mTOR activity and its prognostic significance in human colorectal carcinoma depending on C1 and C2 complex-related protein expression

Tamás Sticz,[1] Anna Molnár,[1] Ágnes Márk,[1] Melinda Hajdu,[1] Noémi Nagy,[1] Gyula Végső,[2] Tamás Micsik,[1] László Kopper,[1] Anna Sebestyén[1,3]

[1]1st Department of Pathology and Experimental Cancer Research, Semmelweis University, Budapest, Hungary
[2]Department of Transplantation and Surgery, Semmelweis University, Budapest, Hungary
[3]Tumor Progression Research Group of Joint Research Organization of Hungarian Academy of Sciences and Semmelweis University, Budapest, Hungary

Correspondence to
Dr Anna Sebestyén, Semmelweis University, 1st Department of Pathology and Experimental Cancer Research, Üllői út 26, Budapest 1085, Hungary; anna@korb1.sote.hu

Received 1 June 2016
Revised 13 September 2016
Accepted 23 September 2016
Published Online First 11 October 2016

ABSTRACT

Aims Tumour heterogeneity and altered activation of signalling pathways play important roles in therapy resistance. The PI3K/Akt/mTOR signalling network is a well-known regulator of several functions that contribute to tumour growth. mTOR exists in two functionally different multiprotein complexes. We aimed to determine mTOR activity-related proteins in clinically followed, conventionally treated colon carcinomas and to analyse the correlation between clinical data and mTORC1 and mTORC2 activity.

Methods Immunohistochemistry was performed with different antibodies on tissue microarray blocks from 103 patients with human colorectal adenocarcinoma. mTORC1- and mTORC2-related activity were scored on different stainings including analysis of the expression of Raptor and Rictor—specific elements of mTORC1 and C2 complexes. The staining scores and clinical/survival data were compared and analysed.

Results Detailed characterisation showed stage and grade independent high mTOR activity in 74% of cases. High mTOR activity was present in mTORC1 and/or mTORC2 complexes; >60% of cases had mTORC2-related high mTOR activity. Based on our analysis, high mTOR activity and Rictor overexpression could be markers of a bad prognosis. Combined phosphoprotein and Rictor/Raptor expression evaluation revealed even stronger statistical correlation with prognosis.

Conclusions The presented staining panel could be appropriate and highly recommended for the accurate specification of mTORC1 and C2 activity of tumour tissues. This could help in the selection of mTOR inhibitors and can provide information about prognosis, which may guide decisions about the intensity of therapy.

INTRODUCTION

The PI3K/Akt/mTOR signalling network is a well-known regulator of several functions that contribute to tumour growth.[1] Recently published articles and reviews describe the potential role of its alteration, especially high mTOR (mammalian/mechanistic target of rapamycin) activity, in contributing to resistance to therapy.[2] mTOR is a serine/threonine protein kinase which exists in two different multiprotein complexes (mTORC1 and mTORC2). These possess specific elements such as Raptor (mTORC1) and Rictor (mTORC2),[3] and play different roles in a variety of cellular functions (eg, proliferation, survival, protein synthesis, metabolism, autophagy, lipid synthesis); moreover, mTOR complexes have different sensitivity to mTOR inhibitors (figure 1). Clinically available mTOR inhibitors, the rapalogs (analogues of rapamycin such as everolimus and temsirolimus are specific mTORC1 inhibitors), do not inhibit mTORC2 directly, although results are conflicting about the effects of prolonged in vivo treatment, which can lead to inhibition of mTORC2 activity as well.[4 5]

mTORC1 primarily promotes phosphorylation of S6K1 and 4EBP1 whereas mTORC2 directly activates other distinct proteins such as Akt by phosphorylating its hydrophobic motif (Ser473). The phosphorylation of target proteins can contribute to different pro-proliferative and anti-apoptotic functions in tumour cells as well.[3] The high frequency of somatic and germline mutations resulting in mTOR pathway activation makes pharmacological inhibition of mTOR a promising therapeutic target for a variety of human cancers.[6 7] New mTOR inhibitors have been developed and are being tested in clinical trials.[8] Besides mTORC1 inhibitor rapalogs, specific ATP-competitive and allosteric mTOR inhibitors and dual inhibitors have also been developed, which can inhibit both complexes. These could be very effective for reversing resistance to treatment related to alterations in the signalling network.[9] mTOR inhibitors have been introduced and approved in the treatment of patients with metastatic renal cell carcinoma, mantle cell lymphoma, pancreatic neuroendocrine tumours and advanced breast cancer with a poor prognosis.[10]

Colorectal cancer is the third most commonly diagnosed cancer worldwide. The conventional therapy for colorectal cancer is surgery and chemotherapy, in certain cases combined with radiotherapy.[11] Tumour heterogeneity and related overactivation of alternative signalling pathways and/or activation of dormant cancer stem cells using cross-talk with the microenvironment[12 13] play an important role in resistance to treatment,[14] both in conventional and targeted therapy. Studying the molecular background of these can assist in improving clinical strategies and will help to introduce new combination therapies in the future.

Clinical trials are still ongoing with different inhibitors—in addition, next-generation mTOR inhibitors—and their combinations in several tumour types such as advanced solid tumours.[15] However, their therapeutic effectiveness remains unclear,[7] and limited data are available about the characterisation of phosphoprotein expression in mTOR signalling and the activity of mTORC1 and mTORC2 complexes. The aim of our study was to determine mTOR activity-related proteins in clinically followed,

To cite: Sticz T, Molnár A, Márk Á, *et al*. *J Clin Pathol* 2017;**70**:410–416.

