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ORIGINAL ARTICLE

The role of the PTEN/mTOR axis in clinical response of rectal cancer patients

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Abstract

Background Preoperative chemoradiotherapy has long been accepted as a method to improve survival and lifetime quality of rectal cancer patients. However, physiologic effects of these therapies largely depend on the resistance of cells to the radiation, type of chemotherapeutic agents and individual responses. As one of the signaling cascades involved in chemo- or radiation- resistance, the present study focused on several proteins involved in pTEN/Akt/mTOR pathway to explore their prognostic significance.

Materials and methods Samples from advanced stage rectal cancer patients were analyzed to detect expression levels of pTEN/Akt/mTOR pathway related proteins pTEN, mLST8, REDD1, BNIP3, SAG and NOXA, together with p53, by RT-qPCR. Kaplan–Meier analysis was used to assess expression-survival relation and correlations among all proteins and clinicopathological features were statistically analyzed.

Results. Except p53, none of the proteins showed prognostic significance. High p53 expression presented clear impact on overall survival and disease free survival. It was also significantly related to pathologic complete response. p53 showed high correlation to local recurrence as well. On the other hand, strong correlation was observed with PTEN expression and tumor response, but not with survival. High associations were also observed between mLST8/REDD1, PTEN and NOXA, confirming their role in the same cascade.

Conclusion The contentious role of p53 as a prognostic biomarker in colorectal cancer was further affirmed, while PTEN and REDD1 could be suggested as potential candidates. Additionally, NOXA emerges as a conjunctive element for different signaling pathways.

Keywords Rectum cancer · PTEN · p53 · mTOR · mLST8 · REDD1 · NOXA

Introduction

Rectal cancer is one of the most common types of cancer all over the world. It is curable by surgery if it is localized within the wall of the colon. However, surgical success is limited in advanced stages and risk of local recurrences is high. Recently the treatment of locally advanced rectal cancer has largely changed to total neoadjuvant therapy that incorporates chemotherapy with chemoradiotherapy at different schedules preoperatively, although long course radiotherapy with concomitant chemotherapy antecedent to surgery is still one of the widely used standard treatments. This multimodal approach has substantially improved local control and reduced metastatic rates. However, survival rates are still far from expected and responses are highly variable among individuals. Therefore, predictive factors are prerequisites to estimate patients' responses and to prevent undesirable side effects.

Various approaches are being used to increase life expectancy and to manage better prognosis. Genes responsible for oncogenesis, tumor suppression, cell cycle and apoptosis are molecular targets to estimate responses to radio- and/or chemo-therapy applications. In concern of genetic predictive markers, our knowledge is very limited. Genes influencing

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rectal cancer progress and response involve several classic actors like p53 or KRAS, but also many relatively new alterations observed in e.g. APC (Wnt-pathway), BRAF, PI3KCA, PTEN (RTK pathway) or SMAD4 (TGF-β signaling) [1, 2].

The PI3K/PTEN signaling pathway is a potential candidate to predict response to therapy, especially for EGFR monoclonal antibodies [3]. PTEN is a tumor suppressor gene encoding a lipid phosphatase that dephosphorylates inositol phosphates and negatively modulates cell growth through the Akt/mTOR axis. Akt has been the focus of intense research in this context, especially for the last decade, since our comprehension about its physiological role expanded to a wide range of pathologies, involving tumor initiation and development in many types of cancer. The main regulator of this system is an upstream phosphorylase to Akt, PTEN, which can stop signaling by removing 3'-phosphate groups. A study reported that PTEN expression detected using immunohistochemical (IHC) staining in 45 colorectal cancer (CRC) patients showed extremely low levels of protein in CRC tissues compared to normal tissues collected from the same subjects [4]. When cytosolic inactive Akt becomes activated, it is recruited to the membrane and bound to PIP3 through its PH domain. Maximal activation requires Ser473 phosphorylation, managed by the kinase activity of mTORC2 complex [5]. Modulation of Akt activity also involves ubiquitylation through multiple lysine residues by E3 ubiquitin-ligases [6, 7].

Apoptosis is another important factor regarding sensitivity or resistance of cells to radiation, therefore it is the major concern of many studies targeting to predict individual responses against different therapy regimens.

The effect of apoptosis results from a balance between pre- and post- apoptotic factors. Our previous studies had focused on the clinical significance of an anti-apoptotic protein, sensitive-to-apoptosis gene (SAG), which belongs to SCF E3 ubiquitin ligases family [8, 9]. It was shown that SAG silencing in different cancer cell lines induces apoptosis via induction of NOXA accumulation, a small, 103 amino acid protein, with a Bcl-2 homology 3 (BH3) motif, characteristic of BH3-only pro-apoptotic proteins [10]. The E3 ubiquitin ligases are considered one of the major components of the ubiquitin—proteasome system. Their high selectivity and specificity for the target substrates and essential roles in cell growth, proliferation, apoptosis and cell cycle regulation make them attractive subjects in cancer research [11].

Several groups reported that the SCF E3 complex also has a role in the ubiquitination and degredation of DEPTOR, a subunit of mTORC1/2 complexes [12, 13]. Since activation of the mTOR signaling pathway is frequently found in cancers, modulation of mTOR complexes, involving the Akt/PTEN pathway, negative regulators of DEPTOR, HIF-1α, REDD1, and modifications by SCF E3 ligases could provide new perspectives to estimate possible outcomes.

