Determination of mTOR kinase activity and investigation of therapeutic options of rapamycin in childhood acute lymphoblastic leukemia

Doctoral theses

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1. INTRODUCTION

1.1. Childhood acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL), which takes 25 % of the newly diagnosed childhood malignancies, is the most common form of cancer occurring in pediatric patients. It forms a heterogeneous group of patients, both from genetic and clinical point of view, with leukemia cells deriving from undifferentiated immature B-or T-progenitor cells. As a result of the intensive combination therapy, nowadays the chances of long-term survival have reached 80-85% of all patients, compared to the 90% lethality in the 60s. However, in spite of the efficacy of the intensive therapy, the treatment still seems to fail in 15-20% of patients, in the background of which both chemotherapy resistance and recurrence can be revealed. Moreover the intensive combination therapy induced acute and late side effects causing further complications.

Results of in vitro, in vivo and clinical phase trials indicate that targeted therapy may enhance chances of recovery among childhood ALL patients, and at the same time the dosage of conventionally applied chemotherapeutics might be reduced, which consequently may develop a treatment with reduced toxicity. The possible therapeutic targets in ALL is primarily the inhibition of intermediates and cell surface receptors of signaling pathways involved in differentiation, proliferation, survival and activation of normal B- and T-lymphocytes; and as such, mTOR kinase has recently attracted remarkable attention as a potential target.

1.2. The mTOR signaling pathway

The mTOR (mammalian target of rapamycin) is a serine-threonine kinase with molecular weight of 289 kD and belonging to the phosphoinositide 3-kinase (PI3K) family, and plays a central role in the regulation of essential cellular functions (growth, proliferation, survival, motility and autophagy). The activity of mTOR is related to two distinct complexes (mTORC1 and mTORC2), which have different structure and function. Both complexes are characterized by the presence of mTOR kinase, mLST8/GβL and DEPTOR. In addition, in the structure of mTORC1 complex, Raptor and PRAS40 are involved, while Rictor, mSin1 and Protor 1/2 are basic protein constituents of complex mTORC2. The basic target molecules of mTORC1 are ribosomal S6 kinase 1 (S6K1) and binding protein 4E (4EBP1). S6K1 phosphorylates ribosomal protein S6, which takes part in the ribosome biogenesis. Following the phosphorylation of 4EBP1 protein, initiation factor eIF4E is released, and having bound to

other factors it results the cap-dependent translation. The mTORC2 phosphorylates AKT and other AGC kinases, thus supporting not only cell survival and proliferation, but also taking part in regulation of cell motility. Even though certain genetic alterations leading to increased mTOR activity have been detected in acute lymphoblastic leukemia, we still have little data available on the precise role of mTOR signaling pathway. The first members of mTOR inhibitors is rapamycin and its derivatives, the rapalogs (temsirolimus, everolimus, deferolimus), which were followed by development of new generation of mTOR inhibitors (inhibiting complexes mTORC1/mTORC2 (mTORK), and as dual inhibitors, capable of blocking PI3K or AKT, in addition to complexes mTORC1 and mTORC2). In recent years a number of clinical trials have been launched related to childhood ALL, examining the effects of mTOR inhibitors in combination with conventional chemotherapy among refractory or relapsed patients.

1.3. microRNA expression

The altered expression levels of microRNAs of the group of short, non-coding RNAs have been described in connection with several hematologic malignancies, including ALL as well. However, concerning miRs related to prognosis of patients, reappearance of relapse and development of chemotherapy resistance, we still have little data available. In our experiments we accomplished the expression analysis of certain miRNAs in samples from childhood ALL patients, which were selected based on previous literature. We examined the expression of the two most common “oncomiR” types, of miR 21 and miR 155; the increased expression of which had been observed in several tumour types. All other miRNAs were selected mainly based on the results of previous studies related to haematological malignancies. The reduced expression of miR 16 had been confirmed in various haematological malignancies. MiR 24 is an important cell-cycle regulator the increased expression of which inhibits cell proliferation, its reduced expression was detected in certain pre B-ALL cases. The lower expression of miR 29b tumour suppressor has been confirmed in AML, osteosarcoma and small cell lung cancer. The increased expression of miR 128b in acute lymphoblastic leukemia and its reduced expression in acute myeloid leukemia, as well as in MLL-AF4 ALL with poor prognosis were also described. MiR 223 is a hematopoietic tissue-specific miRNA, with lower expression in the lymphoid cells and highly increased expression in myeloid cell lines.