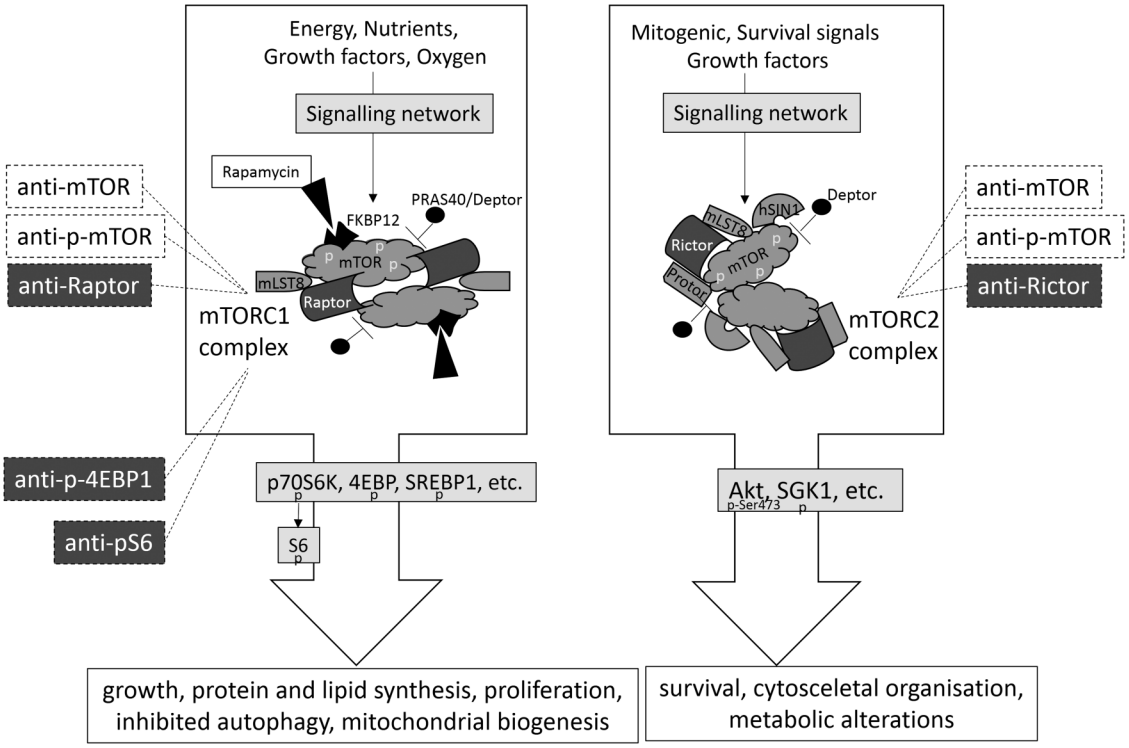

Figure 1 Simplified scheme of the structures and regulatory roles of different mTOR complexes and the discrimination of mTORC1 and mTORC2 complexes using immunohistochemistry. Both large protein complexes of mTORC1 and mTORC2 include mTOR kinase (mTOR), mLST8 proteins. In addition, mTORC1 includes Raptor and mTORC2 includes Rictor. The detection of Raptor and Rictor—the large scaffold proteins regulating mTORC1 and mTORC2 assembly and structure—helps to distinguish the protein complexes. mTORC2 also includes Protor and mSIN1, and both complexes have several negative and positive regulators such as PRAS40, Deptor-mTOR inhibitor proteins. Other distinct characteristics of these protein complexes are their rapamycin sensitivity (FKBP12 protein is necessary for rapamycin binding), upstream signals (growth factors, nutrients and energy supply), activation of mTORC1 and mTORC2 direct/indirect targets (eg, p70S6K, 4EBP1, Akt) and biological functions. Based on these, using different immunohistochemistry stainings we could detect mTOR kinase (anti-mTOR) and its activity (p-mTOR) in different complexes. The amount of Raptor and Rictor helps to distinguish between the complexes, where this activity is realised at tissue level. Moreover, anti-p-4EBP1 and p-S6 (direct and indirect targets) of mTORC1 could also help to prove mTORC1 activity in situ. mTOR, mammalian target of rapamycin; mLST8, mammalian lethal with SEC13 protein 8 TORC subunit; Raptor, Regulatory-associated protein of mTOR; Rictor, Rapamycin-insensitive companion of mammalian target of rapamycin; Deptor, DEP domain-containing mTOR-interacting protein; PRAS40, proline-rich Akt substrate-40 kDa; FKBP12, 12 kDa FK506-binding protein; hSIN1, human Stress Activated Protein Kinase Interacting Protein 1; Protor, protein observed with Rictor; p70S6K, ribosomal S6 kinase-70 kDa; 4E-BP1, eIF4E-binding protein 1; SREBP, sterol regulatory element binding protein; S6, ribosomal S6 protein; SGK, serum- and glucocorticoid-regulated kinase; p, phosphate.

conventionally treated cases of colon carcinoma and to analyse the correlation between clinical data and mTORC1 and mTORC2 activity.

METHODS

Patients

Samples from 103 patients (53 men, 50 women) diagnosed with colorectal adenocarcinoma between 1996 and 2004 at the Semmelweis University were included in our study. All patients underwent surgery and received standard 5-fluorouracil (5-FU) and oxaliplatin combination treatment. The mean age of the patients was 62 years and the median age was 63 years (range 34–78 years). Median survival time was 77 months. Clinical data were available for a minimum follow-up period of 5 years; overall survival (OS) was followed for a 10-year period in all studied cases in our database. Adenocarcinomas were located in the colon (n=72) or the rectum (n=31). According to Dukes' classification (modified by Astler-Coller), 33 tumours were stage B2, 5 were stage C1, 56 were stage C2 and 9 were stage D.

