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Original Article

Expression of phospho-mTOR kinase is abundant in colorectal cancer and associated with left-sided tumor localization

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Abstract: Purpose: To investigate the significance of mammalian target of rapamycin (mTOR) in colorectal cancers. mTOR has recently been suggested as a prognostic biomarker and therapeutic target in an array of human cancers. Findings: phospho-mTOR (p-mTOR) expression was analyzed by immunohistochemistry (IHC) on a tissue microarray containing 1800 colorectal cancers (CRC). Clinical follow-up data were available from all cancer patients. Positive p-mTOR immunostaining was seen in 83.5% of 1640 interpretable CRC and was considered weak in 862 (52.5%) and strong in 508 cases (31.0%). Matching clinico-pathological parameters were available in 1580 cases. p-mTOR staining was more frequent in tubular adenocarcinomas than in the less common histological subtypes (mucinous, medullary, signet cell; *P*=0.0163) and significantly linked to carcinomas of the left-sided colon and rectum as compared to right-sided CRC (*P*=0.0066). There was no significant association between p-mTOR expression and patients' gender, tumor stage, tumor grade or nodal status. In a survival analysis, p-mTOR IHC status of all CRC was unrelated to patient survival (*P*=0.702). In a multivariate analysis including pT, pN, tumor grade, tumor localization and p-mTOR expression, only pT, pN (both *P*<0.0001) and grade (*P*=0.0001) showed prognostic impact, but not tumor localization (P=0.9472) or p-mTOR expression (*P*=0.8879). Conclusion: Our observations indicate that p-mTOR overexpression is abundant in CRC and linked to left-sided tumor localization. The high frequency and overexpression of p-mTOR is providing further rationale for targeting this pathway therapeutically in CRC patients. However, a prognostic role of p-mTOR overexpression in CRC could not be confirmed.

Keywords: Colorectal cancer, phospho-mTOR, tissue microarray, immunohistochemistry

Introduction

Colorectal cancer (CRC) is the fourth most common malignant disease with over one million novel cases and over 500.000 deaths each year worldwide [1]. Although recent advances in the management of the disease have improved outcomes, CRC remains the second leading cause of cancer-related death in Western countries [1]. In advanced metastatic colorectal cancer (mCRC), surgery alone is not curative and therefore adjuvant chemotherapy is needed. New anticancer drugs have improved the standard chemotherapy treatment of CRC and there is much promise in molecular-targeted therapy.

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that belongs to the phosphoinositide 3-kinase (PI3 K)-related kinase family. It constitutes the core of an evolutionarily conserved pathway that regulates cell growth and proliferation in normal human cells [2, 3]. Moreover, mTOR signaling activity is associated with cancer cell growth and survival [4, 5]. Abnormal mTOR expression has been described in a variety of human tumors [6-8] and was found to be associated with poor prognosis [9-11]. In CRC, mTOR overexpression is likely to be involved in the development and progression of the disease and is linked to cancer initiation, invasiveness, and progression [12]. Recently, inhibitors of proteins that are

Table 1. Hospho-mTOR (p-mTOR) immunohistochemistry and clinico-pathological features

			p-mTOR			
			Negative	Weak	Strong	
		N	%	%	%	*P* value
Gender	Male	794	17.4	49.9	32.7	
	Female	786	15.2	55.0	29.8	0.1225
Tumor grade	G1	25	8.0	44.0	48.0	
	G2	1393	15.9	52.9	31.2	
	G3	162	22.2	49.4	28.4	0.1050
Tumor stage	Pt1	68	8.8	50.0	41.2	
	Pt2	255	15.3	52.9	31.8	
	Pt3	1019	16.4	52.1	31.5	
	Pt4	238	19.7	53.8	26.5	0.1972
Nodal status	Pn0	816	15.8	54.0	30.2	
	Pn1	428	16.4	49.8	33.9	
	Pn2	336	17.9	51.8	30.3	0.5672
Tumor type	Tubular	1526	15.9	52.2	31.9	
	Others	54	30.8	57.7	11.5	0.0163
Localization	Coecum, ascending	383	19.1	51.4	29.5	
	Transverse	137	27.7	41.6	30.7	
	Descending	67	19.4	49.5	31.3	
	Sigmoid	410	15.1	54.4	30.5	
	Rectum	582	12.5	54.5	33.0	0.0066
	Total	1580				

involved in mTOR signaling have been under active preclinical or clinical investigation for cancer therapy [13-19].

