Effects of microRNAs on the drug-response of malignant tumours, focusing on PI3K/Akt/mTOR pathway

\- Final project report -

The oncological role of alteration in microRNA (miR) expression and its epigenetic potential have been studied in many aspects, however, the available data about these are rather conflicting. The expressions of elevated mTOR activity associated miRs were studied in the recently ended project. We selected miRs and or onco-miRs for our study which were previously described/characterised in colon carcinomas and referred as potential mTOR activity associated miRs (21,-155, -145, 92a, -100, -144,-199a, -218…). In paralell, the activities of mTORC1 and C2 complexes were also evaluated in many different tumours and statistically analysed their correlation to clinical, therapeutic and survival data of the patients.

To exclude the controversy colon carcinoma cell lines with different mutation profile (EGFR, K-RAS, B-RAF, PI3KCa, P53, mTOR) these were treated with mTOR and EGFR inhibitors (mTORI and EGFRI) or cisplatin and with their combinations in our miR studies. The anti-proliferative effects and the alteration in miR expression profiles were also studied after different treatments.

Many new publications were prepared related to these projects: 12 new publications (8 original publications – 7 in English and 1 in Hungarian, ∑IF: 22.8 –; 5 related reviews; 1 book - book chapters) were already published and 3 new original publications are under review and 3 additional were prepared (submission is going on) – in English.
Our results related to these projects and publications are the following:

1. Large amount of different malignancies has elevated mTOR activity. The certain tumours show individual presence and distribution of mTOR C1 and C2 complexes, These correlate to the prognosis, survival, therapeutic response of the patients and/or the incidence of metastasis in different tumour types (Diffuse Large B-cell lymphomas, Hodgkin lymphomas, acute lymphoid leukemias, colon and lung carcinomas).(1-5; R1-R5; K)
2. In correlation with the elevated mTOR activity the alterations in miR expression profile were also detected; these correlated to clinical data of childhood ALL patients (6)
3. We established miR expression studies on human formalin fixed paraffin embedded colon carcinoma samples and dissected (laser micro-dissection - LMD) tissues, based on these we selected suitable internal standard miRs for such an analysis and we could conclud that LMD of epithelial and stromal components are neccessary to find the source of miR expression changes. We could describe tumour and stromal specific miR expression changes in colon carcinoma samples related to mTOR activity (7).
4. mTOR inhibitors could reduce the expression of different overexpressed miRs *in vitro* in colon carcinoma cells. However, in these cells this effect could be related to the inhibited protein expression and could also reduce the amount of p-body proteins in the treated cells, as well (9).
5. mTOR inhibitors, especially NVP-Bez235, dual inhibitor induced the in vitro effect of cisplatin and could be effective in cisplatin and EGFRI resistant colon carcinoma cells, as well (8).
6. Altered mTORC1 activity related cell signalling failures (e.g. Notch hyperactivation) and microenvironmental changes (galectin-1 production and related Treg cell enrichment) help the progression of Hodgkin lymphomas (HL); The mTORC1 inhibitor rapamycin

analogs inhibit the tumourgrowth, the galectin-1 expression and induce apoptosis in HL cells both *in vitro* and *in vivo* (2, 10, 11, R1, R4).

7. mTOR inhibitory treatments can restore the role of negative regulators, e.g. break through resistance to TGFb effects in TGFb resistant lymphoma cells both *in vitro* and *in vivo* (12).
8. mTOR complexes (rate and activity of mTORC1 and mTORC2) related activity alterations correlate to the metabolic diferences in tumour cells; to study this aspect of mTOR tumour biology we established several new methods, these help to characterise the metabolic alterations (mTOR activity dependent and independent, as well) in experimental and clinical samples. (13, 14, R5, Kf)

The project related new references, reviews and book, book chapters:

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2. Mark A, Hajdu M, Varadi Z, Sticz TB, Nagy N, Csomor J, Berczi L, Varga V, Csoka M, Kopper L, Sebestyen A: Characteristic mTOR activity in Hodgkin-lymphomas offers a potential therapeutic target in high risk disease - a combined tissue microarray, in vitro and in vivo study. ***BMC CANCER* 13:** Paper 250. 12 p. (2013)
3. Nemes K, Sebestyen A, Mark A, Hajdu M, Kenessey I, Sticz T, Nagy E, Barna G, Varadi Z, Kovacs G, Kopper L, Csoka M: Mammalian target of rapamycin (mTOR) activity dependent phospho-protein expression in childhood acute lymphoblastic leukemia (ALL). ***PLOS ONE* 8:**(4) Paper e59335. 11 p. (2013)
4. Krencz I, Sebestyén A, Fábián K, Márk Á, Moldvay J, Khoor A, Kopper L, Pápay J: Expression of mTORC1/2-related proteins in primary and brain metastatic lung adenocarcinoma. **Hum Pathol under review**
5. Sticz T, Molnár A, Márk Á, Hajdu M, Nagy N,Végső Gy, Micsik T, Kopper L,Sebestyén A: mTOR activity and its prognostic significance in human colorectal carcinoma depending on C1 and C2 complex related protein expression, **J Clin Pathol under review**
6. 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,** 2015 Jul;21(3):597-604. doi: 10.1007/s12253-014-9861-z. Epub 2014 Nov 12., 2015
7. Nagy N, Molnár A, Dankó T, Hujber Z, Petővári G, Kopper L, Sebestyén A: miRNA expression study in archive human colon carcinoma tissues, **manuscript in preparation** for Mol Exp Pathol
8. Molnár A, Sticz T, Dankó T, Nagy N, Hujber Z, Végső Gy, Kopper L, Sebestyén A: The effects of rapamycin and related compounds in EGFR inhibitor resistant colon carcinoma cells. **Chem Biol Int under review**
9. Márk Á, Dankó T, Molnár A, Hujber Z, Nagy N, Krencz I, Kopper L, Sebestyén A: The effect of mTOR inhibitors on miR expression in colon carcinoma cell lines. **manuscript in preparation** for Exp Cell Res
10. Márk Á, Hajdu M, Nagy N, Csóka M, Hujber Z, Dankó T, Csomor J, Kopper L, Sebestyén A: mTOR activity related high galectin 1 expression in Hodgkin lymphomas, **manuscript in preparation** for Cell and Tissue Research
11. Nagy N, Hajdu M, Márk Á, Király PA, Tóth M, Dankó T, Csóka M, Sebestyén A: Growth inhibitory effect of rapamycin in Hodgkin-lymphoma cell lines characterized by constitutive NOTCH1 activation. **Tumour Biol**. 2016 Jul 29. [Epub ahead of print]
12. Sebestyén A, Márk Á, Hajdu M, Nagy N, Molnár A, Végső Gy, Barna G, Kopper L: Rapamycin can restore the negative regulatory function of transforming growth factor beta 1 in high grade lymphomas. **Cytokine,** 2015 Jun;73(2):219-24. doi: 10.1016/j.cyto.2015.02.024. Epub 2015 Mar 17.

13. Hujber Z, Jeney A, Oláh J, Szoboszlai N, Baranyai L, Környei J, Petõvári G, Sebestyén A.: Measuring 14C-glucose and 14C-acetate oxidation in tumour cells and tumourous host organism, **Magy Onkol.** 2015 Dec;59(4):292-301.
14. Jeney A, Hujber Z, Szoboszlai N, Fullár A, Oláh J, Pap É, Márk Á, Kriston C, Kralovánszky J, Kovalszky I, Vékey K, Sebestyén A: Characterisation of bioenergetic pathways and related regulators by multiple assays in human tumour cells. **Cancer Cell Int.** 2016 Feb 11; 16:4. doi: 10.1186/s12935-016-0281-x.
R1 Kopper L., Tímár J.: mTOR -komplexek -molekuláris pókok a molekuláris hálókban, **Magyar Onkológia**, 55:287-294, 2011
R2 Kopper László, Sebestyén Anna: Rezisztencia és/vagy progresszió - csőd vagy csak megtorpanás, **Klinikai Onkológia**, 1:41-47, 2014
R3 Kopper L.: Epigenetika és a daganatok, **Klinikai Onkológia**, magyar nyelvű review, 3. évf. 1. szám - 2016. feb
R4 Sebestyen A, Kopper L: A mammalian target of rapamycin (mTOR) és gátlóinak szabályozó szerepe, mellékhatásaik, **Orvostovábbképző Szemle** 21:(Augusztusi különszám) pp. 5-13. (2014)
R5 Sebestyén A, Hujber Z, Jeney A, Kopper L: Tumormetabolizmus, magyar nyelvű review, **Klinikai Onkológia** 3:(2) pp. 127-134. (2016)
K Kopper L, Tímár J, Becságh P, Nagy Z: **Célzott diagnosztika és célzott terápia az onkológiában** 4., Budapest: Semmelweis Kiadó, 2015.
Kf Sebestyén A: **Metabolizmus - metabolom**, Kopper L, Tímár J, Becságh P, Nagy Z (szerk.): Célzott diagnosztika és célzott terápia az onkológiában 4. 524 p., Budapest: Semmelweis Kiadó, 2015. pp. 62-63.