Tissue microarray and immunohistochemistry

Formalin-fixed paraffin-embedded tissue blocks were reviewed and areas were designated for tissue microarray (TMA) blocks. Use of patient biopsy materials and all protocols were approved by the Institutional Ethical Review Board (TUKEB no. 7/2006). A minimum of two cores (2 mm) were selected from different areas of blocks and four TMA blocks were constructed (70 tissue cores/block). In some cases a third or fourth core and other control tissues such as normal colon and lymph node tissues were included.

Antigen retrieval was performed for 20–30 min in 10 mM citrate buffer (pH=6) or Targeted Retrieval Solution (Dako). Slides were incubated with primary antibodies (overnight at 4°C), followed by Novolink secondary detection system (Novocastra), diaminobenzidine substrate and haematoxylin counterstaining. The antibodies used for the characterisation and the proteins related to mTORC1 and mTORC2 complexes and their activity are summarised in figure 1 and table 1.

The intensity of the immunohistochemistry (IHC) reaction (0, negative; 1+, weak; 2+, moderate; 3+, strong positivity) was agreed upon before blind evaluation of the scores. The cut-off for positivity was set at 10% of tumour cells staining for the antibodies related to mTOR signalling based on other published recommendations.[16] Scoring was done by two independent pathologists. For phosphorylated-mTOR IHC, samples with a score of 2+ or 3+ were considered as tumours with high

Table 1 Primary antibodies (immunohistochemistry)

Detected protein	mTOR	p-mTOR	Raptor	Rictor	p-S6	p-4EBP1	p-AMPK
Source cat. #	CST #2983	CST #2976	Nov #110-57455	Bethyl #00429	CST #2211	CST #2855	CST #2535
Dilution	1:150	1:100	1:150	1:500	1:150	1:500	1:100
Retrieval	TRS	Citrate	TRS	Citrate	Citrate	TRS	Citrate

Antigen retrieval was performed for 20–30 min in 10 mM citrate buffer (pH=6) or TRS and was followed by incubation with indicated primary antibodies overnight at 4°C.
Bethyl, Bethyl Laboratories; CST, Cell Signalling Technology; Nov, Novus Biotechnology; TRS, Targeted retrieval Solution (Dako).

mTOR activity and samples with a score of 0 or 1+ were considered as tumours with low mTOR activity. In case of disagreement (less than 10%), a consultative discussion involving a third pathologist gave the final score. Raptor/Rictor dominant expression was determined based on staining intensity. To evaluate Rictor or Raptor dominance, one of the two proteins had to have a higher staining score (a difference of at least one score) to be considered dominantly expressed; their expression was considered balanced if staining intensity was equal. 3DHistech Pannoramic Viewer software and a Nikon E200 microscope were used to evaluate TMAs.

Case distribution was statistically analysed and compared with high/low mTOR activity and Rictor/Raptor expression in colon carcinomas according to Dukes' stage with χ^2 test. OS was estimated using the Kaplan-Meier method. Multivariable analysis of different factors was done using the Cox regression model. In these analyses, PAST software (free software downloaded from http://folk.uio.no) or SPSS software (Statistical Package for the Social Sciences, Chicago, Illinois, USA) were used; $p<0.05$ was considered statistically significant.

RESULTS

Immunohistochemical analysis of proteins indicative of mTOR pathway activity in colorectal carcinomas

Seven IHC reactions (mTOR, p-mTOR, p-S6, p-4EBP1, p-AMPK, Rictor and Raptor) were performed on 103 cases. The evaluation of p-mTOR staining showed high mTOR activity in 76 cases (73.8%) (table 2). p-4EBP1 and p70S6K are well-known direct target molecules of mTOR; however, p-S6 IHC staining proved to be the most sensitive and reliable marker of mTOR (especially mTORC1) activity in biopsies.[17] The co-expression patterns of p-S6, p-4EBP1 and p-mTOR were therefore carefully analysed in all samples.

Table 2 Case distribution related to mTOR activity, Rictor/Raptor expression and 5-year overall survival (OS) of different groups of patients with colorectal carcinoma

		mTOR activity	
	Cases	High	Low
Total	103 (100%)	76 (73.8%)	27 (26.2%)
OS >5 years	56 (54.4%)	35 (46.1%)	21 (77.8%)
Raptor/Rictor expression dominancy			
Rictor dominant expression	51 (49.5%)	39 (76.5%)	12 (23.5%)
OS >5 years	23 (45.1%)	16 (41%)	7 (58.3%)
Raptor dominant expression	14 (13.6%)	8 (57.1%)	6 (42.9%)
OS >5 years	10 (71.4%)	4 (50%)	6 (100%)
Balanced Rictor and Raptor expression	38 (36.9%)	29 (76.3%)	9 (23.7%)
OS >5 years	20 (52.6%)	12 (41.3%)	8 (88.9%)

Strong (2+/3+) cytoplasmic p-S6 staining intensity was found in 74 of the 76 high p-mTOR expressing cases and p-4EBP1 staining was also correlated well with these. Only three cases with high mTOR activity were negative for p-4EBP1; however, a strong correlation was detected between high p-S6 and p-mTOR expression in these samples. Therefore, these three were also considered as cases with high mTOR activity in the final evaluation. We found only two cases where high p-mTOR expression was accompanied by weak p-S6 and no p-4EBP1 staining. Moreover, weak p-S6 and p-mTOR staining (low mTOR activity) was detected in the other part of the samples (n=27). AMPK negatively regulates mTOR activity, which was supported by our cases as well. p-AMPK staining was positive in all tumour tissues with low mTOR activity and AMPK kinase activity was lacking in tumours with high mTOR activity. p-AMPK IHC positivity was detected only in one sample with high mTOR activity.

