plot A). Changes in integrated MDM2-associated APC long red fluorescence (Long Red Fluorescence Integral) and integrated p53-associated FITC green fluorescence (Green Fluorescence Integral) were measured separately over the nucleus (dot-plots D and B) and over a rim of cytoplasm (Long Red Fluorescence Priph Int, Green Fluorescence Priph Int; dot-plots E and C). Graph F represents changes in MDM2-associated APC long red fluorescence and p53-associated FITC green fluorescence measured in individual patient before treatment and after 12 h

Results

Treatment results are presented in Fig. 2. Life-table and logrank analysis revealed that both p-EFS and p-DFS were significantly better for children with good early treatment response (1.0 vs. 0.635 ±0.145; p = 0.002 and 1.0 vs. 0.716 ±0.139; p = 0.008, respectively).

Both groups differed in the intensity of p53 and MDM2 nuclear expression before the prednisone administration, *i.e.* in the group of poor responders, p53-FITC green fluorescence and MDM2-associated APC long red fluorescence were significantly higher than in the group of good responders (101 470 vs. 82 715; p = 0.05 and 80 466 vs. 62 032; p = 0.025, respectively) (Fig. 3).

Changes of p53-associated FITC green fluorescence and MDM2-associated APC long red fluorescence intensity in the group of good early treatment responders are presented in Fig. 4. Statistically significant rise of p53-associated FITC green fluorescence measured in the nucleus (225 670 vs. 241 954; p = 0.05) and in the cytoplasm (82 715 vs. 94 160; p = 0.001) was seen 12 hours after prednisone administration in the group of good early treatment responders, whereas it remained statistically unchanged in both compartments in the group of poor early treatment responders (data not shown).

In the group of good early treatment responders, statistically significant rise of MDM2-associated APC long red fluorescence measured in the cytoplasm was observed 6 and 12 hours after prednisone administration (62 032 vs. 72 923; p = 0.015 and 62 032 vs. 71 574; p = 0.003, respectively), whereas it remained unchanged at the same time points when measured in the nucleus in the same group, and in the cytoplasm and the nucleus in the group of poor early treatment responders.

Discussion

The response to initial GC therapy continues to be one of the most important prognostic factors in childhood ALL [1, 4]. The selection of time points in the present investigation (after 6 and 12 hours) has been arbitrary and resulted from the following premises:

- oral prednisone is a biologically inactive compound and must be transformed into pred-

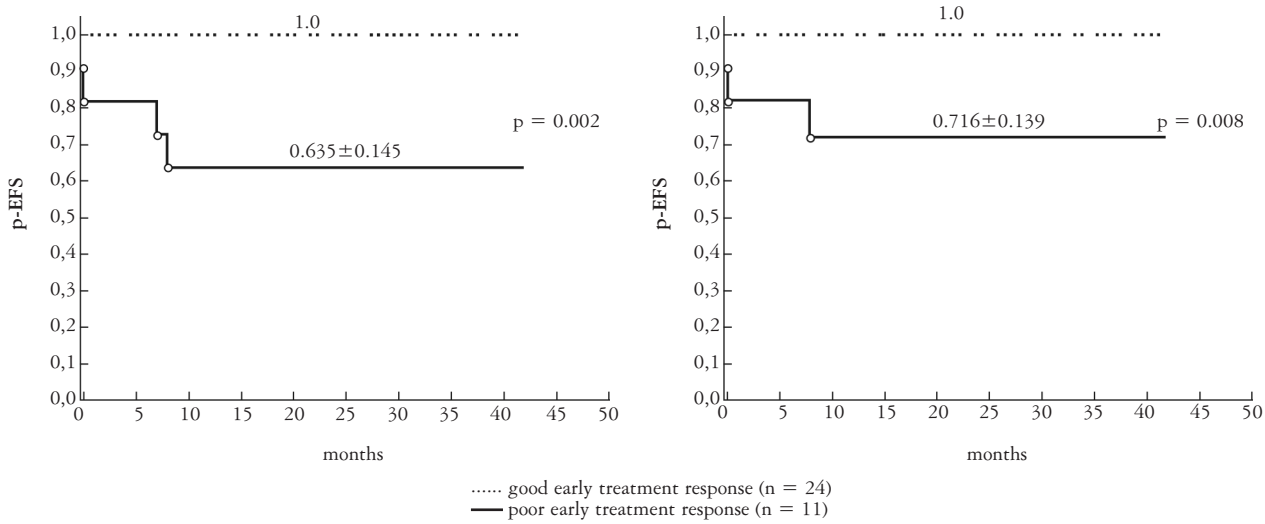

Fig. 2. Probability of event-free survival (p-EFS) and disease-free survival (p-DFS) in respect of early treatment response

