Research Article

Fatma Demet Arslan*, Ayse Kocak, Cengiz Aydın, Emel Ebru Pala, Dilek Oncel, Gulden Diniz, Tayfun Kaya, Levent Ugurlu, Mustafa Degirmenci, Bulent Ozkan, Yasemin Soysal and Harun Muayad Said

Evaluation of potential tumor markers that may predict neoadjuvant treatment efficiency in rectal cancer

[Rektal kanserde neoadjuvan tedavi etkinliğini öngörebilecek potansiyel tümör belirteçlerinin değerlendirilmesi]

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Abstract

Objectives: The recurrence of rectal cancer or its resistance to neoadjuvant treatment develops due to the adaptation to hypoxia, apoptosis or autophagy. Survivin, one of the inhibitors of apoptosis; Beclin 1, which is a positive regulator in the autophagy pathway; and hypoxia-inducible factor-1α (HIF-1α) and carbonic anhydrase-9 (CA9), which are associated with tumor tissue hypoxia, may be related to resistance to treatment. Our aim was to evaluate the potential tumor markers that may help to monitor the response to neoadjuvant treatment in locally advanced rectal cancer (RC).

Methods: Twenty-five patients with locally advanced RC were included in the study. Gene expression and protein levels of Beclin 1, Survivin, HIF-1α, and CA9 were analyzed in fresh tissue specimens and blood samples. The relationships of these markers to tumor staging and regression grade were evaluated.

Results: Higher blood CA9 gene expression levels and lower blood HIF-1α protein levels were found in the response group according to tumor regression grade. After neoadjuvant treatment, tissue Beclin 1 and blood Survivin gene expressions and tissue CA9, blood Beclin 1 and blood HIF-1α protein levels decreased significantly.

Conclusion: Beclin 1, Survivin, HIF-1α ve CA9 may help to predict the effects of the applied treatment approach.

Keywords: Beclin 1; carbonic anhydrase-9; hypoxia-inducible factor-1 alpha; neoadjuvant treatment; rectal cancer; Survivin.

Öz

Amaç: Rektal kanser nüksü veya neoadjuvan tedaviye direnci hipoksi, apoptoz veya otofajiye adaptasyona bağlı olarak gelişir. Apoptoz inhibitörlerinden biri olan Survivin, otofaji yolağında pozitif bir düzenleyici olan Beclin 1 ve

***Corresponding author: Fatma Demet Arslan,** MD, Department of Medical Biochemistry, University of Health Sciences, Tepecik Training and Research Hospital, Gaziler Street, No: 468, 35180, Yenişehir, Izmir, Turkey, Phone: +90 5056468214, Fax: +90 2324330756, E-mail: fatmademet.arslan@gmail.com. https://orcid.org/0000-0003-0766-0303
Ayse Kocak, Yasemin Soysal and Harun Muayad Said, Department of Molecular Medicine, Institute of Health Sciences, Dokuz Eylül University, Izmir, Turkey. https://orcid.org/0000-0002-1510-2937 (A. Kocak). https://orcid.org/0000-0002-7032-2668 (H.M. Said)
Cengiz Aydın, Tayfun Kaya and Levent Ugurlu, General Surgery Clinic, University of Health Sciences, Tepecik Training and Research Hospital, Izmir, Turkey
Emel Ebru Pala and Gulden Diniz, Medical Pathology Clinic, University of Health Sciences, Tepecik Training and Research Hospital, Izmir, Turkey. https://orcid.org/0000-0003-1512-7584 (G. Diniz)
Dilek Oncel, Radiology Clinic, University of Health Sciences, Tepecik Training and Research Hospital, Izmir, Turkey
Mustafa Degirmenci, Oncology Clinic, University of Health Sciences, Tepecik Training and Research Hospital, Izmir, Turkey
Bulent Ozkan, Department of Biostatistics, Faculty of Medicine, Katip Çelebi University, Izmir, Turkey

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tümör doku hipoksisi ile ilişkili olan hipoksi ile indüklenebilir faktör-1a (HIF-1a) ve karbonik anhidraz-9 (CA9), tedaviye direnç ile ilişkili olabilir. Amacımız lokal ileri evre rektal kanserde (RK) neoadjuvan tedaviye yanıtı izlemeye yardımcı olabilecek potansiyel tümör belirteçlerini değerlendirmektir.