Presence of mTORC1 and mTORC2 complexes in colorectal carcinomas

To analyse the activities related to the two different complexes, samples were divided into distinct groups based on cytoplasmic Raptor and Rictor expression (characteristic of mTORC1 and mTORC2, respectively): (1) cases with dominant Rictor expression (n=51, 49.5%); (2) cases with dominant Raptor expression (n=14, 13.6%); and (3) cases with balanced Raptor and Rictor expression (n=38, 36.9%). Interestingly, the two cases characterised by high mTOR kinase activity (indicated by p-mTOR) and no mTORC1-related protein expression (ie, low p-S6 and no p-4EBP1) showed high dominance of Rictor expression, which suggests that mTOR activity was probably due to mTORC2 in these cases. Tumour cells with high mTOR activity showed dominant Rictor expression in 39 samples, balanced Raptor and Rictor expression in 29 cases and dominant Raptor expression in 8 cases (figures 2 and 3).

mTOR activity and complex distribution are independent prognostic factors in colorectal carcinoma

No correlation was found between mTOR activity and patient gender, age or tumour stage by statistical analysis (χ^2 test). However, the patients' survival data and IHC results showed that low mTOR activity significantly correlated with a good prognosis. OS at 5 years was 77.8% in the group with low mTOR activity and 46.1% in the group with high mTOR activity ($p<0.05$). Cases with low mTOR activity and dominant Raptor expression showed the best prognosis, and all these patients had a survival longer than 5 years. Cases with high mTOR activity and dominant Rictor or balanced complex expression showed the worst prognosis, with similar survival rates (5-year OS 41% and 41.3%, respectively). However, the group of patients with high mTOR activity and dominant Raptor expression carried a distinct prognosis: their OS was

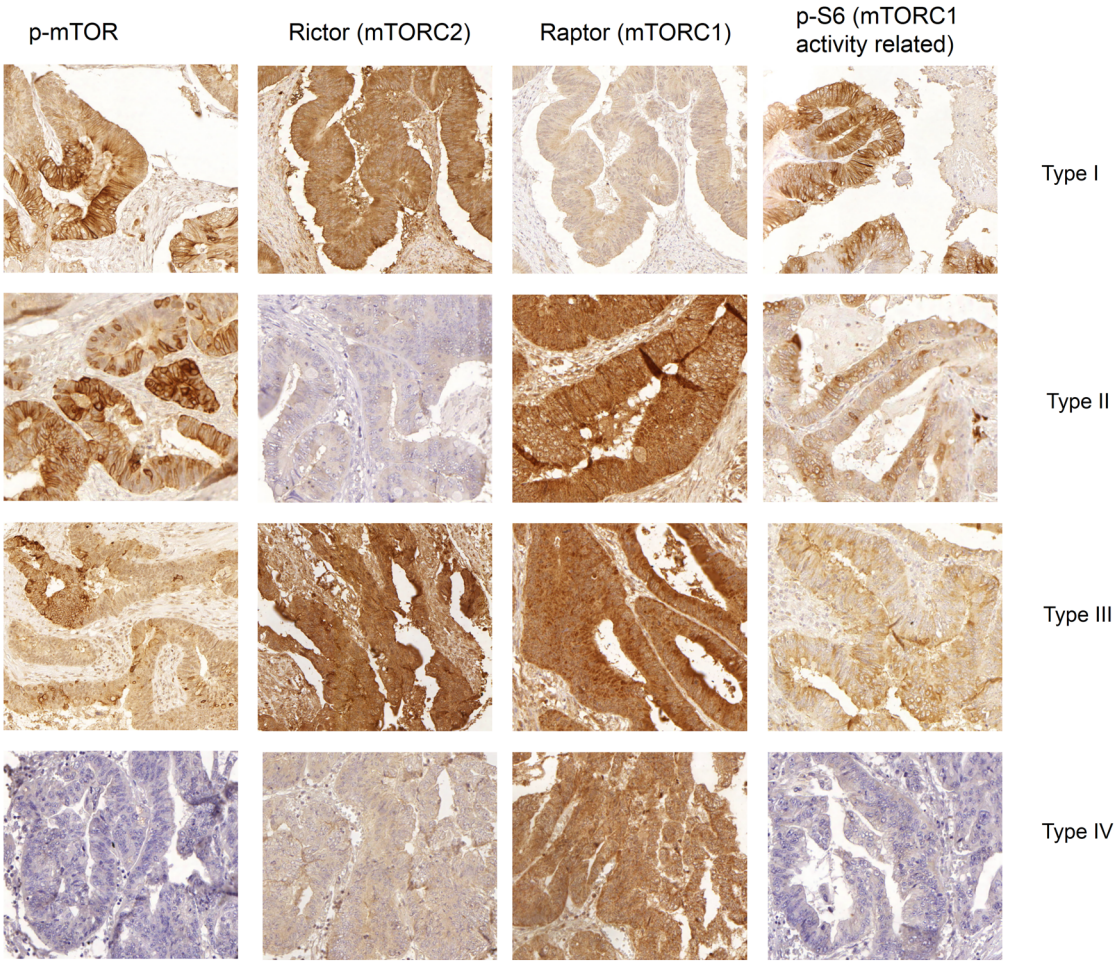

Figure 2 Rictor, Raptor, p-S6 and p-mTOR expression patterns in colon carcinomas. Type I: high mTOR activity with dominant Rictor expression, characteristic of mTORC2 complex activation expression (one representative sample from 39 cases). Type II: high mTOR activity with dominant Raptor expression, characteristic of mTORC1 complex activation (one representative sample from eight cases). Type III: high mTOR activity with balanced Raptor and Rictor expression (one representative sample from 29 cases). Type IV: low mTOR activity (one representative sample from 27 cases). (IHC; Zeiss, Axioscope 2 Plus, 400×).