2. OBJECTIVES OF THE STUDY

There are a number of studies describing the significance of mTOR activity in the field of cancer biology and in targeted cancer therapies. Nonetheless, in pediatric neoplasms, such as in childhood acute lymphoblastic leukemia, there is still very little data available concerning the individual mTOR activity in the leukemia cells and its significance.

Therefore, the aims of the present study were:

1. Description of mTOR signaling pathway activity in childhood ALL
2. Comparative analysis of mTOR activity in leukemia cells isolated from ALL patients and of the prognosis and other therapy related, clinical data
3. *In vitro* analysis of the effect of the mTOR inhibitor rapamycin in cell lines and in isolated primary ALL cells.

In our study expression analysis of certain miRNAs was performed in the available childhood ALL samples and in certain human ALL cell lines as well, in order to determine:

1. The expression level of miRNAs in certain lymphomas and other tumours and its correlations with clinical and prognostic data of patients
2. The changes in miRNA expression during monitoring the efficacy of the treatment.

3. PATIENTS AND METHODS

3.1. Patients

In order to determine the mTOR signaling pathway activity and miRNA expression, as well as to present a statistical evaluation, we used bone marrow and blood samples of 53 primary ALL patients (44 BCP-ALL, 9 T-ALL, 16 females, 37 males). The mean age of patients at diagnosis was 6.3 years (1.8 – 16.4 years). Based on the prognostic factors, 21 patients (40%) were ranged in the low-risk group (SR), 23 patients (43%) in the group with medium risk (IR), and 9 patients (17%) classified in the high-risk group (HR), with their treatments accordingly performed based on the SR, IR or HR branches of the ALL IC-BFM 2002 protocol. Evaluation of the patients' prognosis was performed according to the prognostic factors determined by the treatment protocol, in addition to considering the potential relapses occurring during the follow-up period. The mean time of patients follow-up starting from the diagnosis was 28.44 months (1.5 – 55.2 months). Statistical analyses have been performed in altogether 40 patients with good prognosis and 13 patients with poor prognosis.

3.2. Isolation of primary ALL cells

Lymphoblasts were isolated from bone marrow and peripheral blood samples of childhood ALL patients, after density gradient centrifugation (Histopaque 1077, Sigma). The cells were stored at - 70 ° C until use.

3.3. Cell lines, culture, treatment

During the *in vitro* trials human ALL cell lines (Nalm6, Mn60 – precursor B-ALL; Jurkat, CEM – T-ALL), promyelocytic leukemia cell line (HL60), human lymphoma cell lines (KMH2 – Hodgkin-lymphoma; BHD1 – diffuse, large B-cell lymphoma) were used, as well as primary leukemia cells of childhood acute lymphoblastic leukemia patients. The cell treatment was performed using the following drugs: methotrexate, cytosin-arabinoside, doxorubicin, vincristine, etoposide, methyl-prednisolone, cyclophosphamide and rapamycin.

3.4. Flow cytometry

The apoptosis measurements were performed in FACScan flow cytometer (BD Biosciences), using CellQuest™ (BD) software. The results were evaluated by WinList software (Verity Software House).

3.5. Western-blot

After having separated the protein lysate by SDS-polyacrylamide gel electrophoresis and blotted to PVDF membrane (BioRad), we examined the expression of proteins p-mTOR, p-S6, p-p70S6K and β-aktin. As a secondary imaging system, Vectastain Elite ABC kit (Vector) was used. After the chemiluminescent visualisation (ECL Western Blotting Substrate, Pierce) the membranes were photographed.

3.6. ELISA assay

Proteins p-mTOR, p-S6 and p-4EBP1 were detected by Sandwich ELISA kits (p-mTOR, R&D Systems; PathScan p-S6 ribosomal protein, Ser235/236; PathScan p-4EBP1, Thr37/Thr46, Cell Signaling), according to the manufacturer's instructions. Absorbance and optical density (OD) were measured at 450 nm. As a positive control, the cell line KMH2, causing overexpression of proteins p-mTOR, p-S6 and p-4EBP1 was utilized.

3.7. Immuncytochemistry

We examined the p-mTOR, p-S6, p-p70S6K and p-4EBP1 protein expression in leukemia cells, using Cytospin slides. The detection was performed with Novolink Polymer Detection System (Novocastra), according to the manufacturer's instructions. For visualisation we used DAB substrate-chromogen system (Dako) and for the background staining Haematoxylin stain was used.