Our results with short summary of published and detailed description of under review or recently finished/prepared results

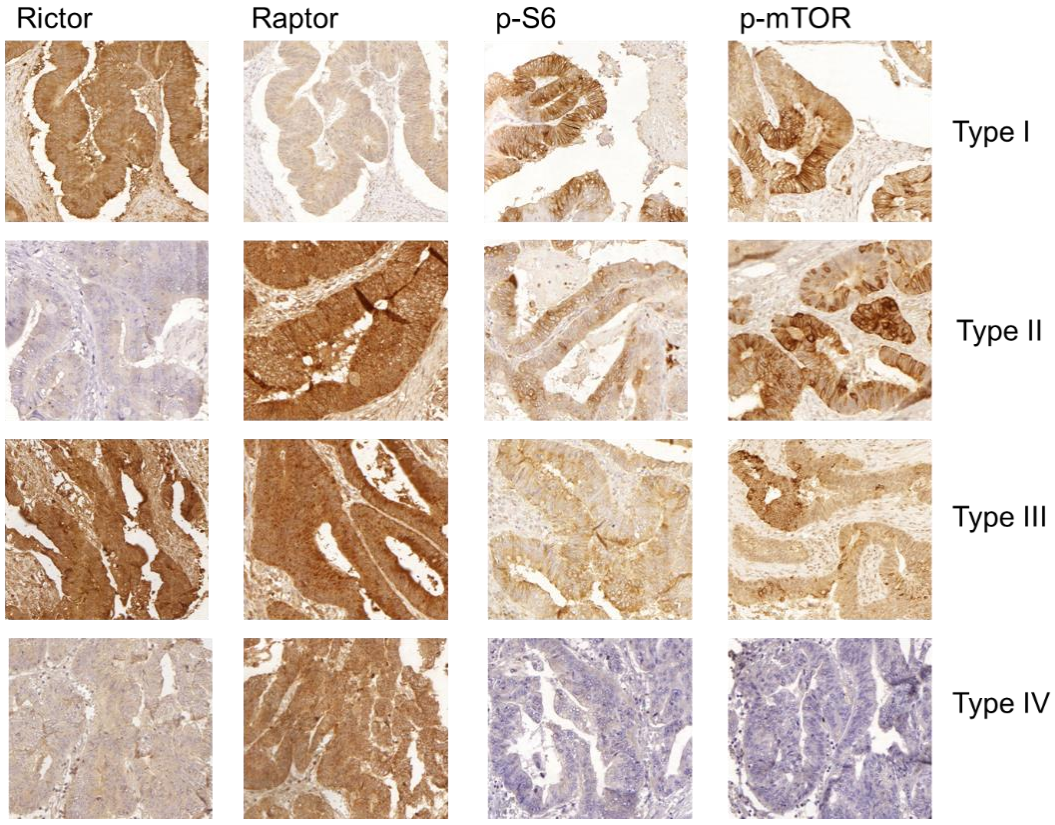
Studies related to activity of mTORC1 and C2 complexes in different malignancies (Statistical analysis the correlations of mTOR activity and clinical data, progression of the diseases)

We characterised the mTOR activity of several lymphoid malignancies, childhood ALLs and other lymphomas, such as DLBCLs and HLs and we analysed the correlation between the mTOR activity status and the prognosis of the diseases/survival of the patients at recently used common therapy. The following was published in three highly qualified and ranked journals (Modern Pathol D1-es - 5/189 in Pathology; BMC Cancer Q1-es - 57/325 in Oncology; Plos One D1-es - 154/1849 in Medicine misc. at Scimago Ranking): high mTOR expression is frequent in lymphoid malignancies. The actual low mTOR activity refers to the prospective good prognosis and therapy response, however, the presence of mTORC2 complex and its activity significantly correlate to worse survival, could be a prognostic marker for bad prognosis. The described high mTORC1 activity with lack of mTORC2 complexes in HL lymphomas correlates with these findings, it is well-known that HLs have good therapeutic results and long time disease free survival response. The almost 80% success rate in the therapy of childhood ALLs also supports the previous statements, moreover, according to our detailed study on leukemias, significant correlations were found between the higher mTORC1 activity of primary ALL cells and prospective relapse and worse prednisolon response. Activated (non-GC) DLBCLs showed similar statistical results in the analysis of mTOR complexes elated activities and clinical, survival data, in these subtypes of DLBCLs high mTORC2 activity is common in human biopsies which correlated to worse prognosis. (1, 2, 3)
Other two publications related to the mTOR complex activities of colon carcinomas and primer and brain metastatic lung carcinomas were recently sent to J. Clin Pathol and Hum Pathol from our group and these manuscripts are under review.

Summary of human colon carcinoma study results (5):

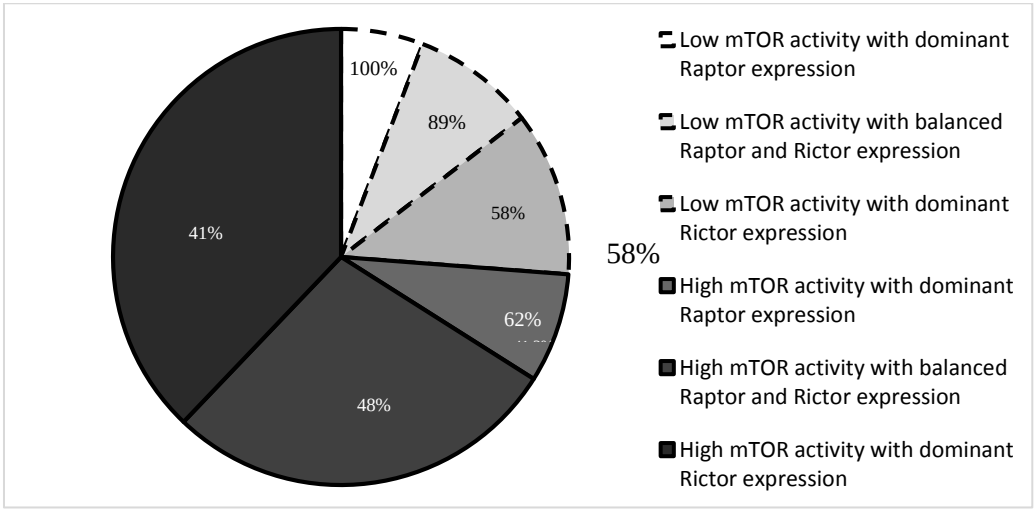
The detailed characterization of different colon carcinomas showed stage and grade independent high mTOR activity in 74% of the cases. The detected high mTOR activity was presented in mTORC1 and/or mTORC2 complexes; more than 60% of the studied cases had mTORC2 related high mTOR activity. Based on our analysis, high mTOR activity and Rictor overexpression could be markers of bad prognosis and the combined phospho-protein and Rictor/Raptor expression evaluation revealed even stronger statistical correlation with prognosis. Our experimentally used staining panel could be appropriate and highly recommended for the accurate specification of mTORC1 and C2 activity of tumour tissues. Moreover, this could help in selecting mTOR inhibitors and can provide information about prognosis, which may guide decisions about the intensity of therapy.

Figure 1. Rictor, Raptor, pS6 and p-mTOR expression patterns in colon carcinomas.



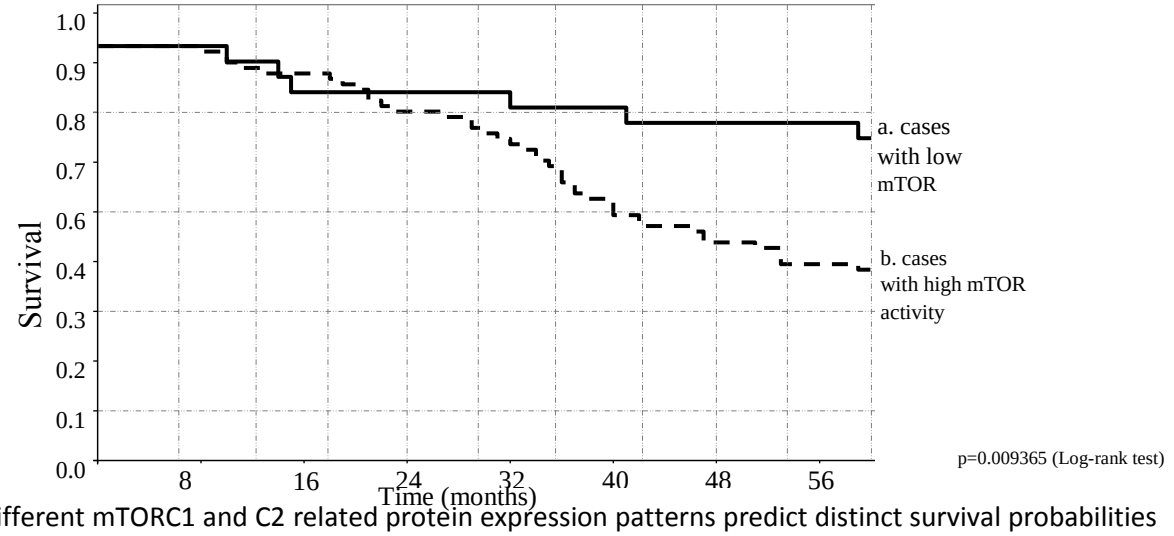
Type I: High mTOR activity with dominant Rictor expression; Type II: High mTOR activity with dominant Raptor expression; Type III: High mTOR activity with balanced Raptor and Rictor expression; Type IV: Low mTOR activity. (IHC of representative cases; Zeiss, Axioscope 2 Plus, 400X) (representative tissue samples)

Figure 2. Patterns of mTOR activity in colon carcinoma samples and 5-year overall survival of patients

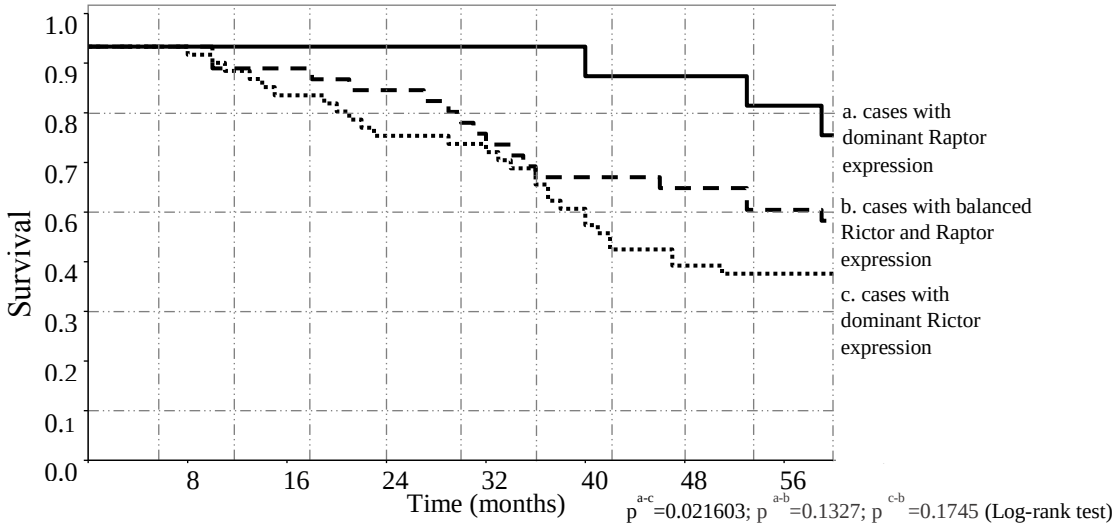


Overall survival data are given in percentages (%) relative to the patient groups with different mTOR activity patterns