Based on these findings, here in this study, we examined gene expression profiles of several proteins related to mTOR and SCF E3 ligase complexes to elucidate prognostic potential of these proteins and their correlation with life expectancies in advanced stage CRC patients. SAG and NOXA as well as mLST8, REDD1, PTEN and p53 gene expression patterns were investigated in a group of patients through a follow-up process up to 10 years (Supplementary Fig. S1). In addition, grouped analyses and correlations were also assessed to understand possible involvements of different interactions on disease progression.

Methods

Patients and therapy

Forty-six patients who were admitted to the Dr. Lutfi Kirdar Kartal Training and Research Hospital for preoperative L5-pelvic chemoradiation with the diagnosis of locally advanced rectal cancer were included in this study. The study was approved by Marmara University School of Medicine Ethics Board and written informed consent was obtained from all patients. Biopsy samples had been taken from the tumor and adjacent normal rectal tissues during the diagnostic procedure. Biopsy specimens were stored at − 80 °C until the day of RNA extraction. Prior to the start of treatment, all patients underwent complete clinical examinations, involving blood counts, liver and renal function tests, and tumor markers. Abdominal-pelvic magnetic resonance imaging (MRI) or computed tomography (CT) was used for clinical staging and supplemented with transrectal ultrasound when needed. Additionally, lung X-rays or chest tomography were evaluated before the start of treatment. Patients were followed up with physical examination and blood examination, abdominopelvic MRI or CT every 3 months for the first 2 years, every 6 months for up to 5 years, and then annually. Follow-up periods of 3-, 5- and 10 years were evaluated for survival analyses with median follow-up time of 6 years.

External beam radiation therapy (EBRT)

All patients were treated preoperatively with long course preoperative chemoradiotherapy (CRT) between the dates 2006 and 2008. EBRT was applied on to the L5-Pelvis field to a total dose of 45–50 Gy in 5 weeks with a four- field technique (AP-PA and lateral opposed fields), using GE Saturn 41 S-700 linear accelerator with 15 MV photon energy. Patients were treated once a day, 5 days a week with a fraction size of 1.8–2 Gy.

Chemotherapy

Chemotherapy was administered as a daily intravenous bolus with fluorouracil and calcium folinate with doses 320–400 mg/m^2/day and 20 mg/m^2/day for 5 days respectively in the first and last weeks of radiotherapy as described previously [8]. At the end of CRT application, patients were scheduled for 4–6 weeks resting period and presented for surgery.

RNA isolation and quantitative real-time PCR (qPCR)

Total RNA from frozen tissues was extracted with the RNeasy Plus Mini Kit (Qiagen). Integrity and purity of RNA samples were tested in spectral readings (260/280 ratio) and genomic DNA contamination was tested by PCR analysis. cDNA synthesis was performed using first Strand cDNA synthesis kit (Roche) according to the manufacturer's instructions (Roche) as described previously [8, 9]. The expressions of all genes were measured by qPCR method using SYBR Green dye in LightCycler 96 system (Roche). Primers used in this study were: β-actin forward 5′-CTG TGC TGT CCC TGT ATG CC-3′ and reverse 5′-GTG GTG GTG AAG CTG TAG CC-3′; mLST8 forward 5′-GGG GAC TCC CAG TAC ATC G-3′ and reverse 5′-TCC AGT CTC CAC ACA CCA GA-3′; REDD1 forward 5′-CCT TTG GGA CCG CTT CTC-3′ and reverse 5′-ATC TGG GGT GGG AGT TCG-3′; PTEN forward 5′-CGA AGC CAT CTT GAA CAC AA-3′and reverse 5′-GTT GCT TGG GAC CTC TCT TG-3′; SAG forward 5′-CGG GAT CCA TGG CCG ACG TGG AAG-3′and reverse 5′- CGA AGC TTT CAT TTG CCG ATT CTT TGG AC-3′; NOXA forward 5′-TGG AAG TCG AGT GTG CTA CTC AA-3′ and reverse 5′-CAG AAG AGT TTG GAT ATC AGA TTC AGA-3′; BNIP3 forward 5′-GCA CAA CAT GAA TCA GGA CAG -3′ and reverse 5′-CAT CTT CTT GTG GCG AAG G-3′; P53 forward 5′-AGG CCT TGG AAC TCA AGG AT-3′ and reverse: 5′-CCC TTT TTG GAC TTC AGG TG-3′.

All reactions were tested by at least two independent experiments, each duplicate, and a third when required. The expression levels were normalized to beta-actin and relative expression analyses and fold-change calculations were accomplished using $2^{-\Delta\Delta CT}$ method. Results were also compared with data evaluation online system provided at Qiagen web site for customers and only double-checked confirmations were chosen for statistical analyses.

After defining two groups relative to median values ("high" if greater than median; "low" if it is smaller or equal to median), expression of all proteins were further investigated for their association with overall and disease free survival times (OS and DFS) or pathological response to understand their prognostic relevance.

