Investigation of new therapeutic targets in pediatric soft tissue sarcomas

Doctoral Thesis

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1. Introduction

Biomarker based-patient selection is in the focus of oncologic research. Our research aimed to find new therapeutic targets for pediatric soft tissue sarcoma patients. This patient group was chosen because no significant progress has been made in the treatment of these malignancies in recent decades, thus, their survival outcomes have not improved significantly. My doctoral dissertation presents the results of the investigation of the anaplastic lymphoma kinase (ALK), mammalian target of rapamycin (mTOR) and metabolic pathways in pediatric soft tissue tumors.

2. Aims

In my doctoral work I aimed

1. to investigate the ALK status of childhood soft tissue sarcoma patients.
- We performed immunohistochemistry to detect the expression of ALK protein,
- and fluorescent in situ hybridization (FISH) to confirm the different alteration of the ALK gene.
- Furthermore, we compared the results of the two methods.
2. In my research I intended to find new therapeutic targets for the therapy of rhabdomyosarcomas (RMSs).
- We aimed to characterize the activity of the mTOR pathway and to detect the expression of the related markers of the mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Furthermore, we aimed to characterize the specimens taken after neoadjuvant chemotherapy and the samples taken during relapse besides the primary samples.
- We studied the metabolic characteristics of RMS cells through the expression of different metabolic enzymes; the glycolysis with the immunopositivity of the phosphofructokinase (PFK) and the lactate-dehydrogenase A (LDHA), the oxidative phosphorylation with the β-F1-ATPase (ATPB), the pentose-phosphate pathway with the glucose-6-phosphate-dehydrogenase (G6PDH) and glutamine consumption with the glutaminase (GLS).
3. After confirmation of ALK or mTOR positivity, we documented the effects and side effects of the administered inhibitor therapy.

3. Methods

3.1. Patients

During the first phase of our investigation, we examined the high risk and relapsed soft tissue sarcoma patients treated at the 2nd Department of Pediatrics at Semmelweis University between 2010 and 2014. We studied 18 cases; 2 alveolar soft part sarcoma, 2 alveolar and 11 embryonal RMS and 3 inflammatory myofibroblastic tumor (IMT).

In the second phase of the doctoral work, we studied the mTOR and metabolic features of RMS patients diagnosed between 2007 and 2017 in Hungary. We investigated 65 samples from 48 patients; 53 embryonal, 7 alveolar and 5 from the botryoid or spindle cell variant. We collected primary and neoadjuvant treated, as well as relapsed samples to characterize the differences in the mTOR and metabolic profile.

3.2. Investigation of ALK Alterations

3.2.1. ALK Immunohistochemistry

We performed the investigations on formalin-fixed paraffin-embed slides with a thickness of 3 μm. Monoclonal 5A4 ALK (Novocastra, Leica) was applied in a 1:10 dilution. A known positive IMT histological sample was used as a positive control. The immunohistochemical results were scored by the following system: + (10–50%), ++ (50–80%) or +++ (80%<).

3.2.2. ALK FISH

FISH was performed on 3 μm thick slides for ALK rearrangement using ALK Dual Color Break Apart probe (Vysis, Abbott Molecular Inc.). To evaluate the sections, we analyzed 100 tumor nuclei in approximately 8–10 fields of vision. The tumor was considered positive if more than 15% of the nuclei were positive with FISH. Rearrangements appeared as split 3′ and 5′ signals. Overexpression was detected in cases where the nuclei showed at least quadrupled signs.

3.3. Investigation of mTOR and Metabolic Pathways Related Proteins

In the second phase of my doctoral work, we constructed 2 tissue microarrays (TMA) (6 × 8 cores, diameter 2 mm) containing double or triple cores per tissue blocks. Representative areas of formalin-fixed paraffin-embedded blocks were selected based on hematoxylin-eosin stainings as well as Myf-4 and desmin immunoassayed slides by an experienced pathologist. Non-neoplastic liver parenchyma, tonsil, and testis were used as normal tissues and immunohistochemical staining controls in each TMA block. Our goal was to make more cost-effective use of our resources and samples by making TMA blocks, and to perform more standardized execution of stainings during comparative analyzes. We used the histological specimens from patients whose treatment was complete and whose sample size allowed a sufficient amount of tumor tissue to remain after removal of the cores. In those cases when these criteria did not meet, we used whole sections to keep the biopsy materials intact for further diagnostic evaluation. We worked on 4 μm thick slides.