Yöntemler: Çalışmaya lokal ileri evre RK'li 25 hasta dahil edildi. Beclin 1, Survivin, HIF-1α ve CA9'un gen ekspresyonu ve protein seviyeleri, taze doku ve kan örneklerinde analiz edildi. Bu belirteçlerin tümör evresi ve regresyon derecesi ile ilişkileri değerlendirildi.

Bulgular: Tümör gerileme derecesine göre yanıt grubunda daha yüksek kan CA9 gen ekspresyon seviyeleri ve daha düşük kan HIF-1α protein seviyeleri bulundu. Neoadjuvan tedaviden sonra, Beclin 1 dokusu ve kan Survivin gen ekspresyonları ve CA9 dokusu, kan Beclin 1 ve kan HIF-1α protein seviyeleri önemli ölçüde azaldı.

Sonuç: Beclin 1, Survivin, HIF-1α ve CA9 uygulanan tedavi yaklaşımının etkilerini tahmin etmeye yardımcı olabilir.

Anahtar Kelimeler: Beclin 1; hipoksi ile indüklenebilir faktör-1 alfa; karbonik anhidraz-9; neoadjuvan tedavi; rektal kanser; Survivin.

Introduction

Rectal cancer (RC) is one of the highest morbidity- and mortality-causing factors in humans worldwide [1]. Radical surgery is the main RC treatment approach, but neoadjuvant treatments can be considered as a complementary approach. After neoadjuvant treatment, 8–20% of RC patients had a complete response, 30–60% had a partial response, and the remainder had resistance to treatment [2, 3]. Cell death was the aim of these treatments. However, resistance to apoptosis is an important factor responsible for inadequate treatment and relapse in gastrointestinal oncology. There is a regulatory association between apoptosis and autophagy. Therefore, combined treatments that include apoptotic and autophagic mechanisms, have been investigated in order to overcome cell death resistance in RC [4–6].

Survivin, an inhibitor of the apoptosis family of genes, acts by blocking caspase activity. The Survivin gene has been found to be overexpressed in RC patients, thereby protecting the tumor cells from cell death and, as a consequence, indicating a poor prognosis [7, 8]. For this reason, Survivin is targeted during treatment. Survivin antisense oligonucleotides have been proven to effectively induce cell death in RC cells *in vitro* [9].

In addition to apoptosis inhibition, autophagy is also required for the survival of cancer cells. Autophagy provides the ability to maintain life under hypoxic conditions [10]. Beclin 1 is induced in response to cellular stress and is a positive regulator in the autophagic pathway to sustain tumor cell survival. Beclin 1 suppression is another therapeutic goal for sensitizing RC cells to apoptosis [11]. Likewise, interaction between Beclin 1 and Survivin has been demonstrated; specifically, the Survivin protein expression decreased and its turnover increased when Beclin 1 was downregulated [12]. This relationship can explain the connection between autophagy and apoptosis.

Adaptation to hypoxia, such as autophagy, is one factor that enhances the ability of tumor cells to survive. Hypoxia is present in many tumor tissues due to structural and functional abnormalities in the vessels and rapid tumor cell proliferation. The tumor cannot grow more than 2 mm^3 in the absence of angiogenesis. Hypoxia is an important triggering signal for angiogenesis induction, and most of the angiogenic factors regulated by hypoxia are under the control of hypoxia-inducible factor-1α (HIF-1α) [13, 14]. One of the genes in which HIF-1α increases gene expression is carbonic anhydrase (CA9) [15]. It has been shown that the CA9 gene encodes CA9 protein and can be strongly induced by hypoxia in tumor cells [16]. CA9 gene expression is found to be high in rectal tumors, especially in high-proliferation areas [17], and it has been found to be an important prognostic factor [18].