Statistical analyses

Survival curves were established according to Kaplan–Meier method (log-rank) and Cox regression analysis was used to evaluate the effects of individual parameters on survival rates. The comparison and correlations between expressions were analyzed using *t*-test and Spearman's correlation analysis. All tests were two-sided and yields were accepted as significant whenever $p < 0.05$. The SPSS v.16 package (SPSS Inc., Chicago, Illinois) and Excel software for Windows (Microsoft Office Professional Plus 2016) were used for statistical analyses.

Results

Clinical characteristics of patients

Demographic and clinico-pathological characteristics of patients are presented at Table 1. Expression profiles of 46 patients (26 males, 20 females) who underwent preoperative CRT therapy and ensuing surgical intervention were inspected for the genes of interest (GOI). Pathological complete response was noted in six patients (13%). Two patients who refused the operation were accepted as pathological complete response in statistical calculation because they had a clinical complete response and also had long survival. One patient lived 22 months at 78 years old and another patient lived 15 months, at the age of 70, died from a cause other than cancer.

Relationship between expression status, clinical factors and survival period

In 46 patients, we analyzed expression of Akt/PTEN/mTOR pathway related proteins in order to determine their effect on the therapy-response after patients were treated with radiation and concomitant fluorouracil and calcium folinate applications as described in "Methods" section. Except few samples, fold-change values were relatively uniform through the samples, where mean fold-change values for mLST8 3.48 ± 1.10, REDD1 12.02 ± 4.09, PTEN 3.69 ± 1.03, SAG 1.06 ± 0.10, NOXA 18.21 ± 9.4, BNIP3 6.41 ± 2.33, p53 1.43 ± 0.74 and corresponding median values were 0.56, 1.0, 0.9, 0.9, 1.8, 0.9 and 0.6, respectively.

During median 6 years follow-up period, five of the patients developed locoregional tumor recurrence (10.9%) and 9 out of 46 developed distant metastasis (19.6%). During the first 3 years, 18 patients (39.1%) died of the disease, ten of these with an age ≥ 70.

Survival rates for 3, 5 and 10 years were 60.9%, 54.3% and 37% respectively. Mean survival time in the pathologic complete tumor (T0) and focal tumor response group (T1)

Table 1 Demographic and clinicopathologic characteristics of patients

	n	%
Gender		
Male	26	56.5
Female	20	43.5
Age		
< 50	20	43.5
> 50	26	56.5
Total	46	100.0
Histopathology		
Adenocarcinoma	35	76.1
Mucinous type	8	17.4
Signet-ring cell type	3	6.5
Total	46	100.0
Pretreatment tumor stage		
T2	7	15.2
T3	33	71.7
T4	6	13.0
Total	46	100.0
Pretreatment nodal stage		
N0	20	43.5
N1	26	56.5
Total	46	100.0
Pretreatment clinical stage		
I	4	8.7
II	16	34.8
III	26	56.5
Total	46	100.0
Pathological stage		
0	8	17.4
I	10	21.7
II	19	41.3
III	9	19.6
Total	46	100.0
Pathological tumor stage		
No tumor found	8	17.4
Focal tumor	6	13.0
Muscularis propria (T2)	5	10.9
Subserosa invasion (T3)	25	54.3
Visceral peritoneum (T4)	2	4.3
Total	46	100.0
Pathological nodal stage		
N0	37	80.4
N1-2	9	19.6
Total	46	100.0
Operation		
Miles	19	41.3
LAR	25	54.3
None	2	4.3
Total	46	100.0
Response		
Downstaging	29	63.0
Upstaging	8	17.4
No change	9	19.6
Total	46	100.0
Local recurrence		
Yes	5	10.9
No	41	89.1
Total	46	100.0
Metastasis		
Yes	9	19.6
No	37	80.4
Total	46	100.0
Vascular invasion		
Positive	17	37.0
Negative	27	58.7
Missing system		
Total	46	100.0
Perineural invasion		
Positive	21	45.7
Negative	23	50.0
Missing system	2	4.3
Total	46	100.0
Overall survival (months)		
5 years 54.3%		
10 years 37%		
Estimate mean 7.090 years	SE ± 0.789 (CI 95%: 5.54- 8.64)	
Disease free survival (months)		
5 years 46%		
10 years 28.5		
Estimate mean 4.437 years	SE ± 0.510 (CI 95%: 3.44–5.44)	

categorized as good response (n = 14) was 8.2 ± 1.4 years and in the partial and stable response group (stage T2-4) (n = 32) was 6.6 ± 0.9 years.

Overall and disease free survivals of each parameter were calculated with Kaplan Meier analysis (log-rank) and all were found to be non-significant, except for p53 expression (p = 0.031) and vascular invasion (p=0.036) (Fig. 1A). Survival relation of each parameter was also evaluated with univariate Cox regression analysis and those with p < 0.1 were pulled for multivariate analysis (Table 2). In accordance with Kaplan Meier analysis, univariate Cox regression analysis showed significant association with p53 and vascular invasion. In multivariate analysis all were non-significant except p53. The patients with low p53 expression showed 2.7 times more death risk than high expression levels.