To investigate the mTOR pathway we applied anti-pmTOR (Cell Signaling, #2976), anti-pS6 (Cell Signaling, #2211) and anti-Rictor (Bethyl, A500-002A) antibodies. The anti-pmTOR antibody recognizes the active form of the mTOR kinase, it is a general marker os mTOR activity. In order to distinguish mTORC1 and C2 activity we performed different immunostainings. The activity of the mTORC1 was detected through the immunopositivity of anti-pS6 staining. As an mTORC2 marker, anti-Rictor antibody was used, which is the scaffold protein of mTORC2. Rictor expression is in correlation with the amount of mTORC2, therefore the activity of mTORC2 was investigated with the co-expression of Rictor, pmTOR and pS6. We performed anti-PFK (Cell Signaling, #8164), anti-ATPB (Abcam, ab14730), anti-G6PDH (Abcam, ab133525), anti-LDHA (Cell Signaling, #3582), anti-GLS (Abcam, ab156876) immunostainings to characterize the metabolic features of RMS cells.

To assess immunohistochemical stainings, we calculated the histo-score (H-score) by multiplying the staining intensity (0, 1+, 2+, 3+) by the percentage of positive cells. Cases with an H-score higher than 100 were considered positive.

3.4. Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics program version 21. We compared our results in relation to PAX-FOXO fusion status, time of sampling (primary, during treatment, recurrent), risk stratification, and survival. The normally distributed samples (Rictor, ATPB, G6PDH results of primary samples) were analyzed according to H-score values (scale variable) using a T-test and a one-way ANOVA test. For our non-normally distributed samples, the Mann-Whiney U test was used. Samples were also compared according to immunopositivity (binomial variable) using Fisher-exact test. The correlation analysis was performed using Spearman-rank correlation. $p < 0.05$ was considered as statistically significant.

4. Results

4.1. Results of ALK Immunohistochemistry and FISH

In order to compare the results, we performed both ALK immunohistochemistry and FISH examinations. In cases in which both primary and relapsed samples of patients were available, we were also able to investigate changes in the ALK status.

ALK amplification was confirmed in one case and translocation in three cases using FISH. Immunohistochemical staining was able to detect the expression of ALK protein in the three cases with ALK translocation. In the amplified case, the expression of ALK protein did not appear, therefore, the role of this detected genetic alteration is uncertain.

In six cases, we studied the relapsed samples of the patient as well. In one case, ALK status changed during the progression and became positive in the relapsed sample. This patient received ALK inhibitor treatment.

4.2. Results of the mTOR Related Protein Expression Analysis

4.2.1. Characterization of the Primary Samples

The pmTOR, marker of mTOR kinase activity, immunostaining showed elevated expression in 56 % (25/45 cases) of the sample. We observed positivity using pS6, marker of mTORC1 activity, in 18% (8/45 cases). Rictor immunostaining, a marker of mTORC2, confirmed elevated Rictor expression in 82% (37/45 cases). In line with our previous findings, pmTOR, pS6 and Rictor stainings showed mainly cytoplasmatic pattern,

however in many cases pmTOR showed nuclear reaction. Both nuclear and cytoplasmic expression of pmTOR can be assessed as positive.

Altogether we detected pmTOR and / or pS6 immunopositivity in 64% (29/45 cases) of the 45 investigated primary RMS cases, which confirmed mTOR activity. Based on the elevated Rictor and low pS6 expression, mTORC2 dominance was observed in the primary RMS cells.

We compared the results of the primary samples according to histological categorization, risk stratification and survival. Significantly higher Rictor expression in the PAX-FOXO fusion positive group was observed compared with the fusion negative group (t-test, $t(43)=-2.41$, $p=0.020$). 80 % (32/40 cases) of the fusion negative cases were positive, meanwhile 100 % (5/5 cases) of the fusion positive cases showed elevated Rictor expression.

4.2.2. Characterization and Comparative Analysis of the Samples Taken After Neoadjuvant Chemotherapy

Histological samples taken from residual tumors removed during chemotherapy (after neoadjuvant chemotherapy) were collected. Nine samples were examined. 33.3 % (3/9 cases) showed elevated pmTOR, 66.7 % (6/9 cases) pS6 and 77.8 % (7/9 cases) Rictor immunopositivity.