Resistance to cancer treatment is an important clinical problem for RC patients. The determination of expression in key genes related to adaptation to hypoxia and the apoptotic–autophagic balance may be useful in predicting response to treatment in gastrointestinal cancers. There is a clinical need to establish molecular biomarkers to separate sensitive and resistant tumors. Such biomarkers can be used to predict the individual tumor response of the patient to multimodal therapy. However, there are very few studies investigating the association of Beclin 1, Survivin, HIF-1α, and CA9 with response to neoadjuvant therapy in RC.

In this study, by analyzing both the gene expression and the protein levels of potential markers (Beclin 1, Survivin, HIF-1α and CA9) that may be helpful in monitoring response to therapy in locally advanced RC patients, the relationship of these markers with tumor staging and regression grade has been investigated.

Materials and methods

A total of 25 patients with locally advanced RC were included in this study. Locally advanced RC (stage II/III) is defined as T3 or T4 rectal adenocarcinoma advanced with mesorectal invasion ≥5 mm, with or

without lymph node metastasis (within no more than 10 cm from the anal canal) or as T1 or T2 rectal adenocarcinoma with lymph node metastasis. Following the National Comprehensive Cancer Network (NCCN) guidelines [19], the patients underwent surgical and neoadjuvant treatment. Patients with T1 or T2 rectal adenocarcinoma without lymph node metastasis or with distant organ metastasis were excluded from the study.

The diagnosis of rectal adenocarcinoma was confirmed in patients by histopathological examination of colonoscopic biopsies. Lymph nodes and distant metastases were scanned by chest and abdominal computed tomography. Pre-operative clinical Tumor-Lymph Node-Metastasis (TNM) staging was assessed by physical examination, magnetic resonance imaging, and biopsy material according to the American Cancer Joint Committee (AJCC) guidelines [20]. Chemotherapy (capecitabine 1,650 mg/m^2/day) protocol was applied simultaneously to external radiotherapy (50.4 Gy/28 fraction) for two months as neoadjuvant therapy. Surgical resection was performed two months after radiochemotherapy. Response to the post-surgical neoadjuvant therapy was assessed according to the tumor regression grade in the AJCC guidelines [20]. Tumor regression was classified into four groups: (0) no residual tumor cells, (1) single cells or small groups of tumor cells, (2) residual cancer with desmoplastic response, and (3) minimal evidence of tumor response. Postoperative pathological TNM staging was performed according to the AJCC guidelines [20].

Tissue total mRNA and protein isolation and measurement

Tissues for molecular analysis were obtained from the endoscopic biopsies, and the tumor bed area of the resection specimens. Tumor tissues were stored at −80 °C until total mRNA and protein isolation were performed. These tissues were homogenized by a mechanical homogenizer (TissueLyser LT, Qiagen GmbH, Hilden, Germany). Total mRNA and protein were isolated from the tissue sample using an isolation reagent (TriPure Isolation Reagent, Roche Applied Science, Indianapolis, IN).

The total amount of mRNA obtained was evaluated with a microvolume spectrophotometer (NanoDrop 2000/2000c Spectrophotometer, Thermo Scientific, ME, USA) at a wavelength of 260 and 280 nm. If the A260/A280 absorbance ratio was 1.8–2.0, it was accepted as pure for mRNA. The total protein levels were measured by the bicinchoninic acid (BCA) method (BCA Protein Assay, Thermo Fisher Scientific, IL, USA). The total protein levels were calculated as μg/μL.

Blood total mRNA isolation

Blood samples were collected into tubes containing EDTA immediately after biopsy and surgery resection. Total mRNA isolation was performed using an mRNA isolation kit (QIAamp RNA Blood Mini Kit, Qiagen GmbH, Courtabeouf, France). mRNA purity measurements in blood samples were performed as in the tissue samples.

Tissue and blood Beclin 1, Survivin, HIF-1α, and CA9 gene expression analysis

The following primers were used for the expression of tissue and blood Beclin 1, Survivin, HIF-1α, and CA9 genes.