nisolone. Maximum serum prednisolone concentration is observed 2–3 hours after oral administration of the drug, and

- apoptosis is a rapid process, lasting between several minutes and several hours. In experimental studies, apoptotic cells are usually detected 6 hours after the beginning of incubation with an apoptosis-inducing agent [26, 27].

In the present study, it was found that nuclear MDM2 expression as well as nuclear p53 expression prior to initiation of the therapy in the group of patients characterized by a poor early response to treatment was higher as compared to the group with a good early response to therapy. It has been suggested that the decreased clearance of leukemic cells in the bone marrow of patients who fail to achieve early remission may be caused by inhibition of apoptosis in these cells, which results from increased expression of endogenous apoptosis inhibitors such as MDM2 or inability to activate apoptosis via p53 increase [6, 11, 13, 17, 28].

MDM2 overexpression can be caused by decreased MDM2 degradation which results in prolonged half-life of MDM2 in leukemic cells [29] or less likely by amplification of *MDM2* gene which is found in less than 1% of ALL [27]. Overexpression of MDM2 protein and/or MDM2 mRNA has been

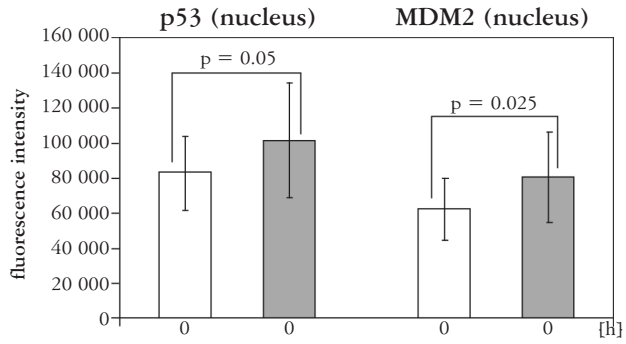

Fig. 3. Changes of p53-associated FITC green fluorescence and MDM2-associated APC long red fluorescence measured in the nucleus before prednisone administration in the group of poor (dark bars) and good (white bars) early treatment responders (mean ± SD)

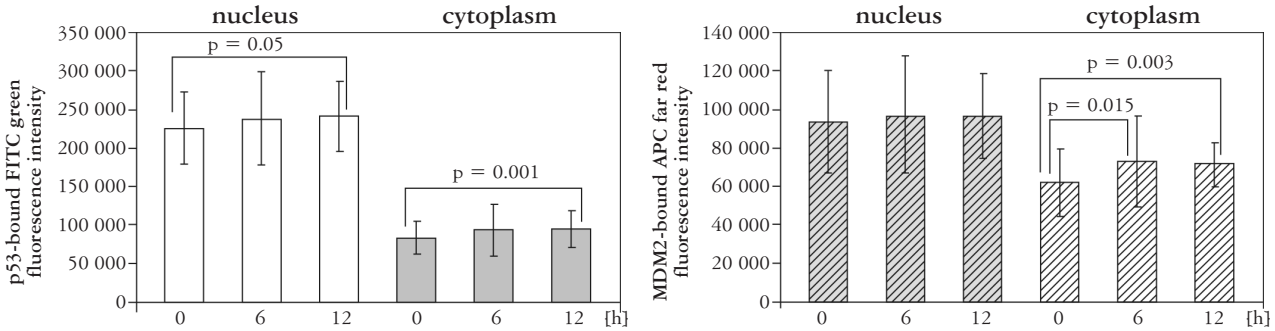

Fig. 4. Changes of p53-associated FITC green fluorescence and MDM2-associated APC long red fluorescence intensities in the nucleus and in the cytoplasm before prednisone administration and 6 and 12 hours after prednisone administration in the group of good early treatment responders (mean ± SD)

Table I. Clinical characteristics of patients.