These samples were also compared to the primary samples from the same patients, this way, eight primary-neoadjuvant tumor pairs were evaluated. The pmTOR expression remained the same (Mann-Whitney U test, $U=143.5$, $p=0.171$), Rictor expression showed to be less intensive (t-test, $t(52)=-2.12$, $p=0.019$), however, pS6 H-score values were significantly higher (Mann-Whitney U test, $U=75$, $p=0.003$). The latter was not due to a diffuse stronger pS6 staining, but to an increase in the number of single-standing sarcoma cells with strong immunopositivity. This observation is not caused by increased mTORC1 activity but by cell division released by the effect of chemotherapy.

4.2.3. Characterization and Comparative Analysis of Samples Taken After Relapse

We also aimed to characterize the changes that occur during recurrence. We had the opportunity to examine the relapsed samples of 11 patients. The pmTOR stained strongly in 64 % (7/11 cases) of all cases, pS6 in 9 % (1/11 cases), Rictor in 55 % (6/11 cases).

Relapsed samples were also analyzed according to histological subgroup, fusion status, survival, and risk stratification. Only the expression of pS6 was significantly higher in the high risk group compared to the non-high risk group (Mann-Whitney U test, U=0, p=0.014). However, the relevance of this result is still uncertain, as low pS6 expression was observed in both groups. Furthermore, higher pmTOR expression was observed in the therapy resistant group compared to the patients cured, even though this result was statistically not significant. Further investigations are required with larger sample sizes.
Moreover, nine primary-relapse case pairs were compared. Although an increased pmTOR expression (Mann-Whitney U test, U=216, p=0.516) next to a preserved Rictor positivity (t-test, t(53)=1.54, p=0.446) and low pS6 expression (Mann-Whitney U test, U=224.5, p=0.635) were observed in the relapsed cases, these observations were not statistically significant.

4.3. Results of the metabolic enzyme expression

4.3.1. Characterization of the Primary Samples

To investigate glucose utilization, we performed PFK and LDHA (markers of glycolysis) and ATPB (marker of oxidative phosphorylation) immunostainings. The primary cases showed elevated expression of PFK in 18 % (8/45 cases), LDHA in 53 % (24/45 cases) and ATPB in 36 % (16/45 cases). These data suggest the importance of the Warburg effect in RMS cells compared to oxidative phosphorylation. Statistical data analysis was performed by histological subgroup, survival, and risk classification. The PAX-FOXO fusion positive cases showed higher PFK expression compared to the fusion negative cases (Fisher-exact test, p=0.033).
G6PDH immunostaining was used to characterize the activity of the pentose-phosphate pathway. Studies on primary histological specimens showed remarkable immunopositivity, 82% of the cases (37/45 cases) showed strong enzyme expression. Based on these results we presume the importance of the pentose-phosphate pathway in primary RMSs. There was no significant difference in histological classification, survival, and risk classification.
The expression of GLS (marker of glutaminolysis) was elevated in 69 % (31/45 cases) of the primary cases. These results suggest a higher glutamine demand of RMS cells. We did not find any significant difference in the comparative analysis.

4.3.2. Characterization and Comparative Analysis of Samples Taken After Neoadjuvant Chemotherapy

Samples taken after neoadjuvant chemotherapy were collected from nine patients and paired with the primary sample in 8 cases. No significant change due to chemotherapy was demonstrated in any of the cases. We detected immunopositivity with PFK staining in 22 % (2/9 cases), with LDHA in 56 % (5/9 cases), with ATPB in 44 % (4/9 cases), with G6PDH in 67 % (6/9 cases) and with GLS in 56 % (5/9 cases).

4.3.3. Characterization and Comparative Analysis of Samples Taken After Relapse

We investigated 11 relapsed sample and compared the results to their primary pair in nine cases. PFK expression was elevated in 27 % (3/11 cases), LDHA in 82 % (9/11 cases), ATPB in 91 % (10/11 cases), G6PDH in 27 % (3/11 cases) and GLS in 46 % (5/11 cases). A significant difference between enzyme expression in primary and relapsed samples could only be demonstrated for G6PDH, the immunopositivity of relapsed cases showed to be lower (t-test, $t(54)=4.06$, $p=0.004$). This suggests that the pentose-phosphate pathway loses its importance during relapse.