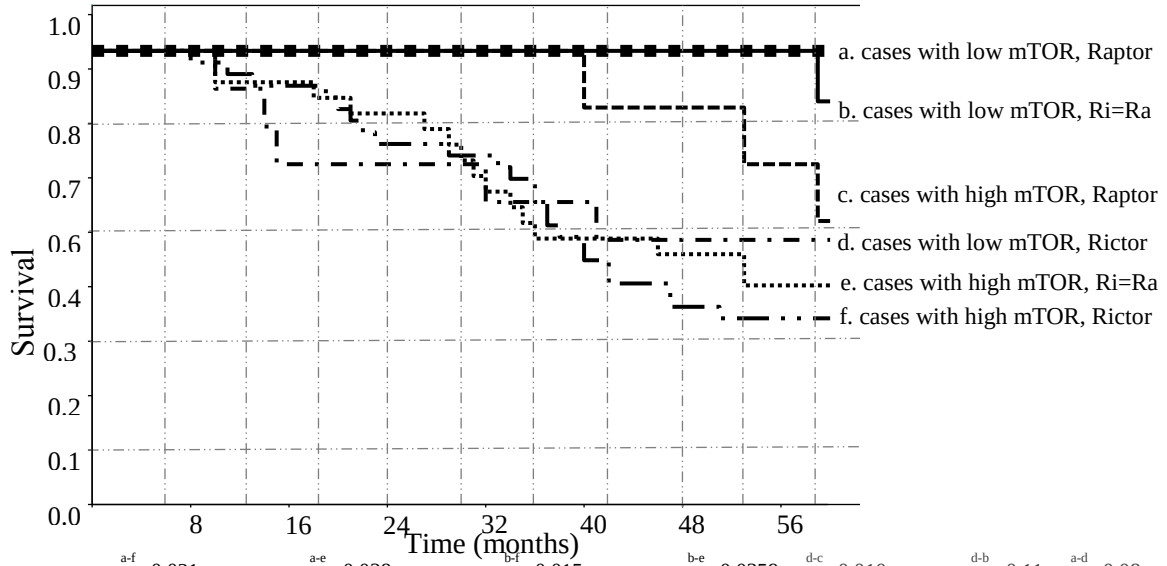
Figure 3. Survival (5-year overall survival) analysis of colon carcinoma patients according to mTOR activity. a. Low and high mTOR activities define patient groups with good and poor survival, respectively (statistically significant, p<0.01)



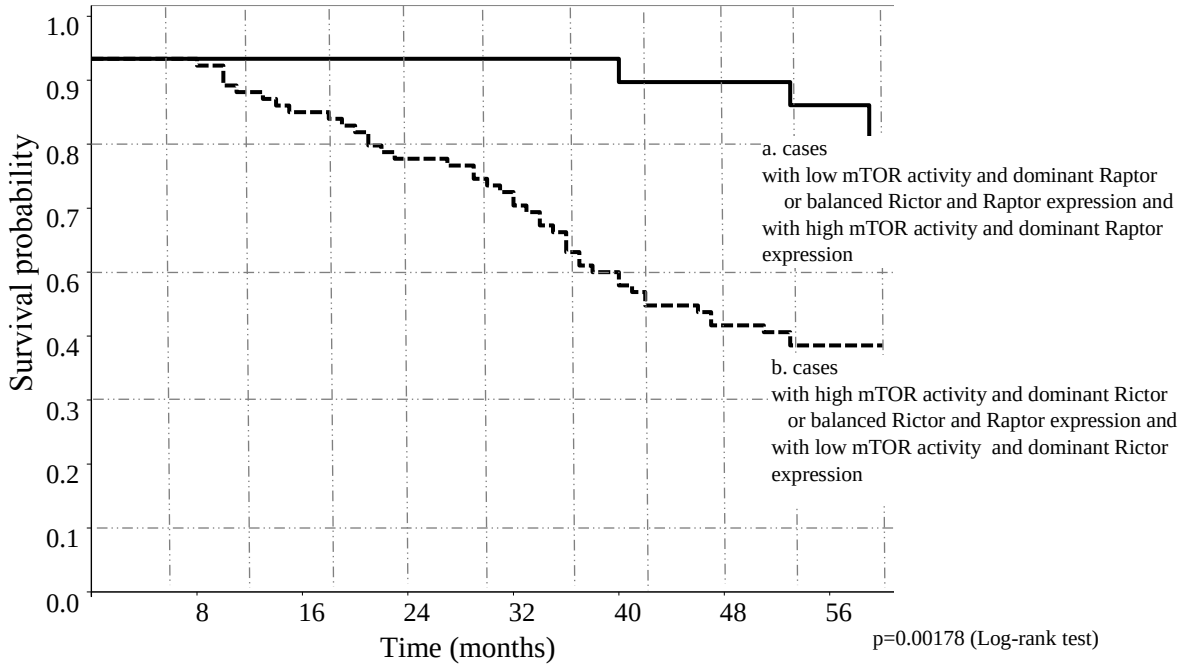
b. Different mTORC1 and C2 related protein expression patterns predict distinct survival probabilities (dominance of Rictor expression/mTORC2 complex, dominance of Raptor expression/mTORC1 complex, or balanced complex expression were distinguished)



c. Patient survival can be refined by combined analysis of mTOR activity and the presence of C1 and C2 complexes where kinase activity can be manifested (low/high mTOR: low/high mTOR activity; Raptor/Rictor: dominance of Raptor/Rictor; Ri=Ra: balanced expression of Rictor and Raptor).



d. Pooling together, groups with similar prognosis (based on combined analysis of mTOR activity and the presence of mTORC1 and C2 complexes/as shown in Figure 3c) segregate good and bad prognostic categories clearly (statistically significant, p<<0.05).



Summary of human primary and brain metastatic lung adenocarcinoma study results (4):

Brain metastases are common complications of adenocarcinomas of lung and are associated with poor prognosis. Although an increasing amount of data indicates that dysregulated activity of mammalian target of rapamycin (mTOR) can influence the metastatic potential of various tumours, the role of mTOR complexes in the development of brain metastases from adenocarcinomas of lung is largely unknown. To estimate mTOR activity, we studied the expression of mTOR related proteins (mTORC1: p-mTOR, pS6; mTORC2: p-mTOR, Rictor) in primary (n=63) and brain metastatic (n=65) lung adenocarcinomas, including 11 paired tissue samples, using immunohistochemistry and tissue microarrays. Correlation with clinicopathological parameters was also analysed. Increased p-mTOR, pS6 and Rictor expressions were observed in 35%, 33% and 40% of primary adenocarcinomas and in 78%, 69% and 62% of brain metastases, respectively. Expression of these markers was significantly higher in brain metastases as compared to primary carcinomas ($p<0.0001$, $p<0.001$, $p<0.001$). The increased mTOR activity in a subset of pulmonary adenocarcinomas and the higher incidence of increased mTOR activity in brain metastases suggest that, in selected cases, mTOR inhibitors may play a role in the treatment of lung adenocarcinomas, particularly to prevent brain metastases in patients with high mTOR activity.

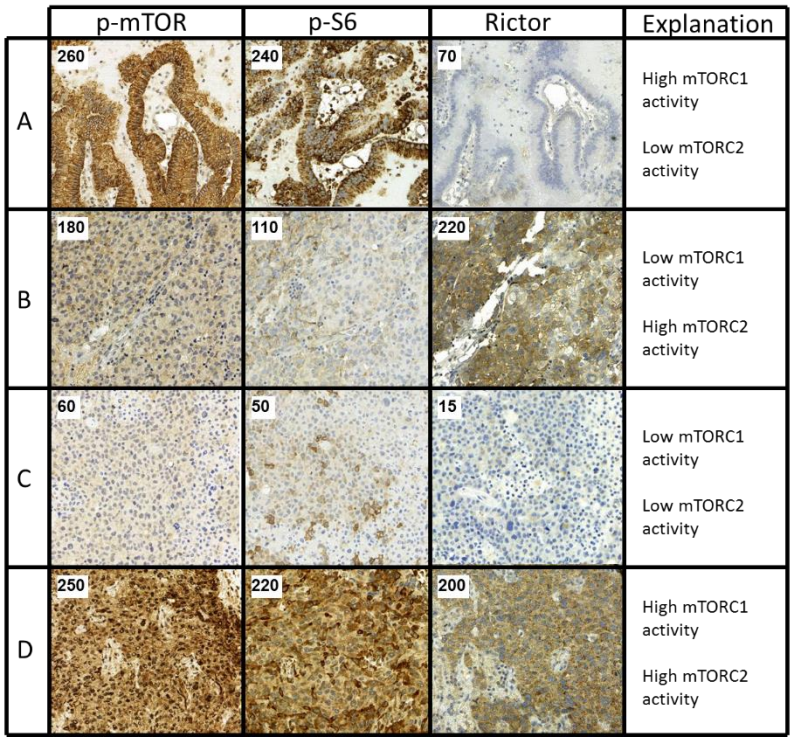


Figure 4. mTORC1 and mTORC2 activities - immunohistochemical stainings and their evaluation. Different pS6, p-mTOR and Rictor score values (in the upper left corners) and the activities of mTORC1 and mTORC2 in BMs of lung ADC cases (A-D) (x400).