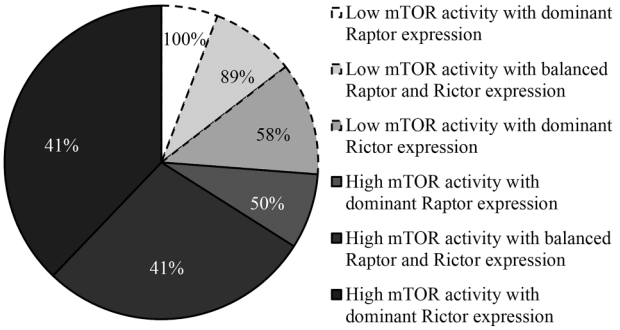

Figure 3 Patterns of mTOR activity in colon carcinoma samples and 5-year overall survival of patients. Overall survival data are given in percentages relative to the patient groups with different mTOR activity patterns.

better (50%) than for the other cases with high mTOR activity but worse than for patients with low mTOR activity (tables 2 and 3). Based on the expression pattern of mTORC1 and mTORC2 complex-related Rictor and Raptor proteins, dominant Rictor expression also seems to have a prognostic relevance as this group of patients had the worst prognosis (5-year OS 45.1%).

Kaplan-Meier analysis confirmed the correlation between worse OS and high mTOR activity accompanied by putative mTORC2 complex activity (ie, mTOR activity with Rictor dominant expression or balanced Rictor and Raptor expression). Our data suggest that these factors may potentially play a role in predicting therapeutic results and patient survival (figure 4). Cox regression analysis (including prognostic variables such as age, gender and histological grade) also indicated that high mTOR activity or high Rictor expression predicted a poor outcome, such as Dukes' stage. Cox regression analysis showed shorter survival increased independently of other variables such as age and gender. However, this analysis confirmed that high mTOR activity is an independent prognostic factor for short survival in patients with colorectal cancer. The results of HR and adjusted HR are displayed in table 4.

DISCUSSION

Previous studies have shown that about two-thirds of colorectal carcinomas display high mTOR activity.[18 19] Our detailed characterisation of the in situ expression of proteins related to the mTOR pathway showed high mTOR activity in 74% of the examined cases, and the evaluation confirmed stage- and grade-independent activity of mTOR signalling in colorectal carcinomas (60–77%). This corresponds well with other results for

Table 3 Case distribution of high/low mTOR activity and Rictor/Raptor expression in colon carcinomas according to Dukes' stage

	High mTOR activity (n=76)			Low mTOR activity (n=27)		
Dukes' stage	**Rictor**	**Balanced Rictor/Raptor**	**Raptor**	**Rictor**	**Balanced Rictor/Raptor**	**Raptor**
B2 (33 cases)	24 (72.7%) 13 (39.4%)	8 (24.3.6%)	3 (9.1%)	9 (27.3%) 4 (12.1%)	1 (3%)	4 (12.27%)
C1 (5 cases)	3 (60%) 3 (60%)	0	0	2 (40%) 1 (20%)	1 (20%)	0
C2 (56 cases)	43 (76.8%) 21 (37.5%)	17 (30.4%)	5 (8.9%)	13 (23.2%) 6 (10.8%)	3 (5.3%)	4 (7.1%)
D (9 cases)	6 (66.7%) 2 (22.2%)	4 (44.5%)	0	3 (33.3%) 1 (11.1%)	1 (11.1%)	1 (11.1%)