4.4. Correlation Analysis

The correlation analysis was performed using Spearman-rank correlation. Altogether 28 correlations were examined. In our study, the detected LDHA and GLS expressions correlated with pmTOR activity. Positive correlations were found between pmTOR and LDHA expression ($rho = 0.403$, $p < 0.05$), as well as between pmTOR and GLS expression ($rho = 0.302$, $p < 0.05$). The relevance of the other significant correlations found is uncertain. (LDHA-PFK ($rho = -0.508$, $p < 0.05$), GLS-Rictor ($rho = -0.306$, $p < 0.05$), GLS-ATPB ($rho = 0.306$, $p < 0.05$))

4.5. Case Studies

The aim of our investigations was to identify patients who may benefit from targeted therapy in the event of relapse or tumor progression. By confirming the ALK and mTOR positivity of the examined tumors, using their inhibitors becomes possible. Among our

patients two children received target therapy. The effects and side effects of the treatment were analyzed retrospectively and compared with the results of our *in situ* studies.

4.5.1. Survival and the Progression of a Studied Alveolar RMS Case

The 8-year-old boy's presentation symptoms were fever and lower limb pain. MRI confirmed the multiplex, osteolytic lesions, which involved the vertebras, the pelvis and all the bones in the knee region. The biopsy confirmed alveolar RMS. We started chemotherapy according to the metastatic arm of CWS 2012 protocol. Good response was established; remission had been achieved. Seven months after the end of treatment relapse was confirmed. He was treated according to the relapse protocol of the CWS 2012 guideline. The child was in bad condition, his pain was difficult to manage, severely limiting the patient's ability to move. He reached 20% on the Karnofsky-Lansky scale, which represents the patient's general well-being.

After confirming ALK positivity we started the ALK inhibitor, crizotinib treatment (200 mg in the morning, 400 mg in the evening). During treatment the lesions significantly regressed. The patient did not experience any serious side effects, only mild, transient vision disorders, taste disturbances, nausea and vomiting at the start of taking the drug. The general well-being of the child improved, his pain disappeared, his activity was not limited by his underlying disease, he reached 100% on the Karnofsky-Lansky scale. In the third month of crizotinib therapy his pain returned, further progression was confirmed. We added topotecan and carboplatin to his therapy. He did not show any response. Based on mild pmTOR and pS6 positivity, 12 cycles of mTOR inhibitor temsirolimus treatment were initiated. We observed continuous tumor progression and treatment was discontinued and palliative care was started. The child passed away three months later.

The samples of this patient were further studied for detailed mTOR characterization retrospectively. High Rictor and low pS6 expression were confirmed. These data suggest mTORC2 dominance, which can be an explanation for the inefficacy of mTORC1 inhibitor therapy.

4.5.2. Survival and the Progression of a Studied IMT Case

The 6-year-old boy's complaints began with right hip pain. Biopsy confirmed IMT. Radical resection resulting in total recovery of the patient was not feasible due to the

infiltrative spread of the tumor. After two months we observed continuous progression and pelvic propagation. Corticosteroid-supplemented chemotherapy was initiated on the high-risk arm of the CWS 2009 protocol. Control MRI showed an unchanged extension of the tumor compared to previous examinations. With immunohistochemistry ALK positivity was detected which was confirmed by FISH as well. Due to the lack of therapeutic response, chemotherapy was discontinued, and crizotinib was initiated at a dose of 250 mg twice daily (2x 280 mg / m2).

Before starting crizotinib treatment, the child's general condition was extremely poor, he experienced severe pain at rest as well. On the Karnofsky-Lansky scale, his condition reached 20-30%. He needed constant care and opioid painkiller at home. Crizotinib treatment resulted a significant reduction in tumor size. His previous complaints improved rapidly, he reached 80% on the Lansky well-being scale by the end of the first month after starting treatment. The initial side effects were vision disorders, taste disturbance, bradycardia, electrolyte abnormalities, but no modification was needed in the therapy.

During the two-year-long treatment the tumor regressed, and no more activity was detectable on the control MRI. This patient, who had become disabled due to his illness, could go to school and even participate at gym classes. Complete surgical removal of the tumor could only have been done with significant truncation, thus the goal was to maintain remission until the child reaches his body size, presumably at the end of adolescence, when prosthesis implantation becomes possible for him.

Because we did not detect any activity on the MRI, we decided to stop the therapy after 25th months. After several weeks his pain returned, and the MRI showed progression. We restarted the treatment immediately.

In April 2019 the MRI confirmed a new lesion next to the followed tumor mass. In June biopsy was performed which confirmed the retained ALK positivity of the tumor. Due to crizotinib resistance we started alectinib, second-generation ALK inhibitor treatment in September 2019 with a dose of 600 mg daily. The lesion showed a 30% regression by the end of the first month of treatment, the child was still asymptomatic and did not experience any side effects.