To further expand our knowledge on the relevance of (phospho-) mTOR (p-mTOR) expression in CRC, we analyzed a tissue microarray including a series of 1800 cancers with clinical follow up and extensive molecular data. Since mTor phosphorylated at Ser 2448 (p-mTOR Ser 2448) is the activated variant of mTOR, we performed p-mTOR immunostaining on our TMA and evaluated the correlation of the activated protein with clinico-pathological parameters.

Material and methods

Patients and tissue microarray (TMA) construction

Two different TMAs with a total of 1800 CRC samples were included in this study. The first TMA was manufactured from resection specimens of 1420 CRC patients at the Institute of Pathology of the University Hospital of Basel). None of the patients received neo-adjuvant or adjuvant therapy. Raw survival data were obtained from the responsible physicians for all of the 1420 patients. The median follow up time was 46 months (range 1-152 months). The second TMA included samples from 380 CRC patients, whose tumor resection specimens were examined at the Institute of Pathology of the University Medical Center, Hamburg-Eppendorf. Also for this TMA, raw survival data were available for all of the 380 patients with a median follow up period of 36 months (range 1-179 months). TMA construction was as described [20]. In brief, hematoxylin and eosin-stained sections were made from each block to define representative tumor regions. Tissue cylinders with a diameter of 0.6 mm were then punched from tumor areas of each "donor" tissue block using a home-made semi-automated precision instrument and brought into empty recipient paraffin blocks. Four µm sections of the resulting TMA blocks were transferred to an adhesive coated slide system (Instrumedics Inc., Hackensack, New Jersey). Patient information and clinical data such as age, sex, localization and type of the tumor, pTNM-stage and carcinoma grade were retrospectively retrieved from clinical and pathological databases (**Table 1**). All tumors were re-classified by two pathologists (PS, AM). Follow-up data were obtained from local cancer register boards or via attending physicians. For statistical analyses, tumor localizations were grouped as follows: right-sided cancer (cecum, ascending colon), cancer of the transverse colon, cancer of the left-sided colon (descending colon, sigmoid colon) and rectum. The utilization of tissues and clinical data was according to the Hamburger Krankenhaus Gesetz (§12 HmbKHG) and approved by our local Ethical Committee.

Immunohistochemistry

Freshly cut TMA sections were immunostained on one day and in one experiment. Slides were

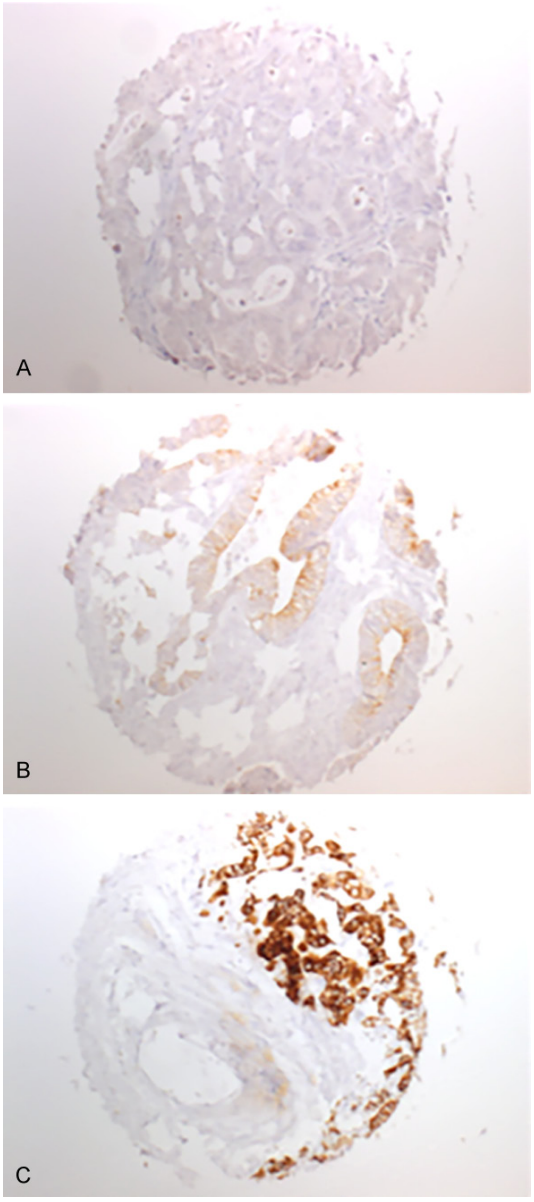

Figure 1. TMA samples of colorectal cancers showing negative (A), weak (B) and strong (C) immunostaining for p-mTOR (magnification 50×).