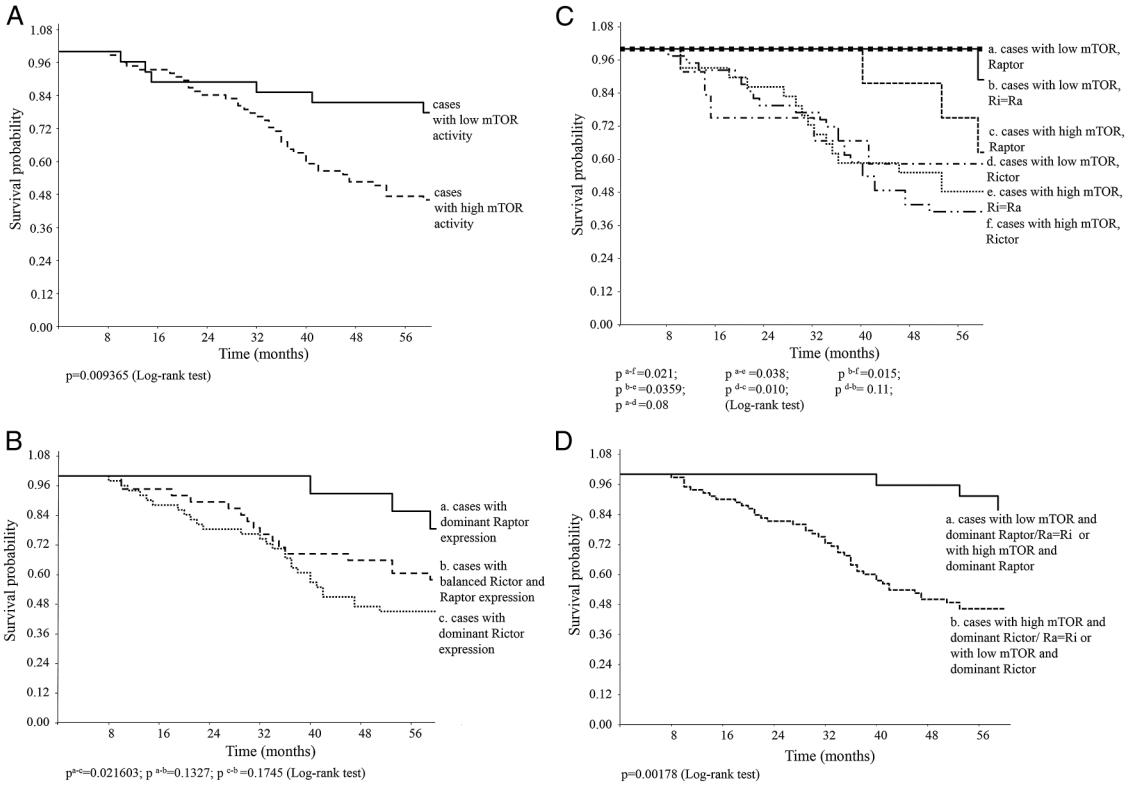

Figure 4 Survival (5-year overall survival) analysis of patients with colon carcinoma according to mTOR activity. (A) Low and high mTOR activity defines patient groups with good and poor survival, respectively (p<0.01). (B) Different mTORC1- and mTORC2-related protein expression patterns predict distinct 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). The statistical significance of the relevant compared pairs was added. (D) Pooling together groups with a similar prognosis (based on combined analysis of mTOR activity and the presence of mTORC1 and mTORC2 complexes as shown in C) segregates good and bad prognostic categories clearly (p<0.05).

stage IIIB colon carcinomas.[20] p-S6 IHC is one of the most sensitive markers of mTORC1 activity in paraffin-embedded tissues.[17] In our study, 97% of the cases with high p-mTOR expression showed high p-S6 expression and the remaining two cases—with low p-S6 expression and high p-mTOR IHC scores—were characterised by high Rictor expression, suggesting that mTOR activity could be related to mTORC2.

The detected high mTOR activity was present with dominant Rictor or balanced Raptor and Rictor expression in 66% of the studied tumours. Others reported mTORC2 activity in colon carcinomas based on p-Akt/Akt1–2 IHC.[19 21] There are several phosphorylation sites of Akt protein, which could be phosphorylated mTOR independently and mTORC2 phosphorylates Akt at Ser473 phosphorylation, earlier studies could not distinguish phosphorylation sites. Moreover, the instability of p-Akt proteins and the specificity of the previously used antibodies make these interpretations somewhat conflicting.[22] Our study is the first to distinguish mTORC1- and mTORC2-related mTOR activity in colon carcinomas. We determined the activity of mTOR kinase (p-mTOR) or mTORC1 (p-4EBP1, p-S6) and the expression of Raptor and Rictor—specific elements of mTORC1 and mTORC2 complexes—in the malignant cells. Our results show

Table 4 Results of Cox regression analysis

	HR (95% CI) p Value	HR[a] (95% CI) p Value
High mTOR expression	2.952 (1.329 to 6.558) 0.008	2.577 (1.145 to 5.803) 0.022
High Rictor expression	2.621 (0.994 to 7.274) 0.064	2.597 (0.923 to 7.311) 0.071
Dukes' stage	2.132 (1.118 to 4.069) 0.022	2.153 (1.125 to 4.118) 0.021
Age	0.996 (0.965 to 1.028) 0.796	1.007 (0.974 to 1.041) 0.701
Gender	1.517 (0.872 to 2.641) 0.14	1.446 (0.823 to 2.541) 0.2

Dukes' stage was defined as B1 and C1-C2-D. High Rictor expression was defined when Rictor ≥Raptor.
HR[a], adjusted HR (adjustment for all other factors included in the table).

that only one marker of mTOR activity or only p-Akt IHC is not sufficient for this distinction.

It was previously reported that mTOR mRNA or protein overexpression is a negative prognostic marker in colorectal cancer.[21 23 24] The presence of the active form and the activity related to the two different complexes—not only the expression—were determined and compared with the prognosis of patients who received conventional therapy in our study. Our analysis showed that high mTOR activity and Rictor overexpression could be markers of a bad prognosis. Combined phosphoprotein and Rictor/Raptor expression analysis revealed an even stronger statistical correlation with prognosis. Survival of patients with low mTOR activity along with dominant Raptor or balanced Rictor and Raptor expression, or with high mTOR activity along with dominant Raptor expression, was significantly longer than for the others (p=0.00178). Based on these results, we suggest testing for protein expression patterns related to mTOR activity for guiding prognosis.

Several authors propose that mTOR inhibitors may increase the effectiveness of treatments and may help overcome therapy resistance in different tumours.[1 25–27] The results of clinical trials show that mTORC1 inhibitors without combination are not sufficiently effective and do not make as great a clinical contribution as single agents.[28–31] It was shown that mTOR activity is frequently related to mTORC2 in colon cancers. However, rapamycin sensitivity of the mTORC2 complex is controversial,[32] and Rictor overexpression may predict low or no response against conventional rapalogs/mTORC1 inhibitors. Chemical improvements are aimed at promoting inhibitors with greater anticancer effects than rapalogs,[33] but the potential side effects of novel inhibitors and combinations should also be considered.[34–36] mTOR activity—especially related to the mTORC2 complex—has also emerged as a driving force behind intrinsic or acquired resistance to targeted drugs used in clinical practice such as HER2 or BRAF inhibitors. Several clinical trials have addressed this issue, and it would be interesting to investigate mTOR activity and Rictor expression in situ in these cases as well.[37 38] Rictor overexpression could also be interesting because Rictor has also been reported in focal adhesion complexes where it may enhance cellular survival and metastatic spread of tumour cells in an mTOR-independent manner.[39]