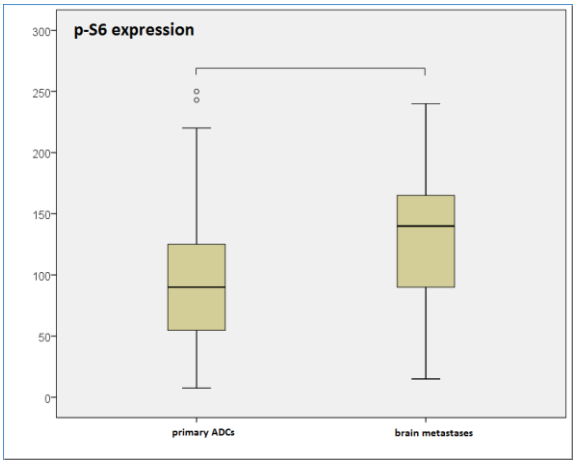


Figure 5. Evaluated expression levels of pS6 in primary LCs and in BMs. Expressions (H-score) of pS6 (p=0.0001) were significantly higher in BMs than in primary ADCs.

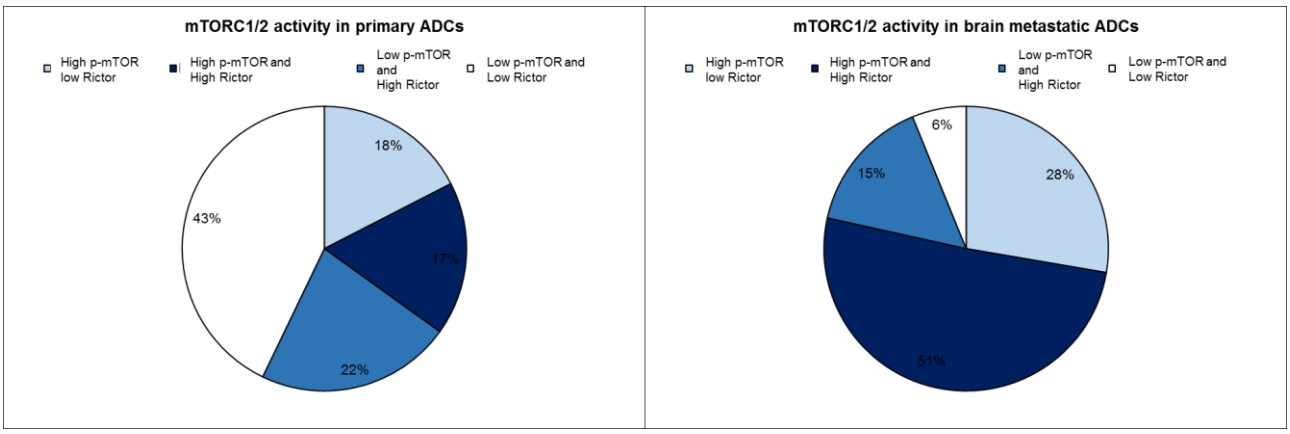


Figure 6. Distribution of the cases with high/low p-mTOR and Rictor expressions. a. – Expressions of p-mTOR and Rictor in primary ADCs. b. – Expressions of p-mTOR and Rictor in brain metastatic ADCs

mTOR activity related miR expression studies in colon carcinomas and ALLs

Expression of miRs in pediatric ALL patients was measured before chemotherapy, at conventional response checkpoints and at relapse. Correlations between altered miR expression and response to prednisolone at day 8 of therapy and long-term prognosis were statistically analysed in our published results. We could detect characteristic low of miR-223 and high of miR-128b expressions (which could inhibit the functions of negative regulatory proteins in PI3K/Akt/mTOR pathways) and their changes during the follow up period. Moreover, we found significant correlation between the lower (80x>) miR-128b expression and the poor prognosis, as well as the poor prednisolone response on day 8 in different ALL cases. A longer disease free time period of patients showed a significant correlation with higher expression levels of miR-128b, these correlated to the mTOR activity status of that patients, as well. Besides these results, it should be considered that both mTOR activity and miR-128b expression were higher in ALL than in normal lymphocytes, however, the highest mTOR activity and the lowest miR-128b correlated to the worse prognosis. It should be considered that both mTOR activity and miR-128b expression were higher in ALL than in normal lymphocytes, however, the highest mTOR activity and the lowest miR-128b correlated to the worse prognosis (6).
To continue these studies we concentrated to other malignancies, and we analysed the mTOR associated miR expression in different archived (formalin fixed) human colon carcinoma samples.

Summary of the results of miR studies in different human colon carcinoma samples and cell lines (7)

Different types of cancer were associated with deregulation of miR expression. In this aspect, one of the most intensively studied disease was the colorectal cancer (CRC) in the recent years, however, the available results are conflicting. In our work, we try to establish the appropriate endogenous control miRs to reproduce previous observations and determine the correct, actual expression level of the target miRs in our samples from both cell culture and fixed human samples. We tested 6 small non coding RNAs as endogenous controls, which were chosen according to selected published results and data bases. Based on our results, RNU6b, RNU49, and miR-16 were selected after significance analysis of c_t values ($p>0.05$). However, the c_t values displayed a wide range with high value in RNU6b and RNU49, they were used for whole FFPE samples which have good quality and quantity of miRs. miR-16 with low c_t value and the least variable endogenous control was suitable in other analyses, in laser micro-dissected (LMD) samples for examples.
Tissue sample pairs (normal and malignant) from colon carcinoma patients and non-malignant colon tissues were also studied in comparison. The expression of 8 selected miRs which related to tumour growth, especially to mTOR signalling pathway were analysed. 75% of these miRs were overexpressed (miR-21, miR92a, miR-100, miR-155, miR-199a and miR-218) in our samples. Three of these six (miR-21, miR-92a, miR-155) miRs showed higher expression changes using RNU6b as reference (more than twofold higher than it was detected in normal tissues) and miR92a, miR-100, miR-218, miR-21, miR-155 showed overexpression using miR16 reference.

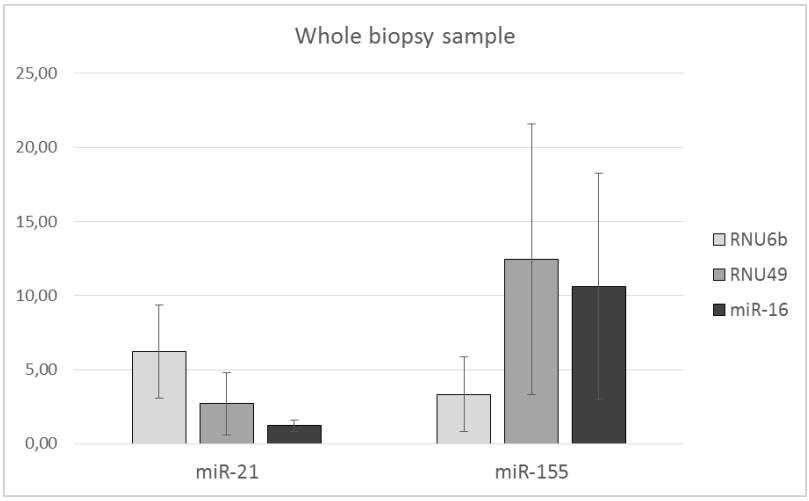


Figure 7. Alterations (fold changes) of the detected *miR-21* and *miR-155*, characteristic onco-miRs expression are depending on the endogenous controls (*miR-16, RNU6b, RNU49*) in the whole biopsy samples of human formalin fixed CRC samples.