3.8. Isolation of microRNAs, cDNS transcription

MiRNAs were isolated with mirVana$_{TM}$ miRNA isolation kit (Ambion) and the samples were stored at -70 ° C until use. The cDNA transcription was performed by stem-loop RT primers (miR-128b, 142-3p, 155, 21, 24, 29b, 223), contained in TaqMan MicroRNA Reverse Transcription Kit and Taqman MicroRNA Assay Kits, according to the protocol.

3.9. Real-time PCR

The amplification of miRNAs was performed by TaqMan Gene Expression Master Mix and by TM-primers (miR-128b, 142-3p, 155, 21, 24, 29b, 223) of TaqMan MicroRNA Assay Kits (Applied Biosystems). The relative expression level of miRNAs was normalized to the level of RNU6B, which was used as an internal control (Applied Biosystems). The data were evaluated using 7500 software v.1.3.0 and DataAssist v.2.0 software (Applied Biosystems).

3.10. Statistics

The data analysis was performed using the paired t-test or Mann-Whitney U test. To compare the categorical variables Chi2 and Fisher's Exact tests were used. The cut-off value of ELISA OD and relative expression of miRNA 128b were determined by ROC analysis. To examine the overall and relapse-free survival, we used the Kaplan-Meier analysis. The multivariate analysis of various prognostic factors was conducted using the Cox regression model. The correlation between p-4EBP1 and miR 128b expression was analysed by Spearman's correlation analysis, which was performed using software programs SPSS 15.1 (SPSS Inc.) and StatSoft STATISTICA 9.0.

4. RESULTS

4.1. Examination of the mTOR signaling pathway activity in human ALL cell lines and isolated childhood leukemia cells

Increased amount of p-mTOR, p-S6, p-p70S6K and p-4EBP1 proteins indicating the mTOR signaling pathway activity was detected in human ALL cell lines and childhood ALL cells, using Western blotting, immunocytochemistry, ELISA, and flow cytometry. Besides, in human ALL cell lines a significantly higher p-4EBP1 and p-S6 expression was detected, while in leukemia cells of childhood ALL patients a significantly higher p-4EBP1 expression was shown, compared to the normal PMNC cells by ELISA.

4.2. Correlation of mTOR activity and clinical data of childhood ALL patients

Examining the correlation between the amount of p-4EBP1 protein and the prognosis of patients, we discovered that expression of p-4EBP1 protein indicating the mTOR activity is significantly higher in bone marrow samples taken from patients with poor prognosis at day 0, compared to that of patients with good prognosis. Based on our results, a cut-off OD value for p-4EBP1 ELISA was determined, which classified the patients in two different groups, patients with good prognosis (OD <1.1, lower mTOR activity) and patients with poor prognosis (OD> 1.1, higher mTOR activity). Considering the clinical data of the two different patient groups, a significant correlation was detected in both cell types (B-and T-ALL) between the increased mTOR activity and the poor prognosis, the prednisolone poor response and the non hyperdiploid karyotype, as well. The correlation between mTOR activity and patient survival time was demonstrated using Kaplan-Meier survival curves. Patients (n=37) characterized with lower mTOR activity (OD<1.1) had significantly better overall and relapse-free survival, compared to patients (n=12) with increased mTOR activity (p=0.00012). Using multivariate analysis, we demonstrated that the high mTOR activity (OD> 1.1) significantly increases the risks of poor prognosis, occurrence of relapse and of poor response to treatment.

4.3. Examination of the mTOR activity in mononuclear cells isolated from follow-up ALL samples collected during treatment

In all cases, regardless of the prognosis, the expression of both p-S6 and p-4EBP1 had reduced until the time before protocol M, with a simultaneous reduction in lymphoblasts. In patients with good prognosis, i.e. lower initial mTOR activity (OD <1.1), the expression of p-4EBP1 decreased in the follow-up samples during treatment and did not increase during the

two years follow-up period either. In patients with poor prognosis, characterized with high initial expression (OD>1.1) of p-4EBP1, protein p-4EBP1 had significantly decreased until the time before protocol M, however at relapse it rose above the initial value, indicating the appearance and increase in the amount of ALL cells with high mTOR activity in the samples.

4.4. Determination of mTOR activity in childhood ALL samples using flow cytometry

In flow cytometric tests the amount of mTOR activity-dependent phosphorylated proteins were analysed using indirect fluorescent staining, applying unlabelled p-S6 and p-4EBP1 antibodies. The increased p-4EBP1 and p-S6 expression were also detected with this method in childhood ALL bone marrow samples (n=3), where the mean fluorescence intensity change (ΔMFI) was 15-20 fold higher than in the non-leukemia control cells.