In summary, C1 and C2 complex-related mTOR activities were detected in colon carcinomas, which indicates that these activities can serve as targets for different therapies. The novelty of our results is that two-thirds of the examined cases also had mTORC2-related activity. Thus, recently completed clinical trials with mTOR inhibitors yielding low success rates or failure may need to be re-evaluated and mTOR activities related to different complexes should be determined before applying mTOR inhibitors.[40–42] Our results suggest that the accurate analysis of tumour tissues and their heterogeneity as well as the specification of mTORC1 and mTORC2 activity should be included in the study design. The IHC panel presented here could be appropriate for these purposes, and we highly recommend using p-mTOR, p-S6, Rictor and Raptor staining and scoring, which should be feasible in the routine diagnostic setting. This panel not only indicates the presence of the complexes to be targeted, which helps selection from different mTOR inhibitors, but provides information about prognosis which may guide decisions about the intensity of treatment. It would also be worthwhile to develop a new staining method for real quantitative analysis of mTORC1 and mTORC2 activity in formalin-fixed paraffin-embedded tissues (by Duolink, for example). Based on these, we suggest that all of the above have to be taken into account, and the success of personalised targeted therapy lies in understanding the relevant context and choosing the right target and therapeutic combination for the right patient.

Take home messages

- The majority of colorectal carcinoma cases have mTORC2 complex-related high mTOR activity.
- mTOR activity and the related complex distribution are independent prognostic factors in colorectal carcinomas.
- mTOR activity related to different mTOR complexes should be determined with p-mTOR, p-S6, Rictor and Raptor IHC staining and scoring before applying mTOR inhibitors in colorectal carcinomas.

Handling editor Cheok Soon Lee

Acknowledgements The authors would like to thank Tibor Krenács, Edit Pars, Zsuzsa Kaminszky and Titanilla Dankó for their technical help and the assistance of oncologists who treated and followed the patients.

Contributors TS collected the biopsy materials and the clinical data with the help of GV. TS performed the IHC stainings and examined the expression of mTOR-related proteins jointly with ÁM, NN and AM. The pathological evaluation was performed by TM, MH and LK. Statistical data analysis was performed by TS, ÁM and NN. AS designed, coordinated and supervised the study and prepared the manuscript, figures and tables with the help of LK, AM, MH and TS.

Funding OTKA (K84262), Hungarian Academy of Sciences (Bolyai and Medinprot support for AS).

Competing interests None declared.

Ethics approval All protocols were approved by the Institutional Ethical Review Board (TUKEB no. 7/2006).