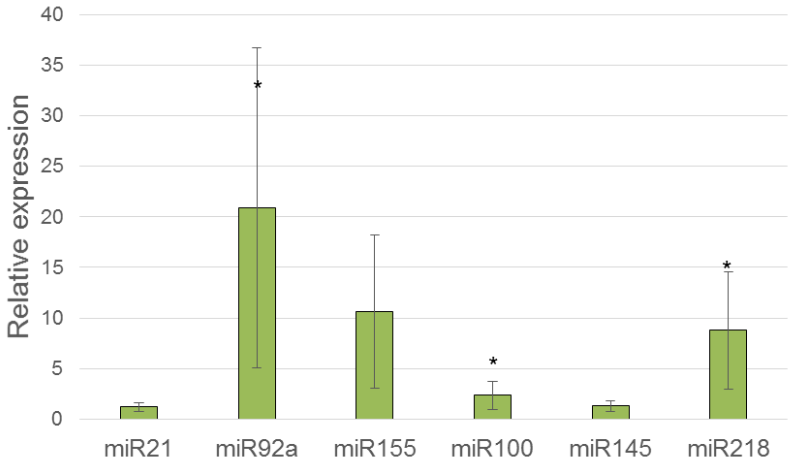


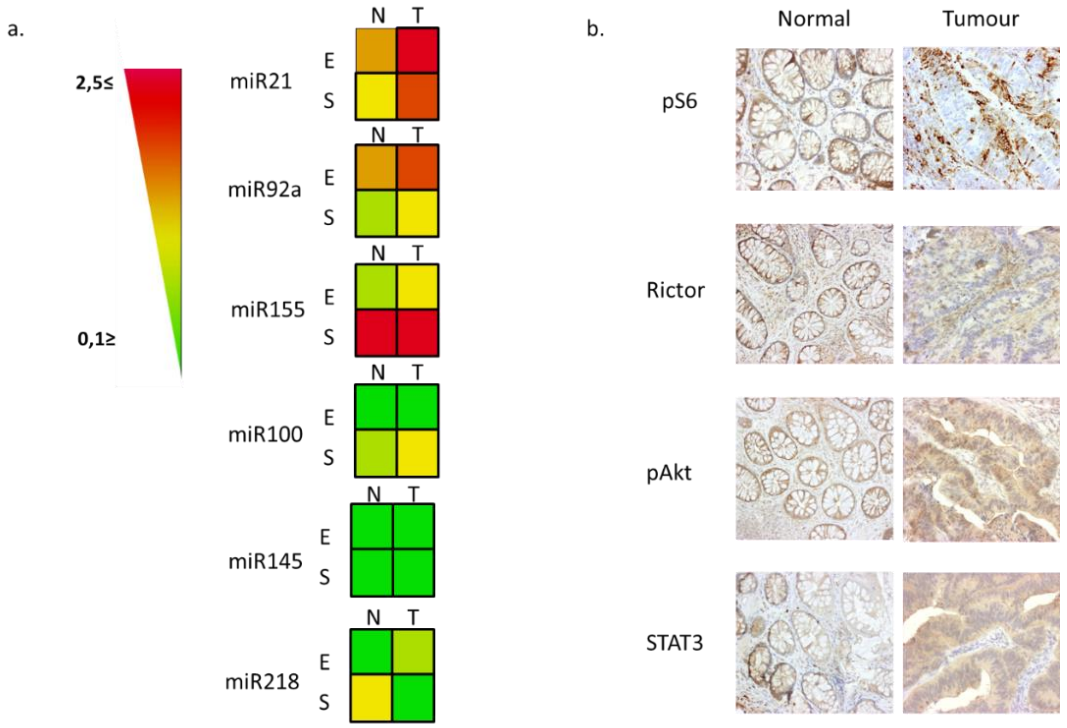
Figure 8. The results of studied miRs in whole CRC biopsy samples. The *miR-92a, miR-100* and *miR-218* showed significant ($p<0.05$) overexpression using *miR-16* reference.

To detect the real source of the expression changes LMD was used to select tumour cells, normal epithelial cells, normal and tumourous stromal elements to study the expression of miRs, as well. The overexpressions of *miR-21, miR-92a, miR-100* and *miR-155* were observed in the tumourous samples. However, these changes were related mainly to the stromal elements and not to the carcinoma cells. The other two miRs showed only few expression changes based our LDM results, we could not detect any significant expression changes of *miR-145.* However, in the case of *miR-218* we could find contradictions between the results from whole biopsies and these LMD data, because the expression of *miR-218* was slightly upregulated in the tumour cells and its expression was reduced in the dissected stromal elements of tumours. The expressions of some related target proteins were also studied by IHC staining in the biopsy materials. pAKT (Ser473), pS6 protein - the indicator of PI3K-mTOR signal activity – and Rictor (characteristic elements of mTORC2) showed elevated expression in tumour tissues. STAT3 (element of JAK-STAT signalling) overexpression was also detected in carcinoma cells.

Table 1. The most important targets of our selected miRs including published data and our results about their expression in CRCs

miRNA	Targets	colon carcinoma	Results in tumour cells	Results in stroma
miR-21	PTEN, TPM1, BCL2 PDCD	over-expression	over-expression	over-expression
miR-92a	BIM, ITGA5, CDH1 (E-cadherin)	over-expression	over-expression	normal level
miR-100	mTOR, IGFR, PLK1, RAP1B	down regulation	down regulation	over-expression
miR-144	mTOR, PTEN, APAF1, NOTCH1, EZH2	down regulation	down regulation	
miR-145	IRS-1, STAT1, c-MYC, p70S6K, N-RAS, HIF1A	down regulation	down regulation	normal level
miR-155	BCL6, BCL2, HDAC4, SOCS-1, SMAD1	over-expression	normal level	over-expression
miR-199a	mTOR, GSKIP, c-MET, SMAD1, SMAD4, MAP3K 11, LIF, HIF1A	no data	normal level	
miR-218	RICTOR, CDK6, PIK3C2A, IKBKB	down-regulation	over-expression	down regulation

Figure 9. miR expression results from LMD samples and the representative IHC staining of tissues samples.a. The areas of tumour (T) and normal (N) epithelial (E) cells and stromal (S) elements of different colon carcinoma tissues were LMD separated. The measured miR expression levels were coded in colour scale (between green and claret which means relative expression is low and high, each colour change carried 0,5 fold relative expression changes. (miR-16 endogenous control were used). b. Increased activity of PI3K/Akt/mTOR and JAK-STAT signalling were detected in CRC tumour tissues. pS6, pAkt (Ser473), Rictor, STAT3 were observed by IHC staining-DAB staining (brown colour)(400x).



The detected changes were shown in a schematic scale, which suggests that these miR expression changes could play a role in the progression of colon carcinoma cases by disrupting the regulation of PI3K/Akt/mTOR signalling in tumour tissues, however, the previously described conflicting results could be explained by the heterogeneity of tumour tissue. (7)

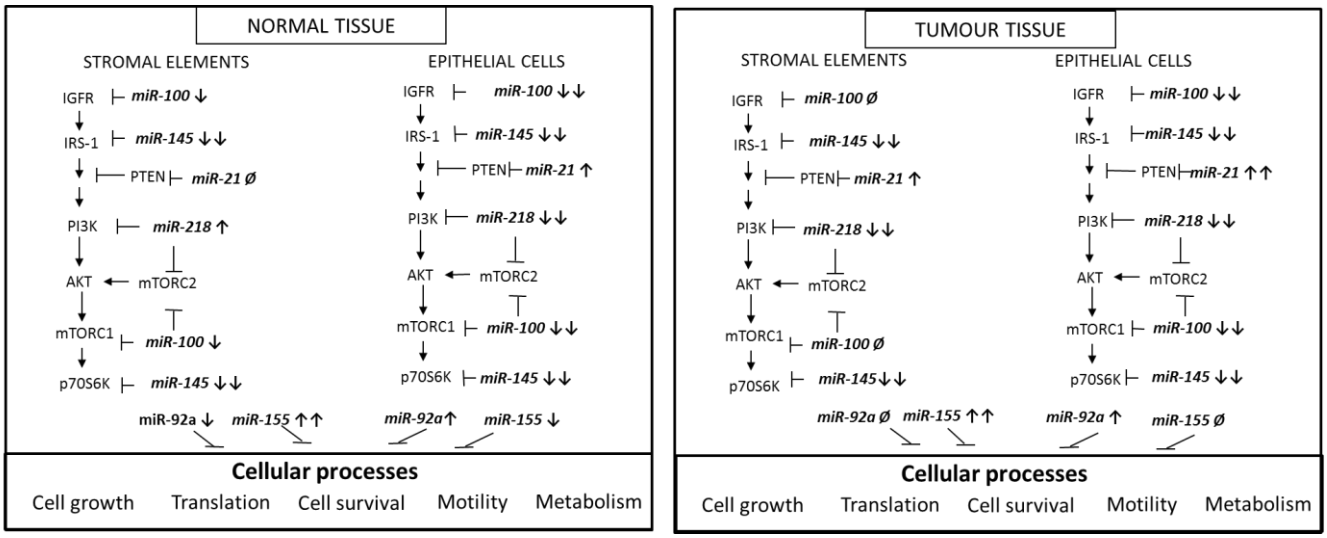
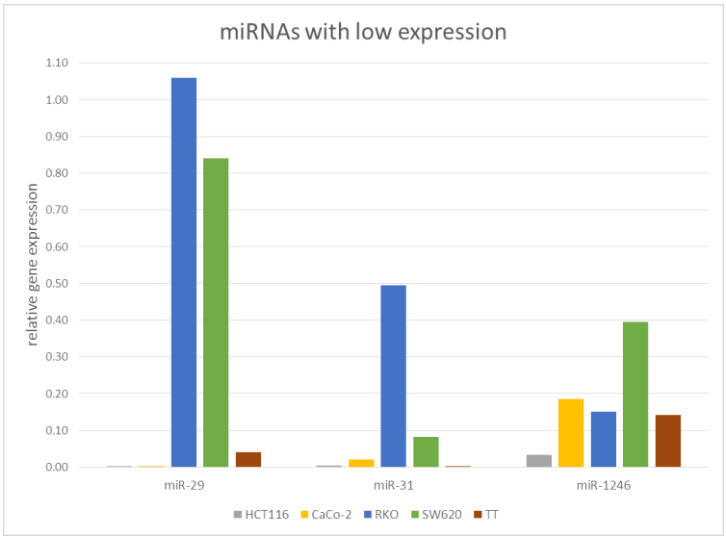


Fig 10. Summary of the miR expression study in LMD colon carcinoma samples

According to the previous expression study, the expression patterns of selected 11 miRs (miR-9, miR-16, miR-24, miR.29, miR31, miR-92, miR-126, miR-142, miR-182, miR-199, miR-1246) were studied in four colon carcinoma cell lines (HCT116, CaCo2, RKO, SW620), beside normal control cells and tumourous colon biopsy specimens, as well. Based on the detected expression levels, the studied miRs could be classified into three groups: a.) lower (miR-29, miR-31, miR-1246); b.) middle-rate/intermediate level (miR-9, miR-16, miR-24, miR-92, miR-182, miR-126); and c.) highly expressed, (miR-142, miR-199) miRs.

Figure 11. Expression level of different miRs in different colon carcinoma cell lines (using RNU49 internal standard the expression level were normalized to normal colon cells; TT: tumour tissue)

a.