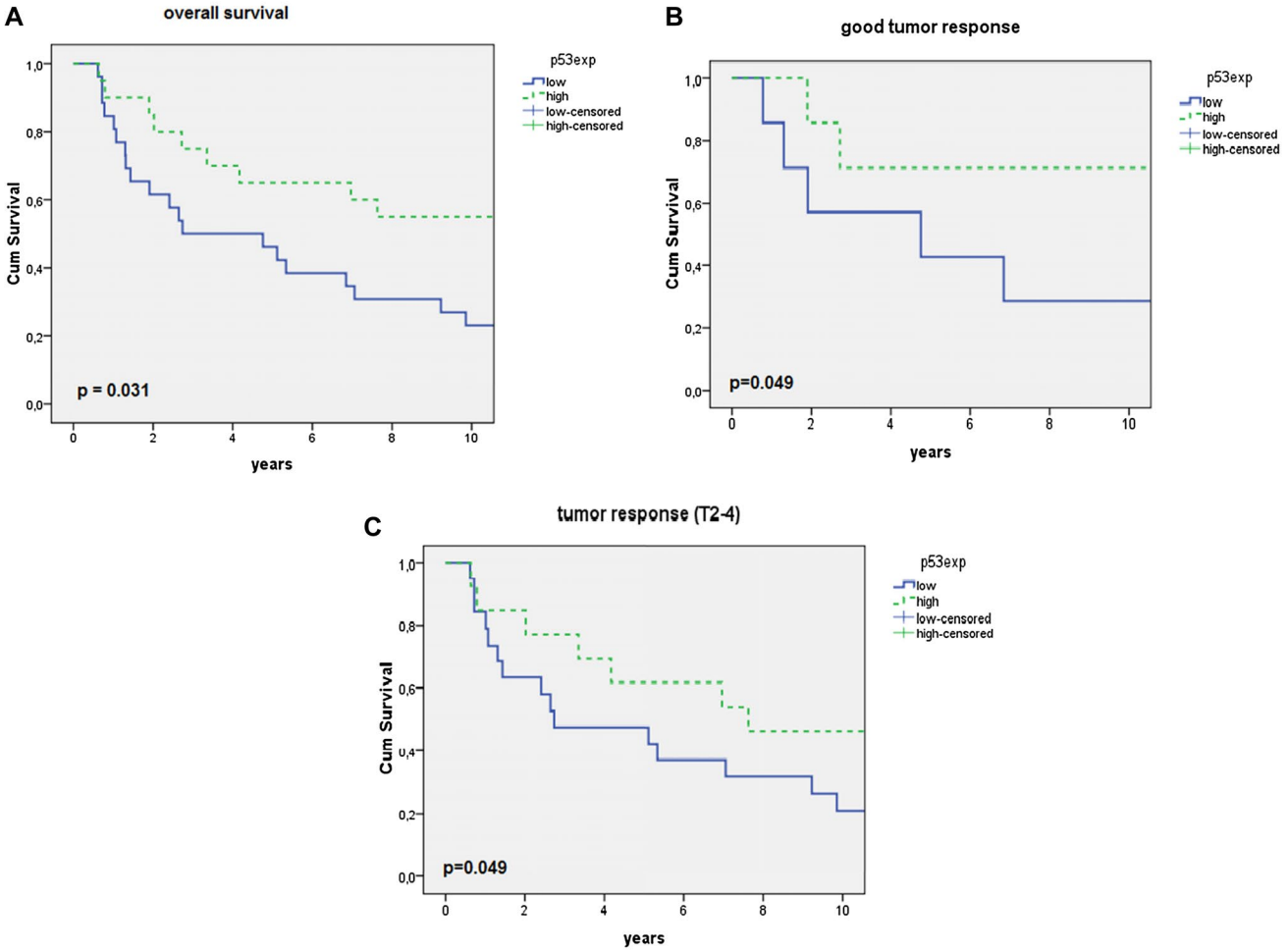

Fig. 1 Kaplan–Meier survival curve based on p53 expression **A** Association of p53 expression with overall survival periods. Kaplan–Meier survival curve reflects the positive association between p53 expression and 10-year overall survival where higher expression in tumor tissues significantly related to longer survival periods, **B** Association of p53 expression with good tumor response (T0–T1) (see text) and **C** partial/none response T2-4 group. Kaplan–Meier curves illustrate the difference between high levels versus low levels of p53 expression for two strata based on good tumor response (T0–T1) and partial/none tumor response to therapy (T2-4)

Mean OS times for high (n = 20) vs low (n = 26) p53 expression were significantly different with 8.9 ± 1.28 years (CI 95% 6.60–11.24) and 5.7 ± 0.97 years (CI 95% 3.77–7.59) respectively (p = 0.031). Similarly, DFS times were 5.7 ± 0.74 years (CI 95% 4.32–7.22) versus 3.4 ± 0.63 years (CI 95% 2.18–4.66) (p = 0.019). p53 was significantly related to pathologic complete response (p 0.034). In addition, it had significant contribution to the observed tumor response to therapy. Mean survival times of patients within the good response group (T0-T1) for high vs low p53 expressions were 10.2 ± 1.91 years (CI 95% 6.54–14.03) vs 5.7 ± 1.75 years (CI 95% 2.34–9.22) and in the group with partial or no response group (T2-4) were 8.1 ± 1.45 years (CI 95% 5.30–10.98) vs 5.5 ± 1.13 years (CI 95% 3.32–7.74), respectively (p = 0.049, Fig. 1B and C).