deparaffinized and exposed to heat-induced antigen retrieval for 5 minutes in an autoclave at 121°C in pH 2.0 in target retrieval solution (Biogenex, San Ramon, USA). Primary antibody specific for mTOR phosphorylated at Ser2448 (rabbit polyclonal phospho-mTOR antibody Ser2448; Cell signaling technology, Germany, Frankfurt am Main; dilution 1:150) was applied at 37°C for 60 minutes. Bound antibody was then visualized using the EnVision Kit (Dako, Glostrup, Denmark) according to the manufacturer's directions. Only cytoplasmic staining was considered. The staining results were categorized into three groups. Tumors without any staining were considered p-mTOR "negative". Tumors with 1+ or 2+ staining in up to 50% of cells or 3+ staining in up to 20% of cells were considered "weakly positive". Tumors with 2+ staining in >50% or 3+ staining in >20% were considered "strongly positive". This categorization was also used in an earlier study of our group [21].

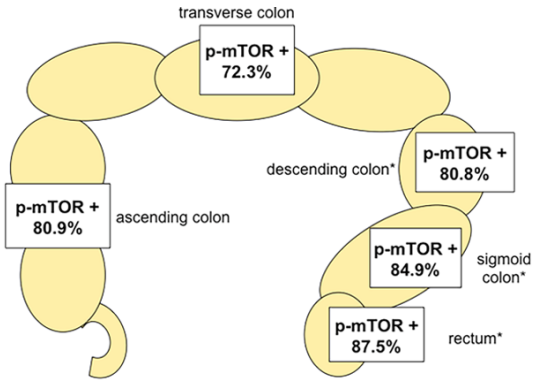

Figure 2. Positive p-mTOR immunostaining (weak and strong) is significantly related to left-sided tumor localization, *P=0.0066.

Statistics

Statistical calculations were performed with JMP® 10.0.2 software (2012 SAS Institute Inc., NC, USA). Contingency tables and the chi²-test were performed to search for associations between molecular parameters and tumor phenotype. Survival curves were calculated according to Kaplan-Meier. The Log-Rank test was applied to detect significant survival differences between groups. Cox proportional hazards regression analysis was performed to test the statistical independence and significance between pathological and clinical variables.

Results

Phospho-mTOR-immunohistochemistry

A total of 116 of 1800 tissue samples (6.4%) were non-informative due to either absence of unequivocal cancer tissue, uninterpretable staining or complete loss of tissue. Normal colorectal epithelial cells typically did not show any staining for p-mTOR. Positive immunostaining was seen in 83.5% of 1640 interpretable CRC. Immunostaining was typically cytoplasmic. Representative examples of p-mTOR stained cancers are shown in **Figure 1**. p-mTOR