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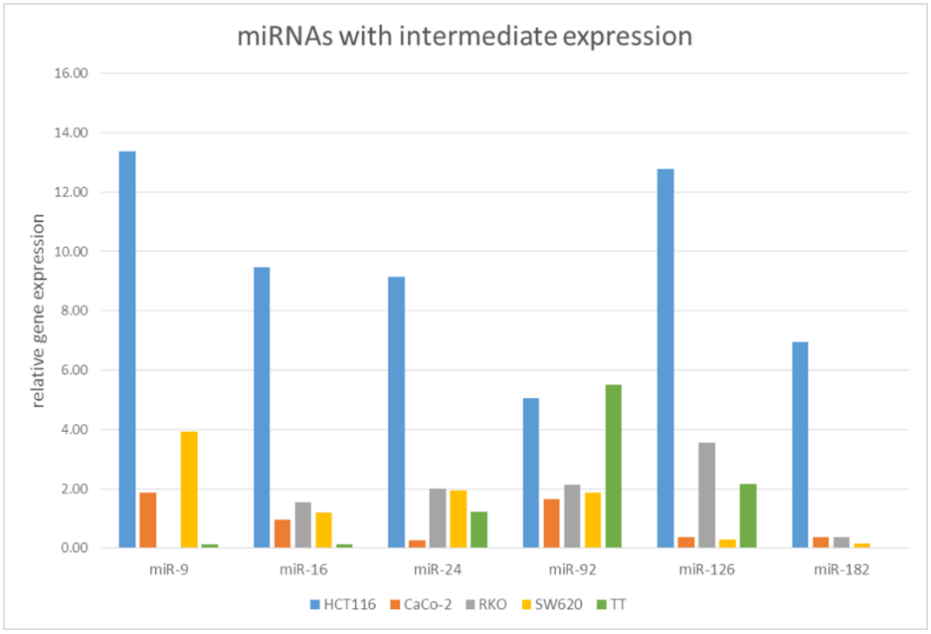
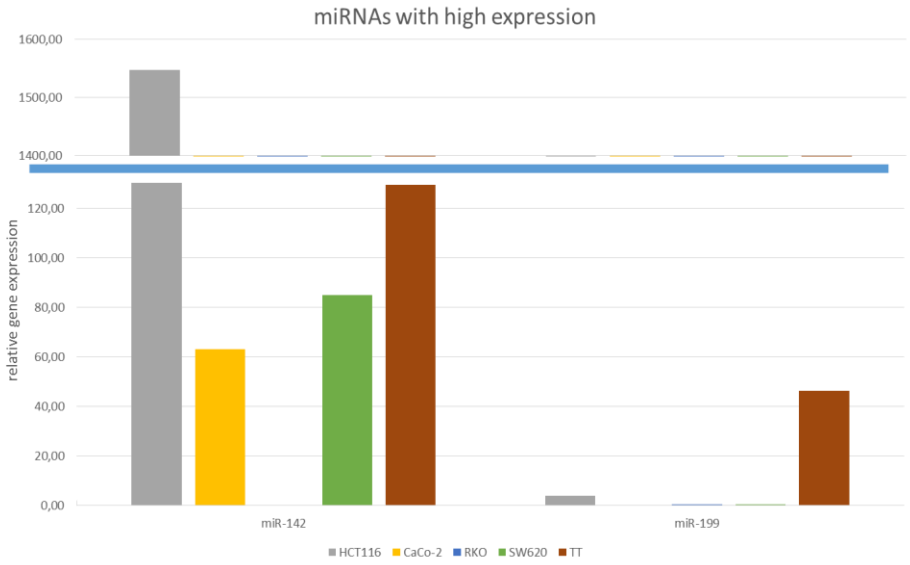


c.

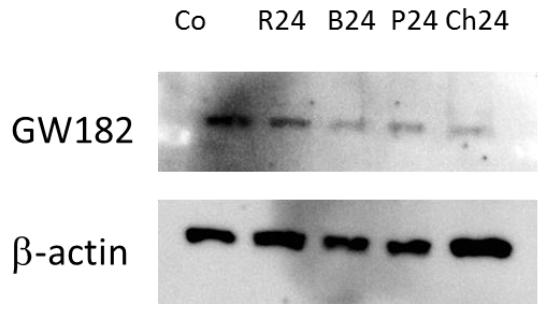


According to previously published data and our miR profile analysis, four miRs (miR-92, miR-126, miR-182 and miR-199) were chosen for further studies to compare the effect of different mTOR inhibitors (mTORIs) - rapamycin, NVP-Bez235, PP242 - on the alterations of the selected miRs in different colon carcinoma cell lines. ***miR-92*** was found to be overexpressed in each of the four colon cell line. 24h rapamycin treatment could reduce the expression level of miR-92 in SW620 cells (54%); nevertheless applying rapamycin for 24h or 72h did not seem to be effective in HCT116. However, 72h NVP-Bez235 or PP242 treatment could reduce miR-92 level significantly in HCT116 (64% and 60%) and in SW620 (85% and 32%). In contrast with RKO, in which every mTORI reduced miR-92 expression in 24h (rapamycin: 24%; NVP-Bez235: 16%; PP242: 13%), none of the tested mTORIs were effective significantly in CaCo2. ***miR-126*** was downregulated in the studied cell colon carcinoma cell lines except for RKO. In this cell line 24h rapamycin or PP242 treatments lowered the miR expression significantly (47% and 36%) and additionally NVP-Bez235 had a similar reducing effect in 72h (68%). In HCT116 it could be observed that applying any of the three mTORIs only 72h treatments caused decreased miR-126 expressions (rapamycin: 45%; NVP-Bez235: 62%; PP242: 27%). Using rapamycin or PP242

resulted in upregulated miR-126, while 72h NVP-Bez235 treatment reduced its level in CaCo2 cell line (77%). Applying rapamycin for 24h and NVP-Bez235 or PP242 for 72h reduced miR-126 expression in SW620 (rapamycin: 69%; NVP-BEZ235: 20%; PP242: 13%). ***miR-182*** levels in the studied tumour cells did not show the expected high expression values especially in HCT116 and CaCo2. Nonetheless 24h rapamycin, NVP-Bez235 or PP232 treatment resulted in reducing expression levels in RKO (rapamycin: 52%; NVP-Bez235: 85%; PP242: 63%) and in SW620 (rapamycin: 34%; NVP-Bez235: 23%; PP242: 41%). Furthermore using PP242 for 72h had the same effect in SW620 (39%).***miR-199*** were detected overexpressed in two cell lines (control HCT116 and RKO). Regarding to the studied mTORIs, they seemed to be effective in the 72h treatment in HCT116 cell line (rapamycin: 51%; NVP-Bez235: 74%; PP242: 40%). Rapamycin influenced miR level decrease both in RKO (24% at 24h; 59% at 72h) and in SW620 cells (74% at 24h; 83% at 72h). In addition, miR-199 was reduced in 24h PP242 treated SW620 cell line (48%), as well.

SW620 and two miRs (miR-92 and miR-199), were selected for further study aimed to observe the effect of cycloheximide, as an effective protein synthesis inhibitor to compare its effect on miR expression effected by mTORIs'. In case of middle-rate/intermediate expressing miR-92, 24h cycloheximide treatment resulted in decreasing miR expression level similarly to rapamycin and NVP-Bez235. Due to 72h cycloheximide treatment, the basically low miR-199 expression level was further reduced. Nevertheless, comparing this result with our previous data, rapamycin and PP242 reduced miR-199 expression in 24h whereas NVP-Bez235 treatment persisted in decreasing expression level in 24h and even in 72h. These effects and their similarity correlate to our Western blot results were the decrease in the amount of P-body proteins (processing bodies for gene-silencing – GW182) were also detected after both cycloheximide and mTORI treatments.

Figure 12. Western blot analysis of p-body protein expression in SW620 colon carcinoma cells after different mTORIs (Rapamycin 50 ng/ml; NVPBez235 1μM; PP242 1μM) or cycloheximide (1μM) treatment (24h) using GW182 antibody from Abcam.



These results indicate that mTORIs mainly reduce the expression of overexpressing regulatory miRs and these could be related to the altered protein synthesis (the mTOR activity is necessary for many protein synthesis), gene silencing p-body amount and metabolic capacity of the inhibited tumour cells. Moreover, these effects are highly dependent on the individual sensitivity of the treated cells. Our results also suggest that mTORI treatment could alter the

expression of many miRs and these effects could help in the anti-tumour effect of the treatments. (9)

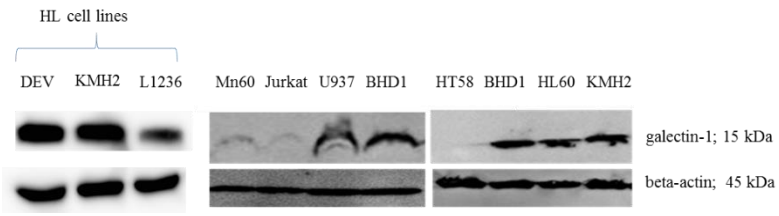
In vitro and *in vivo* effects of mTORIs in colon carcinomas and lymphoma cell lines

The the mTORC1 inhibitory effects of rapamycin, were characterised and published in certain lymphoma cells (11, 12). These results confirmed that mTORC1 inhibitors could be effective in different regulatory failures. TGFb negative regulator role was restored in TGFb resistant lymphoma cells and xenografts (12). We confirmed that rapamycin could effectively inhibit the tumour growth and induce apoptosis in Notch inhibitor resistant HL cell lines which were characterised by constitutive Notch activity and in parallel high mTOR activity (11).