Application of univariate regression analysis showed that a decreased hazard could be attended with high p53 (hazard ratio (HR) = 0.431; CI 95% 0.195–0.949). High NOXA and low REDD1 expressions were also related to lower risk, but results were not significant (Fig. 2A).

When life spans were analyzed against different age groups [Median 57 (range 27–86)] (< 50 years of age (n: 20) vs > 50 (n 26) mean overall survival was 9.0 ± 1.17 years (CI 95% 6.67–11.27) and 5.6 ± 0.96 years (CI 95% 3.69–7.45) respectively (p 0.027). Pretreatment tumor status of patients, however, did not exhibit any statistically significant association to life spans, though mean life spans between T2, T3 and T4 were highly different (mean OS for different stages were 7.7 ± 2.0, 7.1 ± 0.9, 5.3 ± 1.7 years, respectively).

Table 2 Univariate and multivariate analysis of clinicopathologic and genetic parameters

Univariate analysis	Exp (B)	95.0% CI for Exp (B)		Sig.	Exp (B)	95.0% CI for Exp (B)		Sig.
		Lower	Upper			Lower	Upper	
NOXA	0.888	0.428	1.841	0.749				
mLST8	0.936	0.439	1.994	0.864				
p53	0.431	0.195	0.949	**0.037***	0.366	0.157	0.855	**0.020**[a]
PTEN	0.983	0.454	2.128	0.965				
REDD1	1.615	0.747	3.493	0.223				
SAG	1.203	0.574	2.520	0.625				
BNIP	1.241	0.599	2.574	0.561				
Complete response	0.971	0.370	2.548	0.953				
Downstaging response	1.143	0.546	2.395	0.723				
Node response	1.369	0.583	3.212	0.471				
Perineural invasion	0.482	0.225	1.032	**0.060***	0.754	0.317	1.796	0.524
Vascular invasion	0.459	0.217	0.968	**0.041***	0.491	0.212	1.137	0.097
Local recurrence	1.866	0.709	4.910	0.206				
Metastasis	0.571	0.252	1.293	0.179				

Cox regression analysis was conducted for each parameter which was thought to be concerned

*$p < 0.1$ pulled for multivariate analysis. Node Response—N0 vs N+, Complete Response—pathological complete response (T0N0), Downstaging response—downstaged vs others (upstaged and stable). Bold font indicates statistical significance

[a]High expression of p53 has 0.37 times death risk than low expression profiles in multivariate analysis

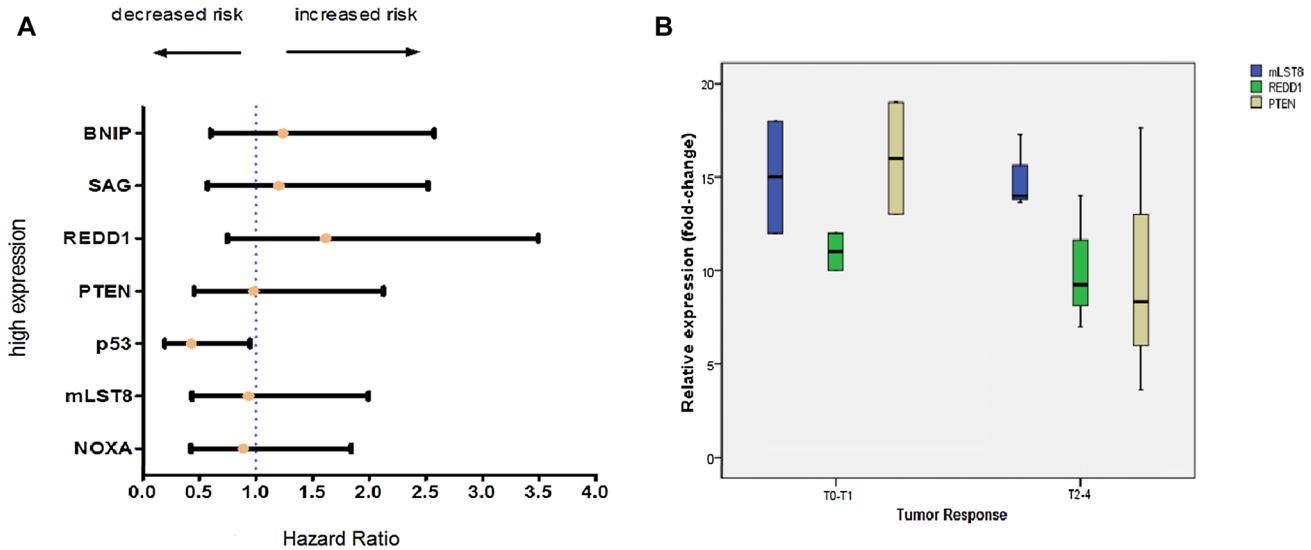

Fig. 2 Estimated risk and response analyses for GOI **A** Forest plot representing hazard ratios and 95% confidence intervals (CI) after Cox regression analyses of variables at the median 6 years follow-up status. Hazard ratios (HR) for each variable was represented with circles, and horizontal error bars correspond to the lower and upper limits CIs of the estimated HR. Values smaller than HR = 1 denotes reduced risk of hazard, **B** Clustered plot for mLST8, REDD1 and PTEN expressions (fold change) relative to good tumor response (T0-1) and partial/none tumor response (T2-4) groups

Correlation analyses of mTOR/PTEN and SAG/NOXA axis

Correlations were computed between the gene expressions to understand their possible relationship (Table 3). mLST8 was chosen since it is a common member of both mTORC1 and mTORC2 complexes. High associations were observed between mLST8/REDD1 and mLST8/PTEN, confirming their role in the same cascade. mLST8 was also associated with BNIP3, and NOXA but not with p53.