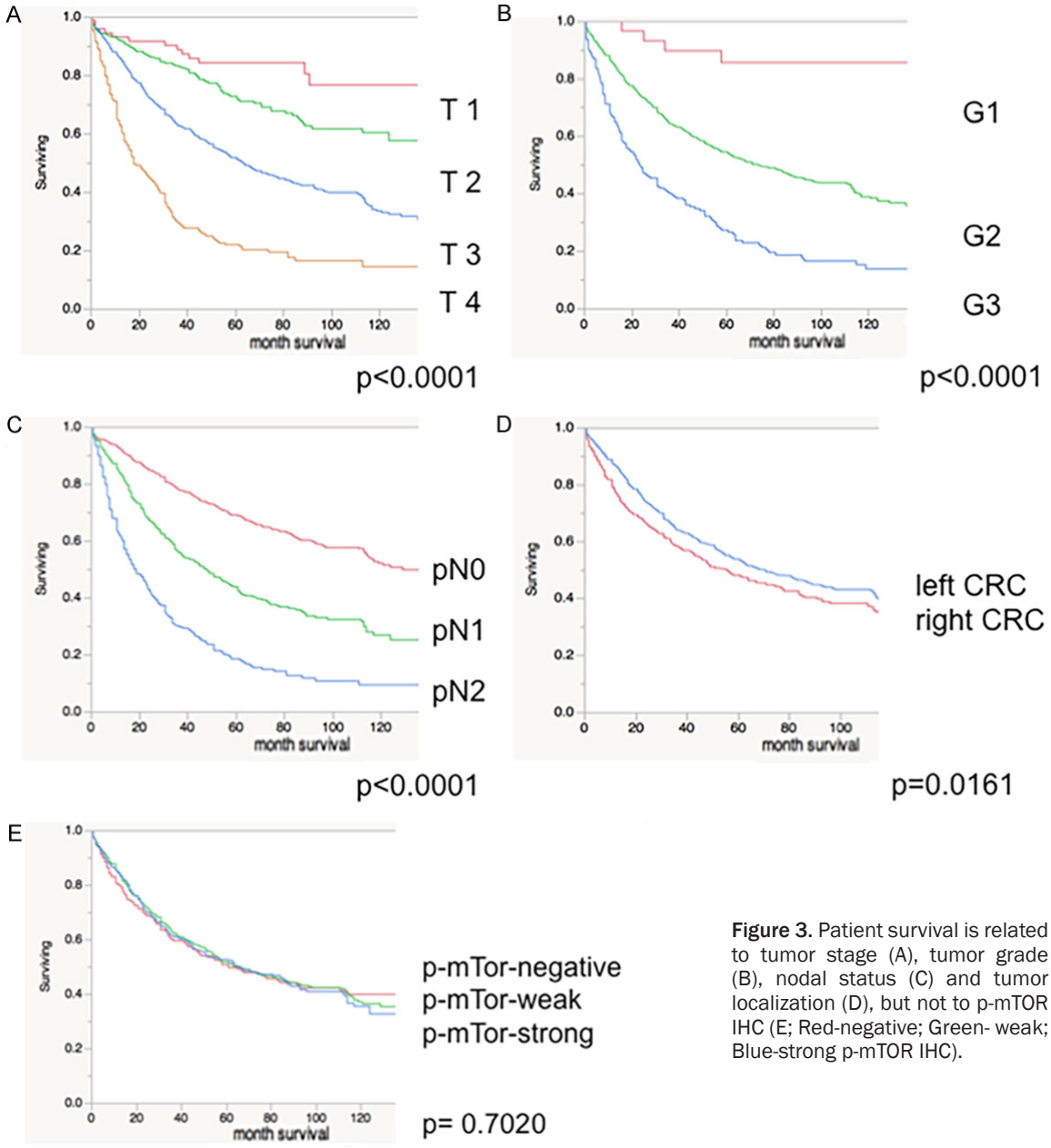

Figure 3. Patient survival is related to tumor stage (A), tumor grade (B), nodal status (C) and tumor localization (D), but not to p-mTOR IHC (E; Red-negative; Green- weak; Blue-strong p-mTOR IHC).

staining was considered weak in 862 (52.5%) and strong in 508 cases (31.0%). Clinicopathological parameters in association with p-mTOR IHC results were available for 1580 cases. The relationship between p-mTOR staining, tumor phenotype and clinical parameters is shown in **Table 1**. p-mTOR staining was more frequent in tubular adenocarcinomas than in the less common histological subtypes (mucinous, medullary, signet cell; P=0.0163, **Table 1**). Moreover, p-mTOR expression levels were significantly related to the tumor localization. p-mTOR expression was more frequent in carcinomas of the sigmoid colon (84.9%) and rectum (87.5%) as compared to right-sided CRC (80.9%, **Table 1**; **Figure 2**; *P*=0.0066). There was no significant association between p-mTOR expression and patients' gender, tumor stage, tumor grade or nodal status (**Table 1**).

Survival analysis

As expected, high tumor grade and stage as well as advanced nodal status were associated with poor patient survival (**Figure 3A-C**; P< 0.0001 each) while histological tumor type was

Table 2. Multivariate analysis of phospho-mTOR expression in colorectal cancer

N Analyzable	*P*-value				
	pT	pN	Grading	Tumour localization	p-mTOR expression
1580	<0.0001	<0.0001	0.0001	0.9472	0.8879

unrelated to clinical outcome (*P*=0.6279, Figure not shown). Left-sided CRC (distal to the splenic flexure) was associated with a better prognosis (*P*=0.0161; **Figure 3D**) as compared to cancers of the right-sided colon. p-mTOR IHC status of all CRC was unrelated to patient survival (*P*=0.7020; **Figure 3E**). These associations also held true in the subset of tubular carcinomas only (data not shown).