Summary of our results about the mTOR related galectin-1 expression and its microenvironmental role (10)

The other function of high mTOR activity and the potential role of mTOR inhibitor effect in the tumour microenvironment were shown in our other recently ended study. High galectin-1 expression was observed in HL cells and cell lines. We also found that mTOR activity is neccessary for galectin-1 production. mTOR inhibitor treatment decreased galectin-1 expression in HL cell lines as well as in HL xenografts. It was already described that elevation of galectin-1 expression can increase the recruitment of regulatory T-cell in the microenvironment of many tumour cells and can promote the local immunosuppression, the tumour growth and the survival/immunescape of tumour cells. Increased galectin-1 expression was found in the majority (65/72) of HL samples, which showed a significant positive correlation with high mTOR activity. High galectin-1 expression was accompanied by increased numbers of regulatory T cells. Galectin-1 expression did not correlate with histological subtypes of HL. However, there was a negative correlation between the stage and the extent of regulatory T-cell infiltration. Based on our results, mTOR inhibitors may have a beneficial therapeutic effect in advanced stage and refractory HL, which is supported by clinical trials. Furthermore, the impact mTOR inhibitors can alter the extracellular matrix and in particular, galectin-1 expression, suggests that these alterations have biological significance in shaping the microenvironment in HL. (10)

Figure 13. Galectin-1 expression in lymphoma/leukemia cell lines (Western blot results**)**



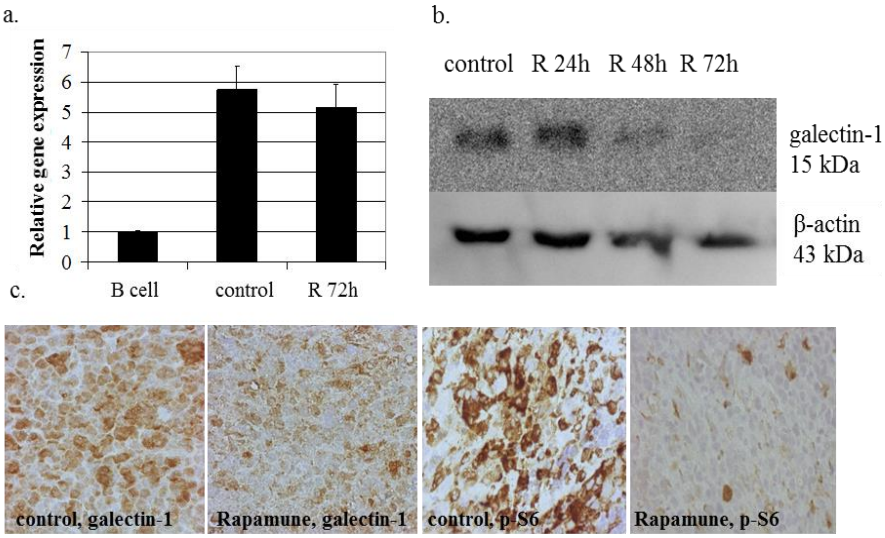


Figure 14. mTOR inhibition decreases galectin-1 protein levels in HL cells *in vitro* and *in vivo*
a: Galectin-1 Real-time PCR results of KMH2 cells (compared to normal B cells; R: rapamycin 50 ng/ml). b: mTOR inhibition decreases galectin-1 protein expression *in vitro* (WB, KMH2,) and c: *in vivo* KMH2 xenografts (Rapamune: 3 mg/kg, IHC, 200x magnification);

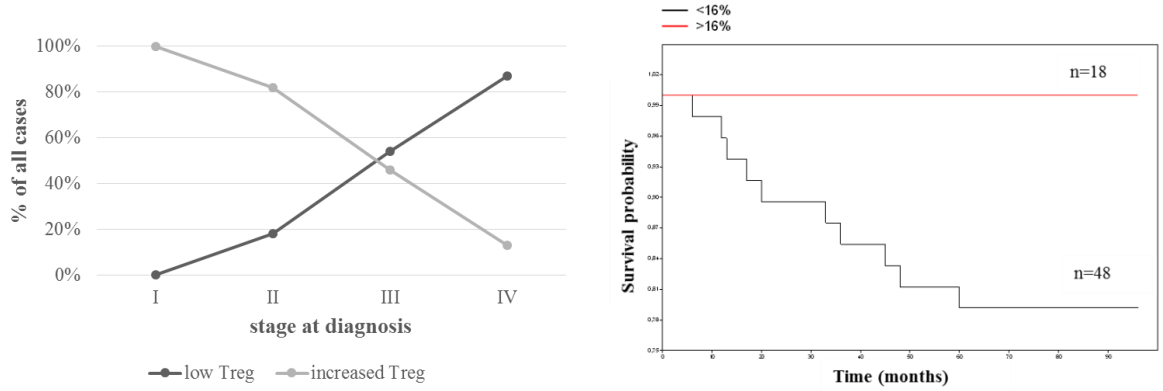


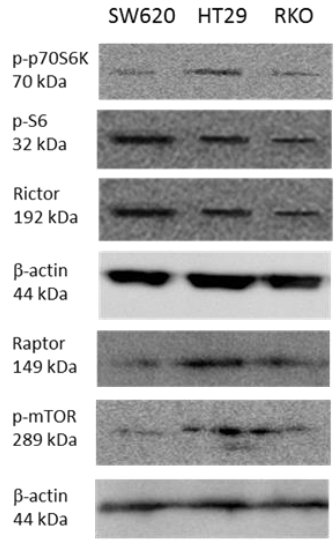
Figure 15. a. Correlation between Treg infiltration and clinical stage in HL. Low Treg : 0.5-5% infiltration. (p=0.001), b. Using 16% as a cut off level for Treg infiltration, a significant difference in survival was observed among HL patients (p=0.0415).

Summary of our results about the effect of mTORIs in different therapy resistant colon carcinoma cells

As in our previously described studies, mTOR inhibitors were found very effective anti-tumour agent in human colon carcinoma models with different mutation profile. The EGFRI resistance of the studied different colon carcinoma cell lines related to their known mutations were confirmed in our experiments. High mTOR activity was detected in the studied different colon carcinoma cell lines. Different techniques such as Western blot, ICC and Duolink staining showed that the elevated mTOR activity could be present in both mTORC1 and mTORC2 complexes. Cell lines showed individual differences in the amount of the Rictor and Raptor similarly to our previously described data related to human colon carcinoma cases. These expression patterns - the expression of the active mTOR kinase (phosphorylated mTOR – p-mTOR) and the mTORC1/mTORC2 complex characteristic Raptor or Rictor, mTORC1 activity related expression of phosphorylated p70S6K and its target protein ribosomal S6 (pS6) -

correlated to the mTOR inhibitor sensitivity differences. It was also found that cells which express higher amount of Rictor (+++) were less sensitive to mTOR, especially mTORC1 inhibitors (GC3, HCT116 and HT29). Rapamycin and the other dual or mTORC1 and C2 inhibitors showed significantly inhibited proliferation in the other - more sensitive – cell cultures. Moreover, in the most mTORI sensitive cell line - RKO - both dual and mTORC1-C2 inhibitors inhibited the proliferation significantly higher than rapamycin. In these cells the mTORC1 activity related protein, the phosphorylated S6 (pS6) level was reduced rapidly after 24h rapamycin and NVP-Bez235 treatment, as well. However, in the other, less sensitive HT29 cells the pS6 protein level could only be reduced by NVP-Bez235 (dual inhibitor) treatment and the significant reduction only appeared after 72h treatment where the Rictor expression was also downregulated.

a.

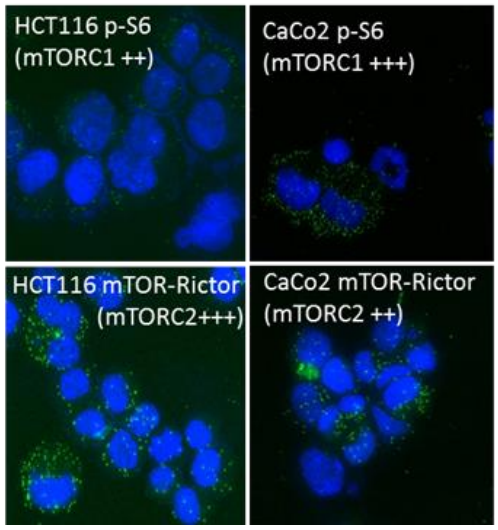


b.

ICC	SW620	HT29	RKO
p-S6	+	+++	++
Rictor	++	+++	++
Raptor	+	++	+

ICC	SW480	GC3	Colo205	HCT116	CaCo2
p-S6	+	++	++	++	+++
Rictor	+	+++	+	+++	+++
Raptor	+	++/+++	+	++	++

c.



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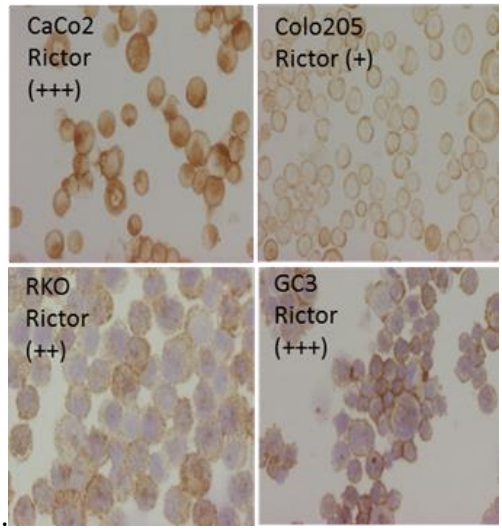


Figure 16. Different mTOR activity profile of colon carcinoma cell lines (a. Western blot results, b. ICC evaluation, c. Duolink staining, d. representative ICC stainings)