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

1 Brown KK, Toker A. The phosphoinositide 3-kinase pathway and therapy resistance in cancer. *F1000Prime Rep* 2015;7:13.
2 O'Brien NA, McDonald K, Tong L, *et al*. Targeting PI3K/mTOR overcomes resistance to HER2-targeted therapy independent of feedback activation of AKT. *Clin Cancer Res* 2014;20:3507–20.
3 Laplante M, Sabatini DM. mTOR signalling in growth control and disease. *Cell* 2012;149:274–93.
4 Zaytseva YY, Valentino JD, Gulhati P, *et al*. mTOR inhibitors in cancer therapy. *Cancer Lett* 2012;319:1–7.
5 Harada K, Miyake H, Kumano M, *et al*. Acquired resistance to temsirolimus in human renal cell carcinoma cells is mediated by the constitutive activation of signal transduction pathways through mTORC2. *Br J Cancer* 2013;109:2389–95.
6 Meric-Bernstam F, Gonzalez-Angulo AM. Targeting the mTOR signalling network for cancer therapy. *J Clin Oncol* 2009;27:2278–87.
7 Wang XW, Zhang YJ. Targeting mTOR network in colorectal cancer therapy. *World J Gastroenterol* 2014;20:4178–88.
8 Benjamin D, Colombi M, Moroni C, *et al*. Rapamycin passes the torch: a new generation of mTOR inhibitors. *Nat Rev Drug Discov* 2011;10:868–80.
9 Zhang YJ, Duan Y, Steven Zheng XF. Targeting the mTOR kinase domain: the second generation of mTOR inhibitors. *Drug Discov Today* 2011;16:325–31.
10 Li J, Kim SG, Blenis J. Rapamycin: one drug, many effects. *Cell Metab* 2014;19:373–9.
11 Ferlay J, Soerjomataram I, Dikshit R, *et al*. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 2015;136:E359–86.
12 Kharaishvili G, Simkova D, Bouchalova K, *et al*. The role of cancer-associated fibroblasts, solid stress and other microenvironmental factors in tumour progression and therapy resistance. *Cancer Cell Int* 2014;14:41.
13 Colak S, Medema JP. Cancer stem cells—important players in tumour therapy resistance. *FEBS J* 2014;281:4779–91.
14 Ramos P, Bentires-Alj M. Mechanism-based cancer therapy: resistance to therapy, therapy for resistance. *Oncogene* 2015;34:3617–26.
15 Czarnecka AM, Kornakiewicz A, Lian F, *et al*. Future perspectives for mTOR inhibitors in renal cell cancer treatment. *Future Oncol* 2015;11:801–17.
16 Liu YH, Xu FP, Rao JY, *et al*. Justification of the change from 10% to 30% for the immunohistochemical HER2 scoring criterion in breast cancer. *Am J Clin Pathol* 2009;132:74–9.
17 Li S, Kong Y, Si L, *et al*. Phosphorylation of mTOR and S6RP predicts the efficacy of everolimus in patients with metastatic renal cell carcinoma. *BMC Cancer* 2014;14:376.
18 Wang D, Chen J, Guo F. Clinical significance of mTOR and p-mTOR protein expression in human colorectal carcinomas. *Asian Pac J Cancer Prev* 2011;12:2581–4.
19 Gulhati P, Cai Q, Li J, *et al*. Targeted inhibition of mammalian target of rapamycin signalling inhibits tumorigenesis of colorectal cancer. *Clin Cancer Res* 2009;15:7207–16.
20 Wen M, Li B, Cao X, *et al*. Clinical significance of aberrant mammalian target of rapamycin expression in stage IIIB colon cancer. *Oncol Lett* 2014;8:1080–6.
21 Johnson SM, Gulhati P, Rampy BA, *et al*. Novel expression patterns of PI3K/Akt/mTOR signalling pathway components in colorectal cancer. *J Am Coll Surg* 2010;210:767–76, 776–8.
22 Vassilakopoulou M, Parisi F, Siddiqui S, *et al*. Preanalytical variables and phosphoepitope expression in FFPE tissue: quantitative epitope assessment after variable cold ischemic time. *Lab Invest* 2015;95:334–41.
23 Alqurashi N, Gopalan V, Smith RA, *et al*. Clinical impacts of mammalian target of rapamycin expression in human colorectal cancers. *Hum Pathol* 2013;44:2089–96.
24 Wu S, Sun C, Tian D, *et al*. Expression and clinical significances of Beclin1, LC3 and mTOR in colorectal cancer. *Int J Clin Exp Pathol* 2015;8:3882–91.
25 Cheaib B, Auguste A, Leary A. The PI3K/Akt/mTOR pathway in ovarian cancer: therapeutic opportunities and challenges. *Chin J Cancer* 2015;34:4–16.
26 Sebestyén A, Márk Á, Hajdu M, *et al*. Rapamycin can restore the negative regulatory function of transforming growth factor beta 1 in high grade lymphomas. *Cytokine* 2015;73:219–24.
27 Martz CA, Ottina KA, Singleton KR *et al*. Systematic identification of signalling pathways with potential to confer anticancer drug resistance. *Sci Signal* 2014;7:ra121.
28 Pusceddu S, Tessari A, Testa I, *et al*. Everolimus in advanced solid tumours: when to start, early or late? *Tumouri* 2014;100:e2–3.
29 Costello BA, Borad MJ, Qi Y, *et al*. Phase I trial of everolimus, gemcitabine and cisplatin in patients with solid tumours. *Invest New Drugs* 2014;32:710–16.
30 Naruse T, Yanamoto S, Yamada S, *et al*. Anti-tumour effect of the mammalian target of rapamycin inhibitor everolimus in oral squamous cell carcinoma. *Pathol Oncol Res* 2015;21:765–73.
31 Francipane MG, Lagasse E. mTOR pathway in colorectal cancer: an update. *Oncotarget* 2014;5:49–66.
32 Sabatini DM. mTOR and cancer: insights into a complex relationship. *Nat Rev Cancer* 2006;6:729–34.
33 Zhang J, Jiang W, Liu W, *et al*. Effective targeting of colorectal cancer cells using TORC1/2 kinase inhibitors in vitro and in vivo. *Future Oncol* 2016;12:515–24.
34 Hassan B, Akcakanat A, Sangai T, *et al*. Catalytic mTOR inhibitors can overcome intrinsic and acquired resistance to allosteric mTOR inhibitors. *Oncotarget* 2014;5:8544–57.
35 Yang H, Rudge DG, Koos JD, *et al*. mTOR kinase structure, mechanism and regulation. *Nature* 2013;497:217–23.
36 Willemsen AE, Grutters JC, Gerritsen WR, *et al*. mTOR inhibitor-induced interstitial lung disease in cancer patients: comprehensive review and a practical management algorithm. *Int J Cancer* 2016;138:2312–21.
37 Mohd Sharial MS, Crown J, Hennessy BT. Overcoming resistance and restoring sensitivity to HER2-targeted therapies in breast cancer. *Ann Oncol* 2012;23:3007–16.
38 Johnson GL, Stuhlmiller TJ, Angus SP, *et al*. Molecular pathways: adaptive kinome reprogramming in response to targeted inhibition of the BRAF-MEK-ERK pathway in cancer. *Clin Cancer Res* 2014;20:2516–22.
39 Liu L, Chen L, Chung J, *et al*. Rapamycin inhibits F-actin reorganization and phosphorylation of focal adhesion proteins. *Oncogene* 2008;27:4998–5010.
40 Wainberg ZA, Soares HP, Patel R, *et al*. Phase II trial of everolimus in patients with refractory metastatic adenocarcinoma of the esophagus, gastroesophageal junction and stomach: possible role for predictive biomarkers. *Cancer Chemother Pharmacol* 2015;76:61–7.
41 Park JH, Ryu MH, Park YS, *et al*. Successful control of heavily pretreated metastatic gastric cancer with the mTOR inhibitor everolimus (RAD001) in a patient with PIK3CA mutation and pS6 overexpression. *BMC Cancer* 2015;15:119.
42 Chiarini F, Evangelisti C, McCubrey JA, *et al*. Current treatment strategies for inhibiting mTOR in cancer. *Trends Pharmacol Sci* 2015;36:124–35.

mTOR activity and its prognostic significance in human colorectal carcinoma depending on C1 and C2 complex-related protein expression

Tamás Sticz, Anna Molnár, Ágnes Márk, Melinda Hajdu, Noémi Nagy, Gyula Végso, Tamás Micsik, László Kopper and Anna Sebestyén

J Clin Pathol 2017 70: 410-416 originally published online October 11, 2016
doi: 10.1136/jclinpath-2016-203913