Multivariate analysis

In a multivariate analysis including pT, pN, tumor grade, tumor localization and p-mTOR expression only pT, pN (both *P*<0.0001) and grade (*P*=0.0001) showed prognostic impact, but not tumor localization (*P*=0.9472) or p-mTOR expression (*P*=0.8879; **Table 2**).

Discussion

The results of this study show that p-mTOR (Ser2448) is abundant in CRC (83.8%) and that it is associated with left-sided tumor localization.

The high frequency of p-mTOR found in this study is comparable to previous results. Wang et al. described about 61% p-mTOR positive CRC [22]. Interestingly, Cai et al. had previously reported that p-mTOR overexpression is predictive of poor outcome in stage II CRC [23]. In contrast, we could not find a significant association between p-mTOR expression and prognosis, despite other features, such as tumor grade and tumor stage, being linked to outcome in this patient cohort. This observation held true when only stage II CRC and p-mTOR expression was examined (data not shown). However, the differences in p-mTOR expression between tumors of different grade and stage were quite small in terms of absolute numbers. The strong significance seems to be caused by the high number of analyzed cases in our study (n=1580) providing strong statistical power. The strong association between classical prognostic features such as tumor stage, tumor grade and nodal status and prognosis in our patent set provide indirect proof for the validity of our clinical data. Therefore, we believe that our findings argue against a clinical utility of p-mTOR expression as a prognostic biomarker in colorectal cancer patients.

Interestingly, p-mTOR expression levels were significantly higher in left-sided than in right-sided CRC (**Table 2**; **Figure 2**). This finding obtains support in a study by Alqurashi et al showing that high levels of mTOR RNA were found more frequently in left-sided CRC [12]. The reason for this observation is unclear. It could be hypothesized that pathways leading to increased p-mTOR expression are less often activated in patients with hereditary-non-polyposis colon cancer syndromes (HNPCC) or sporadic colorectal cancers exhibiting microsatellite instability (MSI), which are more often localized in the right colon. This observation does not rule out other causes for the left colon preference of p-mTOR. CRCs that arise on the left or right side exhibit substantial differences in gene expression and signal transduction patterns. In fact, it is expected that every alteration occurring more frequently in the left colon than in the right colon should be statistically associated with lower MSI frequency, as MSI is known to preferably occur in the right colon [24].

Based on the high frequency of p-mTOR positivity seen in this study and the concordance of our results with previous data, it appears unlikely, that our TMA-based approach, analyzing only limited amounts of tumor tissue per patient (1 spot of 0.6mm diameter) has led to a significant number of false negative cases.

The high frequency and overexpression of p-mTOR provides further rationale for targeting this pathway therapeutically in CRC patients. It has been reported that concomitant BRAF and PI3K/mTOR blockade is required for effective treatment of BRAF (V600E) colorectal cancer [25]. Recently, it has been suggested that combinations of drugs targeting BRAF (and/or MEK) and eIF4F-a downstream complex of the PI (3) K-AKT-mTOR pathway-may overcome most of the resistance mechanisms arising in BRAF (V600E)-mutant cancers, including CRC [26]. Positive results with mTOR antagonists in renal carcinoma and several lymphoma subtypes have recently led to the initiation of a variety of

new clinical trials with other types of tumors, including gastric, endometrial, and non-small cell lung cancer as well as sarcoma and neuroendocrine tumors [27]. Regarding CRC, the treatment with everolimus, an oral inhibitor of mTOR, showed efficacy in patients with metastatic colorectal cancers in phase I studies [28, 29]. In a phase II study, among patients receiving everolimus, those with a KRAS mutation experienced significantly shorter median overall survival compared with those with wild-type KRAS [30]. In another phase II study, the oral combination of tivozanib and everolimus was well tolerated, with stable disease achieved in 50% of patients with refractory, metastatic colorectal cancer [31]. Future studies may be needed evaluating everolimus in combination with other agents or in patients with dysregulation of the PI3K/Akt/mTOR pathway.

Although the presence of a target on tumor cells does not guarantee successful therapy by itself, our data at least indicate that mTOR might play a functional role in CRC and could potentially identify patients benefitting from therapy with mTOR antagonists.

In summary, our data show that p-mTOR expression occurs frequently in CRC with a clear preference for left-sided tumors. The high frequency and overexpression of p-mTOR provides further rationale for targeting this pathway therapeutically in CRC patients. Despite a relationship between p-mTOR expression and unfavorable tumor phenotype, previous reports on a possible prognostic role of p-mTOR in CRC could not be confirmed.

Disclosure of conflict of interest

None.

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