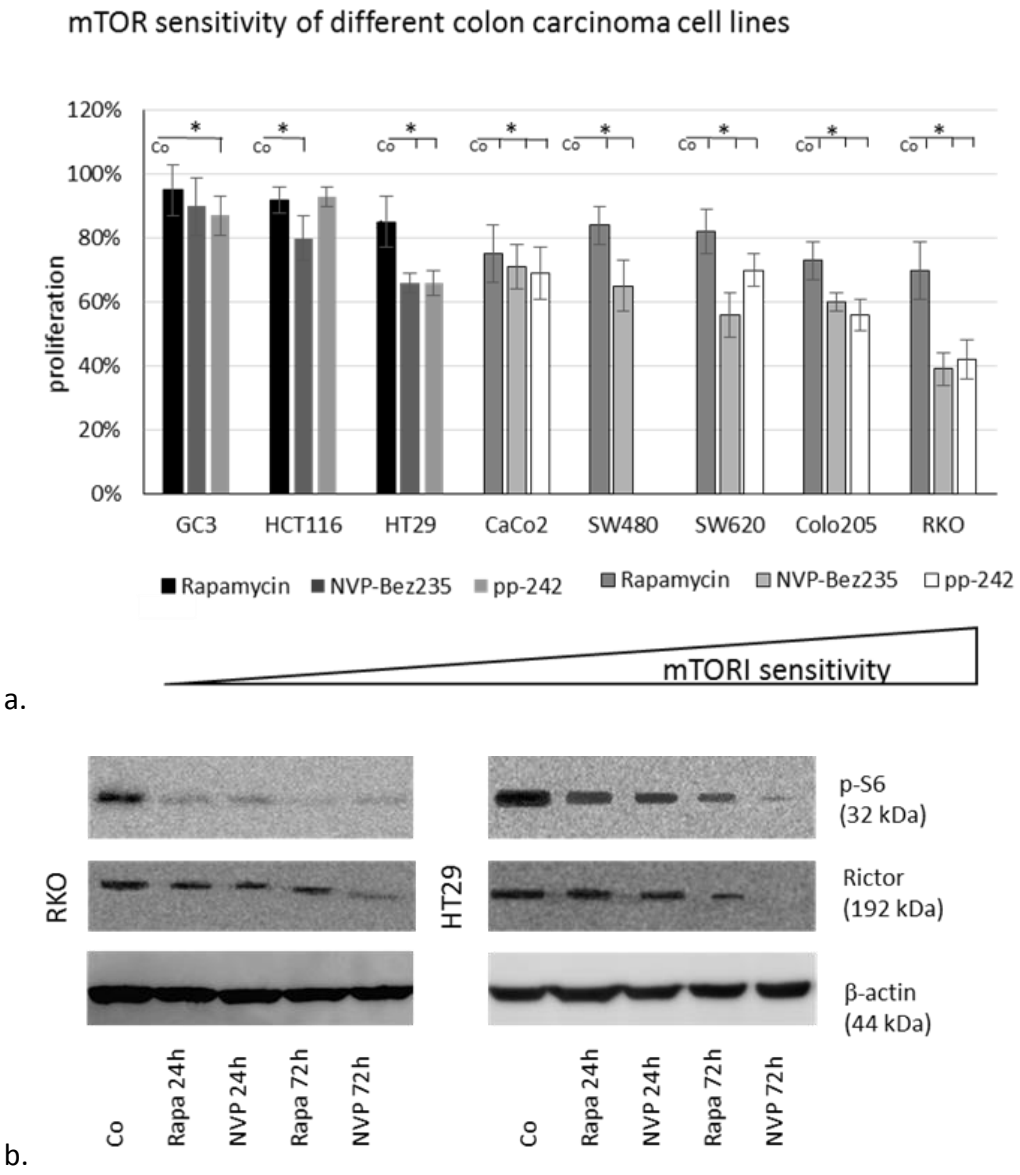
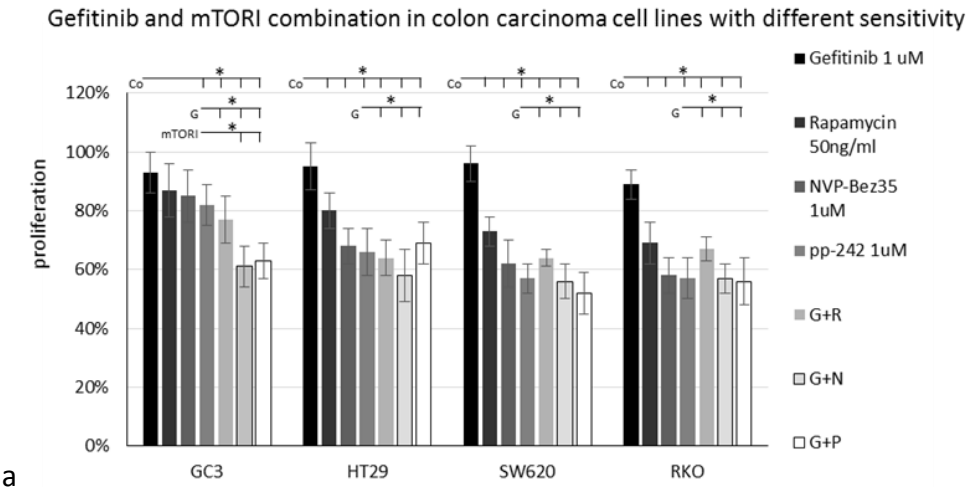
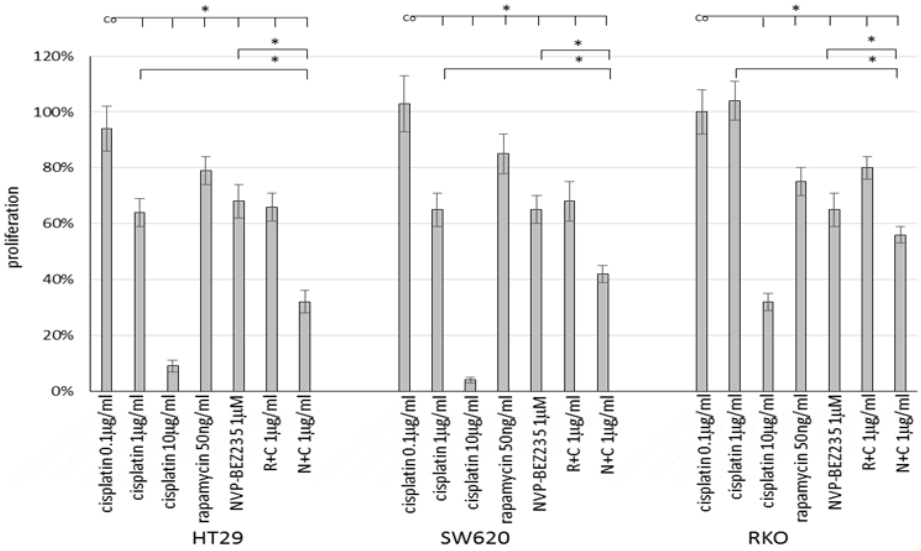


Figure 17. mTOR inhibitor sensitivity of different colon carcinoma cell lines (a) and the effect of these inhibitors on the expression of mTOR activity markers after *in vitro* treatments in two colon carcinoma cell lines

It was also detected that rapamycin and especially other more effective mTOR inhibitors such as NVP-Bez235 (mTOR and Akt kinase dual inhibitor) and PP-242 (mTORC1 and C2 inhibitor) in combination with EGFRI could be more effective in the less mTORI sensitive and EGRI resistant colon carcinoma cells (GC3, HCT116 and HT29). However, the mTORI and EGFRI combination could have no more additional effects compared to the mTORIs in the mTORI sensitive other cell lines. mTOR inhibitors, especially NVP-Bez235, dual inhibitor induced the effect of cisplatin and could be effective in cisplatin and EGFRI resistant RKO colon carcinoma cells, as well. Moreover, the dual mTOR and Akt kinase inhibitor could induce the effect of cisplatin significantly in both HT29 and SW620, less mTOR sensitive and EGFRI resistant cell lines.



a



b

Figure 18. Rapamycin induces the effect of gefitinib and cisplatin in certain colon carcinoma cells. a. Dual mTOR inhibitor NVP-Bez235 and mTORC1-C2 inhibitor PP242 with gefitinib could be effective in anti-tumour growth of GC3, EGFR inhibitor and rapamycin resistant colon carcinoma cells and in other less mTORI sensitive cells. b. mTOR inhibitors, especially NVP-Bez235 (mTOR and Akt kinase dual inhibitor) induce the effect of cisplatin and could be effective in cisplatin and EGFRI resistant RKO colon carcinoma cells (representative results of 72h experiments, 50ng/ml rapamycin, 1µM NVP-Bez235, 1µM -242, 1µM gefitinib; Alamar Blue test).

Based on these results, the new mTORIs and their combinations could be effective in EGFR or other chemotherapy resistant colon carcinoma cases especially *in vivo* where the long-term treatments could induce apoptosis, as well. As we detected in the case of lymphoma xenograft model. To plan and manage these combinations the exact determination of mTOR activity in the tumour tissues is necessary beside their mutational status, as well. Such an *in situ* characterisation will help to predict therapy resistance and find more effective therapeutic protocols in the near future. However, the potential side effects must also be considered. (8).

The metabolic effects of mTOR activity were also studied in our related additional studies with the recently used cellular and xenograft models. For these works establishing measurements

of different metabolites and substrate utilization were done both in our *in vitro* and *in vivo* models (13). We started the metabolic characterisation of different colon carcinoma cell lines and tried to find adequate IHC markers beside mTOR activity markers for such a characterisation in tumour tissues, as well. The different mTOR activities were accompanied by different metabolic characteristic and environmental adaptability in the studied cells (14). Our aims are to continue these mTOR activity related studies and to establish a new Tumourmetabolism research group/team.

Short summary the significance of our new results:

Useful experience were acquired both in the miR expression study methods and in mTOR activity related descriptive and experimental studies. We could publish 8 original and 5 review articles and further 6 original articles are under review or near to submission. These results were presented in many national and international conferences. Related to our works and to targeted therapy a book and many book chapters were written and the connected students could finish their theses. Nemes K. earned PhD degree and other two students's PhD theses are in progress (Nagy N; Sticz T). Many other graduated students could also work in our projects. They finished their diploma theses (Varga V., Molnár A., Dankó T., Krencz I. Buthi N. etc.) and their TDK works earned several awards (e.g. OTDK 1st prize and ProScientia Prize of Molnár A).

Summarizing the results of the recently ended project, it contributed to select therapeutic targets for treatments of patients with several malignancies and to develop/establish new methods for predicting more effective treatments and therapy design. Moreover, this project promoted many publicatons; the scientific development, PhD degree earning of graduated and under-graduated students and their entrance into professional scientific work, as well.