With regard to apoptotic axis, we also observed close association between SAG and NOXA proteins (Spearman

Table 3 Spearman correlations between clinical parameters and expression profile of GOIs in PTEN/Akt/mTOR cellular growth /proliferation and SAG/NOXA apoptotic pathways

	Local recur	Met	Vascular invasion	Path comp resp	Tumor resp	LN resp	Pre tumor	Stage resp	REDD1 expr	PTEN expr	SAG expr	NOXA expr	BNIP expr	P53 expr
REDD1 expr		0.370 **0.015***								0.348 **0.018****	0.137 0.362	0.441 **0.002****	0.696 **0.000****	− 0.050 0.743
PTEN expr				0.393 **0.010****	0.447 **0.003****	0.446 **0.003****					0.175 0.244	0.480 **0.001****	0.391 **0.007****	0.263 0.077
mLST8 expr						0.354 **0.019***		0.336 **0.026***	0.564 **0.000****	0.435 **0.003****	0.137 0.362	0.528 **0.000****	0.522 **0.000****	− 0.050 0.743
SAG expr			0.401 **0.007****									0.429 **0.003****	0.175 0.244	− 0.027 0.859
BNIP expr	0.349 0.017*	0.493 **0.000****					− 0.449 **0.002****							0.175 0.244
P53 expr	0.306 0.038*													
NOXA expr		0.318 **0.031***					− 0.364 **0.013***						0.480 **0.001****	0.011 0.940

GOI gene of interest, *Local Recur* local recurrence, *Met* metastasis, *Path Comp Resp* pathological complete response (T0N0) vs others, *Tumor Resp* pathologic tumor response as good (T0-T1) vs T2-T4, *LN Resp* N0 vs N1-2, *PreTumor* pretreatment tumor stage as T2,T3,T4, *Stage Resp* downstaged vs others (upstaged, nochange)

*Correlation is significant at the 0.05 level (2-tailed). Bold font indicates statistical significance

**Correlation is significant at the 0.01 level (2-tailed)

correlation coefficient (r_s) 0.429, p 0.003). Although mean survival time was 7.9 ± 1.39 years (CI 95% 5.21–10.68) for NOXA high expression together with SAG low expression and it was 6.0 ± 1.5 years (CI 95% 3.10–8.99) for the opposite combination, the effect on survival with regard to their expressions was not statistically significant (log-rank, p 0.480). Interestingly, SAG expression correlated with vascular invasion (r_s 0.401, p 0.007) (Table 3). In the case of vascular invasion, mean survival time of patients with low SAG expression was 6.4 ± 1.45 years (CI 95% 3.58–9.27) whereas patients with high SAG expression had mean survival of 1.9 ± 0.50 years (CI 95% 0.92–2.89) (log-rank p 0.047).

BNIP3 has shown correlations with REDD1, PTEN and NOXA, as well. Similarly, mLST8, PTEN and REDD1 expression displayed strong correlation with NOXA expression. Interestingly, we did not detect any correlation with p53, the closest correlation was for PTEN (p = 0.077) as listed in Table 3.

Pathologic complete response had strong correlation with PTEN expression fold-change values (p = 0.010), similarly with tumor responses (p = 0.003) (Table 3). Median fold-change for PTEN expression were 16.0 for good tumor response (T0-T1) group, while it was 8.3 for T2-4 group (Fig. 2B). When analyzed in combination with PTEN expressions, patients who showed good tumor response (T0-T1) to therapy had longer life span if they have a high PTEN expression than those have low expressions (10.8 vs 7.3 years, respectively; p = 0.082). There was a negative correlation between tumor response and REDD1 expression with a considerable trend toward significance (r_s − 0.271; p 0.079), and it was found to be correlated significantly with metastasis (r_s 0.370; p 0.015). A correlation was found between p53 and local reccurence (p = 0.038). Similarly, BNIP3 had strong correlation with local reccurence (r_s 0.306; p 0.017) and metastasis (r_s 0.493; p = 0.000) (Table 3).

Discussion

In recent years, cancer therapy strategies tend to employ multifactorial approaches to improve the outcomes since developments indicate that the success of neoadjuvant therapies relies on the intricate molecular signaling pathways and effects could be increased if these interactions could be resolved for each individual. Therefore, in addition to clinical factors such as lymph node invasion, tumor size or vascular invasion, many genetic factors are under study for their possible prognostic value.

In rectal cancer progress, one of the best known molecular factors is p53 tumor suppressor protein. Studies on the prognostic effect of this protein are highly controversial, possibly due to different technical approaches used in the studies, as well as unique properties of p53 and shortages such as small patient groups with inconsistent clinicopathologic features [14, 15]. The method used for expression analysis is an issue, since mutations in p53 gene extends the protein half life and therefore yields to nonfunctional but higher expression levels of protein with poor prognosis in IHC -based studies. In TRGAted evaluation based on TCPA data, difference between high/low expression were only detectable after approximately eight years. High protein expression profile had lower survival probability, in accordance with other IHC results, and no prognostic significance was observed. Other reports based on transcriptional data, however, points out that defective p53 or reduced function of it cause to a higher rate of recurrence or reduced response to CRT and strong expression of p53 or p53 induced p21 is associated with better responses [16–18].