4.5. The apoptosis induction of rapamycin combined with chemotherapeutic agents in human ALL cells

The apoptotic effect of rapamycin treatments and combination treatment with rapamycin (doxorubicin, etoposide, vincristine, methotrexate, methyl-prednisolone, cytosin-arabinoside, cyclophosphamide) were performed in human ALL cell lines, as well as in isolated leukemia cells of childhood ALL patients, *in vitro*. The antiproliferative effect of the 72-hour rapamycin treatment were detected in all examined human ALL cell lines, while in Jurkat (T-ALL) cell line were found an additional increase in the rate of spontaneous apoptosis. In case of Jurkat and Nalm6 (B-ALL), in the combination treatment, rapamycin was proved to enhance the apoptotic effect of each applied chemotherapeutic agent. In leukemia cells isolated from childhood ALL patients, rapamycin increased the effect of different chemotherapeutic agents to varying extent (10-91%). In two patients the induction of apoptosis by rapamycin and combination treatments was not significant (<l0%), while in three patients rapamycin alone enhanced the apoptosis and increased the apoptosis induction of etoposide, vincristine and methyl-prednisolone.

4.6. Rapamycin reduces the amount of mTOR activity-dependent phosphorylated proteins in human ALL and lymphoma cells

The effect of rapamycin on mTOR activity in human ALL cells was monitored by quantitative analysis of phosphorylated target proteins using Western blot, immunocytochemistry and ELISA, while the amount of constituent proteins of mTOR complexes (Raptor, Rictor) was determined by immunocytochemistry. In human ALL cell

lines the mTOR activity decreased following the treatment, in Nalm6 cells we showed low Raptor and high Rictor levels, while in Jurkat cells increased Raptor and lower Rictor expression was detected. In bone marrow cells isolated from childhood ALL patients (n = 4) and treated with rapamycin *in vitro*, the expression of p-4EBP1 significantly decreased in samples of patients with good prognosis, while in samples of patients with poor prognosis the p4EBP1 expression increased.

4.7. miRNA expression analysis in human leukemia cells

In the examined miRNAs (miR: 16, 21, 24, 29b, 128b, 142-3p, 155, 223) a significant overexpression of miR 128b was revealed in human ALL cell lines. The "oncomiR" 21 showed no increased expression in any human ALL cell lines, and higher expression of "oncomiR" 155 was detected only in the CEM (T-ALL) cell line. The T-ALL cell lines were characterized by overexpression of miR 16, while in B-ALL cell lines low expression of miR 223 was found. In samples of childhood ALL patients (n = 24) at day 0. reduced expression of miR 29b, miR 21 and miR 223 was detected in both cell types, while the expression of miR 128b significantly increased in all cases. Moderately increased expression of miR 155 was shown only in cells of B-ALL patients.

4.8. Examination of miR 128b expression, clinical data and prognostic factors in childhood ALL patients

Both in B-and T-ALL significantly higher expression of miR 128b was detected in bone marrow cells of patients with good prognosis at day 0, compared to patients with poor prognosis. The correlation between the clinical data of patients and the expression of miR 128b were further analyzed, and a significant correlation was shown between the reduced expression of miR 128b and poor prognosis, prednisolone poor response, as well as between the increased expression of miR 128b and a relapse-free survival of patients.

4.9. Examination of changes in miRNA expression in mononuclear cells isolated from peripheral blood and bone marrow in follow-up samples of patients

The expression levels of the studied miRNAs had changed in samples of childhood ALL patients collected during chemotherapy; miRNAs with low initial expression showed increasing expression, and the expression of overexpressed miRNAs decreased at time before protocol M. In the bone marrow cells from follow-up samples of patients with good and poor prognosis the expression of miR 128b significantly decreased, while the expression of miR

223 significantly increased until the time before protocol M, compared to the samples before treatment. In patients with good prognosis the expression of miRNAs showed no changes during the two-year follow-up period, while in bone marrow samples from patients with poor prognosis and relapse the expression of miR 128b and miR 223 increased (miR 128b) (according to the increase in number of leukemia cells in bone marrow samples), and decreased (miR 223) at relapse.

5. CONCLUSIONS

5.1. We characterized the mTOR signaling pathway activity and its correlation with the prognosis of patients in childhood ALL cells

a. We have confirmed by several methods the characteristically high activity of mTOR in human ALL cell lines and isolated childhood ALL cells.

b. We have detected a significantly higher mTOR activity in leukemia cells of patients with poor prognosis, compared to patients with good prognosis.

c. A significant correlation has been revealed between the degree of mTOR activity in ALL cells and the poor prednisolone response and the presence of non-hyperdiploid karyotype.