- HIF-1α – Forward primer: '5-TTCCAGTTACGTTCCTTCGATCA-3'
- HIF-1α – Reverse primer: '5-TTTGAGGACTTGCGCTTTCA-3'
- CA9 – Forward primer: '5-TCTCGCTTGGAAGAAATCGC-3'
- CA9 – Reverse primer: '5-CTGAAGTCAGAGGGCAGGAG-3'
- Beclin 1 – Forward primer: '5-GGCTGAGAGACTGGATCAGG-3'
- Beclin 1 – Reverse primer: '5-CTGCGTCTGGGCATAACG-3'
- Survivin – Forward primer: '5-AGAACTGGCCCTTCTTGGAG-3'
- Survivin – Reverse primer: '5-CTTTTTATGTTCCTCTATGGGGTC-3'
- β-Actin – Forward primer: '5-CGGGGCCAATCAGCGT-3'
- β-Actin – Reverse primer: '5-GCCGCTGGGTTTTATAGGG-3'

The guanidinium thiocyanate-phenol-chloroform extraction method was used with TriPure Isolation Reagent (Roche Applied Science, Indianapolis, IN) for mRNA isolation as described above, and cDNA was synthesized from the template mRNA using a cDNA synthesis kit (QuantiTect Reverse Transcription kit, Qiagen GmbH, Hilden, Germany). The β-Actin gene was selected as the housekeeping gene. Levels of mRNA were analyzed using the real-time quantitative polymerase chain reaction (RT-qPCR) method by a thermal cycler device and analysis software (Rotor Gene Q and Q-Rex Software, Qiagen, Valencia, CA, USA). The RT-qPCR cycle consisted of denaturation for 15 s at 95 °C, annealing for 30 s at 53 °C, and extension for 30 s at 72 °C. Each cycle was repeated 40 times. The results were presented as the fold change (FC) of expression according to the delta delta cycle threshold (ΔΔCt) value [21]. These experiments were performed in triplicate.

Blood Beclin 1, Survivin, HIF-1α, and CA9 protein level analysis

Blood samples were collected into tubes with a gel separator immediately after biopsy and surgical resection. Blood samples were centrifuged at 1,500 rpm for 10 min, and the separated serum was stored at −20 °C until analysis. The serum Beclin 1, Survivin, HIF-1α, and CA9 protein levels were determined using enzyme-linked immunosorbent assay (ELISA) kits (Bioassay Technology Laboratory, Shanghai, China). The intraassay and interassay coefficients of variation of the ELISA kits were <8.0% and <10.0%, respectively. The assay ranges of the Beclin 1 (Catalog number E2011Hu), Survivin (Catalog number E1612Hu), HIF-1α (Catalog number E0422Hu), and CA9 (Catalog number E2273Hu) kits were 0.2–60.0, 5.0–1,000.0, 0.05–15.0, 0.05–30.0 ng/mL, respectively. The analytical sensitivities of the Beclin 1, Survivin, HIF-1α, and CA9 kits were 0.1, 2.49, 0.01, and 0.021 ng/mL, respectively. The results were presented as Beclin 1 levels ng/mL, Survivin levels ng/L, HIF-1α levels ng/mL, and CA9 levels ng/mL. The ELISA experiments were performed in duplicate.

Tissue Beclin 1, Survivin, HIF-1α, and CA9 protein level analysis

Tissue Beclin 1, Survivin, HIF-1α, and CA9 protein levels were analyzed by the Western blot method. For this, a vertical electrophoresis system (Mini-PROTEAN® Electrophoresis System, Bio-Rad Laboratories, CA, USA) with sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was used, and 30 μg protein per well was loaded in each well in SDS-PAGE. The wet transfer method was applied to transfer the proteins to the membrane. The blots were incubated overnight at 4 °C with a 1:500 dilution of appropriate primary antibody