5. Conclusion

Summarizing the results of our research:

1. Examining pediatric soft tissue sarcomas;

- we confirmed different ALK alterations and the presence of ALK protein. 50 % of alveolar RMSs, 67 % of IMTs were ALK positive, meanwhile neither the embryonal RMSs, nor the alveolar soft part sarcomas showed immunopositivity.
- ALK translocations were detected in ALK immunopositive cases. In another case, we detected amplification without any ALK protein expression, so the clinical significance of this difference is uncertain.
- Based on our results, we suggest the use of immunohistochemistry to detect the re-appearing ALK protein in the tumor cells and to perform FISH test to confirm the ALK positivity.

2. According to the *in situ* protein analysis of mTOR related markers and metabolic enzymes of rhabdomyosarcoma cases our conclusions;

- we detected elevated mTOR activity in the primary RMS cases with an mTORC2 dominance. The activity of TORC2 was significantly higher in PAX-FOXO fusion positive cases compared to the fusion negative group. Chemotherapy did not alter the mTOR activity, however in relapsed, therapy-resistant patients we detected elevated mTOR activity next to a preserved mTORC2 dominance compared to cured patients.
- We detected the metabolic adaptation of RMS cells:
    - The low PFK, ATPB and strong LDHA immunopositivity suggest the presence of Warburg-effect.
    - According to the enhanced expression of G6PDH we assume the importance of the pentose-phosphate pathway.
    - The intensive GLS immunopositivity confirms a high glutamine demand in RMS cells.
    - The detected a correlation between pmTOR-LDH and pmTOR-GLS expression suggesting the importance of mTOR activity in metabolic adaptation.

- The metabolic pathways have not changed after chemotherapy. G6PDH showed a significant decrease in relapsed tissue samples

3. In two cases we documented the efficacy and side effects of target therapy:

   - ALK inhibitor treatment is effective in IMT cases (the followed IMT patient is in remission next to crizotinib and later alectinib treatment, all side effects were tolerable), however for other sarcomas only a partial response can be obtained (the child treated with alveolar RMS only showed partial response to crizotinib)

   - In the treatment of other sarcomas, in case of therapy resistance or relapse, the use of mTOR inhibitors may arise, but in these cases the activity of the mTORC2 complex may be an important factor in the ineffectiveness of mTORC1 inhibitor, rapalog therapy.

In case of IMTs, ALK inhibitor therapy may be effective, and currently ALK immunohistochemistry is performed as part of the routine diagnostics. All children diagnosed with ALK-positive IMT can receive ALK inhibitor treatment if required with individual off-label approval. ALK inhibitor treatment did not show to be effective in case of RMSs. Presumably, ALK does not play an important role in tumorigenesis even if its expression and genetic abnormality in tumor tissue can be demonstrated. Furthermore, due to the increased mTORC2 activity we detected, it is worthwhile to accurately characterize mTOR activity in high-risk cases, as this group of patients may benefit from a new mTOR inhibitor. Enzymes of the altered metabolism may become new therapeutic targets in the future.

6. Publications

Publications related to dissertation:

- **Felkai, Luca** ; Krencz, Ildikó ; Kiss, Dorottya Judit ; Nagy, Noémi ; Petővári, Gábor ; Dankó, Titanilla ; Micsik, Tamás ; Khoor, András ; Tornóczky, Tamás ; Sápi, Zoltán et al., Characterization of mTOR Activity and Metabolic Profile in Pediatric Rhabdomyosarcoma, **CANCERS** 12 Paper: 1947 (2020)
  IF: 6.126 *

- **Felkai, L** ; Banusz, R ; Kovalszky, I ; Sapi, Z ; Garami, M ; Papp, G ; Karaszi, K ; Varga, E ; Csoka, M, The Presence of ALK Alterations and Clinical Relevance of Crizotinib Treatment in Pediatric Solid Tumors, **PATHOLOGY AND ONCOLOGY RESEARCH** 25 : 1 pp. 217-224. , 8 p. (2019)
  IF: 2.826 *

Publications not related to dissertation:

- Bots, Bianka ; Eipel, Olivér ; Terkovics, Lotte ; **Felkai, Luca** ; Csóka, Monika ,Lágyrészszarkómás gyermekek kezelési eredményei a Semmelweis Egyetem II. Sz. Gyermekgyógyászati Klinikáján**,** **MAGYAR ONKOLÓGIA** 62 : 4 pp. 222-229. , 8 p. (2018)
  IF: -