In our long-term follow-up study, p53 was the only predictor for survival. Patients with higher expression of p53 had clearly longer life span and disease free survival. A high expression status also had significant contribution to the tumor regression. A well-conducted meta-analysis study by Chen et al. compiling substantial number of data from a large population concludes that gene expression analysis is a better approach to estimate therapy outcome, especially when neoadjuvant CRT was conducted [16].

Here, in this work, our main objective was to search for predictive roles of several proteins in the PI3K/Akt/mTOR and SAG/NOXA apoptotic pathways, where there is very limited information about their transcriptional status and its relation to clinical responses since their activities mostly regulated by phosphorylations or epigenetic modifications. Biomarkers chosen in this context were partly a result of our lately concern in mTOR inhibitors and partly due to our previous prognostic studies involving apoptotic proteins, especially based on SAG, through a population of rectal and cervical cancer patients [8, 9]. There is a close connection between DEPTOR degradation (DEPTOR is a natural inhibitor of mTORC1 and mTORC2) and SCF E3 ubiquitin ligase, resulting in mTOR activation, cell growth and proliferation [12]. In this frame, the prognostic role of these proteins was investigated at transcriptional level. Data was evaluated for the CRT responses, 3-, 5- and 10- year survival rates and correlations among the mRNAs of all proteins of interest.

PI3K/Akt/mTOR pathway is an important signaling system linking receptor tyrosine kinases, which are receptive molecules for especially growth factors, cytokines and hormones, to cellular responses for growth, proliferation or metabolism. Phosphatidylinositol-3-kinase (PI3K) is a lipid kinase involved in the initial stages of this pathway with different cell activities, especially cellular growth and proliferation. PTEN, on the other hand, is a lipid phosphatase, an antagonizer for PI3K activity, helps to maintain regulation of the pathway. Dysregulations in PI3K signaling could

initiate cancer formation or induce tumor development and metastatic spread. Many studies have confirmed that deletion or mutations in PTEN are effective factors in cancer due to the resultant uncontrolled activity of PI3K/Akt signaling pathway [19–21].

As a prognostic factor, PTEN deficiency was reported in relation to poor survival [22, 23]. Mechanisms for PTEN regulation are complex, generally proceed through post-translational mechanisms, but also involve transcriptional and post-transcriptional factors as well. Therefore, most of the reports are based on protein quantifications using immunohistochemistry, instead of transcriptional inspections**.** Among many positive and negative regulators, common players in the development of cancer, such as tumor suppressor protein p53, c-Jun oncogene, nuclear factor kappa B (NF-κB) and transforming growth factor beta (TGF-β) are able to change PTEN gene expression [24–26]. PTEN gene alterations due to deletions or gene disruptions were shown to reduce patient survival, at least in a group of the rectal cancer patients [27]. Interestingly, even though the cytoplasmic PTEN expression was shown to be uncorrelated with mRNA expression, a study observed that mRNA levels have greater influence in prognosis compared to protein expression. Low levels of PTEN mRNA were found to be associated with poorer prognosis and shorter survival times in diffuse large B-cell lymphoma patients, with a considerably large cohort of 747 cases [28]. A meta-analysis containing 10,231 patients from 27 studies exhibits that PTEN loss significantly corresponds to worse DFS and OS in breast cancer [29]. Tumors with good-response to CRT revealed a distinct correlation with high PTEN expression in our findings; however, we were not able to find significant prognostic effect of PTEN neither for overall nor disease-free survival after evaluation with both univariate Cox regression and Kaplan–Meier analyses. TCPA data showed a similar result, where higher expression of the PTEN resulted in higher survival probability between 5 and 8 year follow-up periods, though not statistically significant.

Since the PI3K/Akt/mTOR pathway has a distinctive role in multiple aspects of cancer development, we also focused on mTOR complexes. Mammalian target of rapamycin (mTOR) is a serine-threonine kinase involving two enzyme complexes, mTORC1 and mTORC2, acting downstream of PI3K/Akt/PTEN and play a central role in positively regulating cell growth, survival and other cellular functions. mTOR regulation/deregulation mostly based on phosphorylations, therefore, the relation between transcriptional expression levels and prognosis were evaluated only in a limited number of studies. Several studies report a relation between high mTOR expression and poor clinical outcome [30–32]. Downstream effectors of mTOR, the 4E-binding protein 1 (4EBP1) and the p70 ribosomal S6 kinases (S6K1 and S6K2), were reported as factors correlated with poor outcome [33]. Since mTOR is currently known to participate in several pathways involving cancer development and mTOR inhibitors are focus of many clinical trials, we aimed to see contribution of mLST8 mRNA expression levels (target of rapamycin complex subunit LST8 or G protein beta subunit-like, GβL or Gable), which is a common subunit of mTORC1 and mTORC2, to the outcomes after treatment. Considering mLST8 might be a critical scaffolding element to formation of mTOR complexes and very little is known about its role in cancer initiation and development, we investigated its prognostic significance. mLST8 was found to be upregulated in several human colon and prostate cancer cell lines [34]. Its silencing can disrupt both mTORC1 and mTORC2 complex formations. Since current clinical research shows great efforts to develop more effective mTOR inhibitors, what is so called TORKinibs, to be able to inhibit both mTORC1 and mTORC2 activity, mLST8 could have a substantial role in the management of these complexes.