- We have shown that to a certain extent higher mTOR activity, as an independent prognostic factor, tends to increase the risk of poor prognosis (poor response to treatment and/or relapse incidence) in childhood ALL.
- We have confirmed that changes in mTOR activity follow the proportion of leukemia cells in bone marrow samples of patients; that is, in case of effective treatment show a reducing pattern, while at relapse an increasing one.
- We have demonstrated that in peripheral blood samples mTOR activity can be detected by ELISA or flow cytometry, and it correlates with the results of bone marrow samples.

5.2. We have accomplished the role of mTOR activity in proliferation and survival of human ALL cells in vitro

a. We have successfully confirmed the antiproliferative and apoptosis-inducing effect of mTOR activity inhibiting rapamycin treatment in human ALL cell lines, *in vitro.*

b. We have demonstrated that the mTOR inhibiting treatment is capable of enhancing the effects of chemotherapeutic agents in human ALL cells, *in vitro*.

5.3. We examined the expression of certain miRNAs and its correlation with the prognosis of patients in human ALL cells

a. We have demonstrated that overexpression of "oncomiR" 21 and 155 (in general overexpressing in human tumours), is not characteristic of human ALL cells, while the overexpression of miR 128b and the low expression of miR 223 are generally characteristic for human ALL cell lines and childhood ALL cells.

b. We have confirmed that the rate of expression of miR 128b in cells of different ALL patients correlates with the prognosis, the prednisolone poor response and the survival data. Accordingly, significantly higher expression occurs in ALL patients with good prognosis, good prednisolone response and long relapse-free survival, and consequently the leukemia cells of patients with poor prognosis are characterized by lower expression of miR 128b.
c. We have demonstrated that the expression of miR 128b and miR 223, due to their leukemia cell specificity, effectively follow the alterations in the amount of leukemia cells in bone marrow samples.

6. LIST OF OWN PUBLICATIONS

- *Dissertation-related publications:*

Nemes K, Csóka M, Nagy N, Márk Á, Váradi Zs, Dankó T, Kovács G, Kopper L, Sebestyén A. Expression of certain leukemia/lymphoma related microRNAs and its correlation with prognosis in childhood acute lymphoblastic leukemia. *Pathol Oncol Res.* 2014; doi: 10.1007/s12253-014-9861-z. **IF: 1,806**

Nemes K, Sebestyén A, Márk Á, Hajdu M, Sticz T, Nagy E, Barna G, Váradi Zs, Kovács G, Kopper L, Csóka M. Mammalian target of rapamycin (mTOR) activity dependent phospho-protein expression in childhood acute lymphoblastic leukemia (ALL). *Plos One*. 2013;8:e59335. **IF: 3,534**

- *Publications independent of the dissertation:*

Bárdi E, Csóka M, Garai I, Szegedi I, Müller J, Györke T, Kajáry K, **Nemes K**, Kiss C, Kovács G. Value of FDG-PET/CT Examinations in Different Cancers of Children, Focusing on Lymphomas. *Pathol Oncol Res.* 2014;20:139-43. **IF: 1,806**

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Hegyi M, Gulácsi A, Cságoly E, Csordás K, Eipel OT, Erdélyi DJ, Müller J, **Nemes K**, Lautner-Csorba O, Kovács GT. Clinical relations of methotrexate pharmacokinetics in the treatment for pediatric osteosarcoma. *J Cancer Res Clin Oncol.* 2012;138:1697-702. **IF: 2.914**

- *Citable abstracts:*

Sebestyén A, **Nemes K**, Márk Á, Váradi Zs, Hajdu M, Sticz T, Kopper L, Kovács G, Csóka M. Mammalian Target of Rapamycin (mTOR) Activity Dependent Protein Expression and Rapamycin Sensitivity in Pediatric Acute Lymphoblastic Leukemia. *Eur J Cancer*. 2011;47:(Suppl. 1) p. S641.

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Csóka M, **Nemes K**, Márk Á, Hajdu M, Kopper L, Sebestyén A. Expression of certain "oncogenic"microRNAin human acute lymphoblastic leukemia cells. *Pediatr Blood Cancer.* 2010;55:(5) pp. 851-852.

Csóka M, Bárdy E, **Nemes K**, Szegedi I, Kiss C, Kovács G. The role of FDG-PET/CT in follow-up of children with lymphoma. *Pediatr Blood Cancer.* 2010;55:(5) p. 870.