in 5% skim milk/tris-buffered saline with Tween. The antibodies used at these dilution rates were Beclin 1, Survivin, HIF-1α, and CA9 polyclonal rabbit antibodies (Cell Signaling Technology Inc., MA, USA). After the primary incubation, the membranes were washed with tris-buffered saline with Tween; the blots were incubated at 1:1,000 with the appropriate horseradish peroxidase-conjugated mouse anti-rabbit secondary antibody (Cell Signaling Technology Inc., Danvers, MA, USA) at room temperature for 1 h. As a control for equal protein loading, the membranes were stripped and re-incubated overnight at 4 °C for 1:500 dilution with β-Actin primer antibody (Cell Signaling Technology Inc., MA, USA). Protein bands were detected using an enhanced chemiluminescence (Luminata Forte Western HRP Substrate, Millipore, MA, USA) kit for 20 min. Protein bands were visualized with chemiluminescence-sensitive X-ray film (Kodak MXB Film, Carestream Health Inc., Essex, UK). Protein band densities were evaluated using IMAGE-J Analysis Software (National Institute of Health, MD, USA). Data were quantified and normalized according to β-Actin (Cell Signaling Technology Inc., MA, USA). The results were presented as Beclin 1/β-Actin, Survivin/β-Actin, and CA9/β-Actin ratios. These experiments were performed in triplicate, and the Western blotting images were presented as representative of one image.

Statistical evaluation

Data were evaluated using Microsoft Office Excel 2007 and Statistical Package for Social Sciences (SPSS) Statistics 20.0. The tumor stage (T1, T2, T3, T4) or lymph node stage (N0, N1, N2) were determined during clinical staging. If there was regression by at least one stage during pathological staging, it was defined as downstaging. Tumor regression grades 0 and 1 were defined as the "response group"; 2 and 3 were defined as the "non-response group." After tumor regression grading, the differences for gene expression and protein levels in blood and tissue samples between the two groups were analyzed by the Mann–Whitney test. The results were defined as median and 25th–75th percentile.

Blood and tissue samples taken after biopsy and surgical resection were considered as samples before and after neoadjuvant therapy, respectively. The differences in gene expression and protein levels in each patient's blood and tissue before and after neoadjuvant therapy were evaluated by Wilcoxon test. The results were defined as median and 25th–75th percentile. The relationships between Beclin 1, Survivin, HIF-1α, and CA in blood and tissue were analyzed using the Spearman correlation test. For all p values, <0.05 was considered statistically significant.

Results

The mean age ± standard deviation of the patients was 57.8 ± 10.4. The gender of the patients was predominantly male (n=20, 80%). According to the tumor regression grade, the response group comprised 40% of the patients, and the non-response group, 60%. After neoadjuvant therapy, 36% of the patients had downstaging according to the T stage, and 72% of them had downstaging according to the N stage.

The differences between the response and non-response groups were investigated (Table 1) for gene expression and protein levels. Higher blood CA9 gene expression levels (p=0.013) and lower blood HIF-1α protein levels (p=0.043) were found in the response group (Figure 1).

When the relationship between T or N downstaging and gene expression or protein levels was investigated, no statistically significant change was found in gene expression and protein levels in patients who had T or N downstaging.

Changes in gene expression and protein levels before and after neoadjuvant therapy were also investigated (Table 2). Tissue Beclin 1 (p=0.004) and blood Survivin gene expression (p=0.017) and tissue CA9 (p=0.021), blood Beclin 1 (p=0.030), and blood HIF-1α (p=0.040) protein levels had statistically decreased after neoadjuvant therapy (Figure 2).

Positive correlations were found solely between CA9 and HIF-1α (r=0.480, p=0.018) gene expression and between CA9 and Beclin 1 (r=0.664, p=0.003) gene expression in tissue. However, no similar association was found in blood samples. Interestingly, when the protein levels were examined, there was a negative relationship between Survivin and Beclin 1 in the tissue (r=−0.590, p=0.002), whereas a positive correlation was found between blood Survivin and HIF-1α (r=0.722, p<0.001), between blood Survivin and CA9 (r=0.549, p=0.005) and between blood HIF-1α and CA9 (r=0.753, p<0.001) (Table 3).

Discussion

Although there are many studies about the role of tumor markers in predicting the response to neoadjuvant therapy in RC, there are few studies focusing on four different genes (Beclin 1, Survivin, HIF-1α, and CA9). In our study, Survivin, an inhibitor of the apoptosis family; Beclin 1, a positive regulator of the autophagic pathway; and HIF-1α and CA9, related to tumor tissue hypoxia, were investigated. All may be associated with mechanisms of resistance to treatment and response to treatment.