	GOOD EARLY TREATMENT RESPONSE (N = 24)	POOR EARLY TREATMENT RESPONSE (N = 11)
Age	22-192 months (mean 93.29 months, median 98.5 months) < 1 year – 0 patients > 10 years – 9 patients	6-176 months (mean 81.1 months; median 70 months) < 1 year – 1 patient > 10 years – 2 patients
Gender	13 boys 11 girls	7 boys 4 girls
Initial WBC (/μl)	1300-247 900/μl (mean 28 821/μl; median 6950/ml)	1000 – 498 000/μl (mean 105 291/μl; median 56 000/ml)
Diagnosis	T-ALL – 4 patients, B-ALL – 20 patients, including 16 patients CD10(+)	T-ALL – 2 patients, B-ALL – 9 patients, including 6 patients CD10(+)
t(9;22), t(4;11)	not detected	not detected

detected in numerous neoplastic diseases, including 15-25% of ALL (especially in relapse), myeloproliferative syndromes, sarcomas and lymphomas [23, 31-33]. MDM2 overexpression in leukemic cells is associated with a tendency to early relapse and with resistance of leukemia to the employed treatment, and thus with poor prognosis [23, 33].

It is unlikely that increased p53 expression prior to initiation of therapy has resulted from a mutation of the *TP53* gene followed by accumulation of the mutated p53 protein in the cell, since *TP53* mutations occur in less than 5% of ALL in children at the time of diagnosis and in 16-28% of children with relapsed ALL [34, 35]. A higher nuclear p53 protein level in the cells can be related to the translational and posttranslational modifications, *i.e.* phosphorylation, acetylation, methylation or decreased rate of MDM2 – ubiquitin dependent proteolytic degradation [10]. It is also possible that loss of $p33^{ING1b}$ may contribute to increased p53 expression at diagnosis and cells resistance to apoptosis because deletion of chromosome 13q that contains $p33^{ING1b}$ – a protein that has the ability to modulate *TP53* transactivation of the cyclin dependent kinase inhibitor WAF1, is common in ALL [36].

In the group of patients with good early response, we found the rise of cytoplasmic and nuclear p53 expression after 12 hours of therapy. This indicates an increase in p53 synthesis in the cytoplasm and the subsequent translocation of p53 protein to the nucleus, where it acts as an activator of genes that inhibits the cell cycle in G1 phase and as an initiator of apoptosis. In the same group of patients, no initial MDM2 overexpression was demonstrated and no increased nuclear MDM2 expression was observed, whereas cytoplasmic MDM2 expression significantly increased 6 and 12 hours after treatment initiation. The increased expression of MDM2 in the cytoplasm, probably related to its synthesis, may be secondary to the increased nuclear and cytoplasmic expression of p53 protein, since in this group of patients a significant increase of p53 was demonstrated in the nucleus and cytoplasm at the 12 hour after treatment initiation. The lack of increase of both nuclear and cytoplasmic p53 expression after 6 and 12 hours of therapy in patients characterized by the poor early response to treatment may result from MDM2 overexpression in the nuclei prior to therapy.

In conclusion, the assessment of MDM2 expression may supplement methods that determine the response to therapy, possibly allowing for the identification of other risk groups in ALL and in consequence leading to more intensive treatment in groups of patients with poor prognosis and to reduced toxicity of therapy in patients with favourable prognosis. New compounds – direct inhibitors of MDM2 such as nutlin and inhibitors of the interaction between p53 and MDM2 may "sensitize" cells to better response to therapy [20, 24, 25, 37-41]. The presented results are preliminary and undoubtedly require confirmation in further studies of large groups of patients with longer follow-up.

Acknowledgements

The authors acknowledge the excellent technical assistance of Ms Lucyna Pawluch.

This study has been funded by the State Committee for Scientific Research (KBN) – project No. PBZ-KBN-091/P05/2003.

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