mTOR complexes coordinate and organize cellular growth and proliferation not only in response to growth factors, but also in response to nutrients (mTORC1 is the rapamycin and nutrient-sensitive part, but not mTORC2), hypoxia or energy stresses. Understanding tumor biology under hypoxia is an important determinant of angiogenesis and tumor spread. REDD1 (regulated in development and DNA damage responses 1) is one of the hypoxia and DNA damage induced genes that lead to the inhibition of S6K1 and 4E-BP1 phosphorylation, two endpoint effectors of mTOR, and after it completed its function, it is rapidly degraded by the ubiquitin—proteasome system [35–37]. BNIP3 is another hypoxia induced gene. It is a pro-apoptotic gene from Bcl-2 family and it is able to inhibit cellular growth through suppressing mTOR pathway [38]. Epigenetic alterations seem more effective in transcriptional regulation of BNIP3 mRNA expression. In tumors, BNIP3 is silenced via epigenetic mechanisms, such as promoter hypermethylation and histone deacetylation [39]. A study reported that they were able to detect aberrant methylation of BNIP3 in 66% of primary colorectal and 49% of primary gastric cancers, but not in normal tissue samples collected from areas adjacent to the tumors [40].

There are very few available data about the prognostic relevance of REDD1. A study conducted on paraffin-embedded ovarian cancer tissues using IHC found a positive correlation for REDD1 and p-Akt expressions and late-stage disease and also showed that overexpression of these genes resulted in reduced DFS and OS [41]. Similar results were also presented by other studies [42, 43]. BNIP3 was more widely studied in this perspective and high BNIP3 reactivity using IHC found to be linked with poor post-operative outcome in a study involving 72 endometrial cancer patients. In invasive breast cancer patients involving 40 subjects, loss of

BNIP3 expression was related to poor prognostic outcomes, but interestingly not related to hypoxia response [44]. In our study, high expression of both proteins yielded lower survival rates, as other studies reported, and though the differences were eminent, they were not statistically significant. There was a strong correlation between REDD1 and BNIP3. These proteins were also in strong correlation with NOXA, a BH3-only protein involved in the apoptotic pathway. NOXA was of interest due to its co-regulation with sensitive-to-apoptosis (SAG) protein that we previously reported for its prognostic value after 2-year follow-up period [8].

SAG is a RING box protein, also named as Rbx2 or ROC2 and it has a dual function: an E3 ubiquitin ligase activity and an antioxidant activity or ROS scavenger. As an anti-apoptotic protein, it has a protective role in the cell against apoptotic events induced by hydroxyl radicals, hypoxia, radiation, stress etc [45–47].

SAG silencing was found to induce apoptosis through accumulation of NOXA [47]. The promoter of the NOXA gene, on the other hand, was found to be directly regulated by p53 upon X-ray exposure or DNA damage. Our correlation analysis supported the reverse association between SAG and NOXA expression and furthermore pointed to the high correlation of NOXA with the mTOR pathway related proteins, signifying its potential interactivity in this pathway.

Here in this work involving 46 patients with locally advanced rectal carcinoma, basal mRNA expression levels of PI3K/mTOR pathway proteins PTEN and mLST8, hypoxia induced REDD1 and BNIP3, apoptotic and mTOR pathway regulators SAG and NOXA, and a well-known tumor suppressor p53 has been investigated for their contribution to mid- and long-term survival and prognosis. In our findings, there were strong correlations between mLST8 and PTEN, REDD1 and NOXA expressions, but none of them found to be related to the clinical outcome in the long term, except p53. Strangely, there was no correlation of none of the proteins under interest with p53 expression. Though NOXA is a known p53-regulated gene, we didn't observe any correlation between the mRNA levels. In regular conditions, constitutive NOXA mRNA expression was noted as low in tissues and protein expression is induced upon damage [48]. This induction is mostly p53-regulated, but it sometimes proceeds also in a p53-independent manner, especially in the GI tract, as reported by several studies [49, 50]. Therefore, lack of correlation could be due to low basal levels of both proteins or contribution of alternative p53-independent pathways.

Patients who well responded to therapy and displayed retrieval in terms of tumor response status were found to be associated with p53, together with PTEN and a potential association may be suggested for REDD1.

In conclusion, the current data indicate that none of the proteins, except p53, has valuable prognostic role to estimate clinical outcome, possibly due to the pre-mentioned post transcriptional or epigenetic regulations more effective in the regulation of these molecules. p53 expression was recorded as the only significant factor to estimate longer life span and responses to therapy. Tumor response, however, suggests a relation with PTEN and REDD1 expression levels. Another observation was reduced risk of hazard for high NOXA and p53 expressions. Therefore tumor suppressor p53, hypoxia induced REDD1, lipid phosphatase PTEN and pro-apoptotic NOXA proteins could be suggested as potential predictive elements for therapeutic applications.

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Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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