It is known that complete response rates of RC patients after neoadjuvant treatment are between 8 and 44%. Approximately 40% of patients experienced tumor downstaging [22]. In our study, according to the tumor regression grade, the response group was found to be 40% of RC patients. The percentage of those with T downstaging was 36%. Similar results previously published by other researchers support the effectiveness of applied neoadjuvant therapy [22].

Table 1: Gene expression and protein levels of Beclin 1, Survivin, HIF-1α and CA9 according to tumor regression grade before neoadjuvant treatment.

			Response group			Non-response group	
n, %			10 (40%)			15 (60%)	
Gene expression	**25%**	**Median**	**75%**	**25%**	**Median**	**75%**	**p-Value**
Tissue Survivin (FC)	97.34	6,585.15	11,113.30	44.02	127.56	1,213.54	0.244
Tissue Beclin 1 (FC)	8,659.09	130,166.62	2,232,146.06	60.55	4,269.94	1,715,264.94	0.415
Tissue HIF-1α (FC)	279.17	515.56	35,119.87	45.57	278.20	14,664.09	1.000
Tissue CA9 (FC)	11.33	505.83	13,193.14	3.57	47.42	1,358.22	0.412
Blood Survivin (FC)	0.001	0.002	0.183	0.005	0.032	1.266	0.107
Blood Beclin 1 (FC)	0.22	21.41	107.78	0.75	19.97	359.54	0.881
Blood HIF-1α (FC)	0.000	0.006	0.039	0.001	0.012	2.505	0.302
Blood CA9 (FC)	13.93	29.04	10,734.74	0.02	5.62	16.45	**0.013***
Protein levels	**25%**	**Median**	**75%**	**25%**	**Median**	**75%**	**p-Value**
Tissue Survivin	0.73	0.89	0.92	0.72	0.81	1.00	0.285
Tissue Beclin 1	1.44	1.55	1.69	1.09	1.26	1.56	0.849
Tissue HIF-1α	UD	UD	UD	UD	UD	UD	UD
Tissue CA9	1.07	1.47	1.68	0.98	1.09	1.36	0.569
Blood Survivin, ng/L	52.69	69.50	97.63	66.51	88.76	138.74	0.121
Blood Beclin1, ng/mL	2.79	4.50	9.04	2.56	6.98	9.22	0.558
Blood HIF-1α, ng/mL	0.31	0.52	0.75	0.46	1.01	1.30	**0.043***
Blood CA9, ng/mL	0.02	0.09	0.48	0.02	0.39	1.61	0.319

FC, Fold change; UD, Undetermined. *p-Value <0.05 was considered to be statistically significant. Tissue protein levels given as Beclin/β-Aktin, Survivin/β-Aktin, HIF-1α/β-Aktin, CA9/β-Actin ratio.

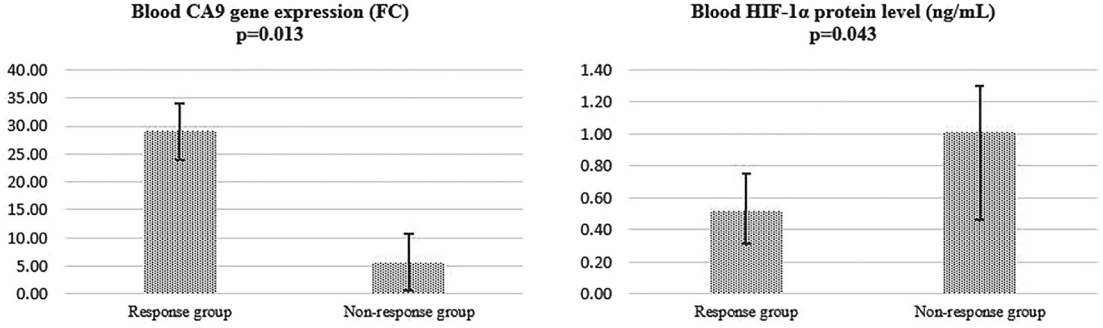


Figure 1: CA9 gene expression and HIF-1α protein levels in blood according to tumor regression grade before neoadjuvant treatment. The bars and error lines are presented as median and interquartile values.

According to our results, Beclin 1 and Survivin gene expression and protein levels in blood and tissue were not associated with tumor regression grade or T and N down-staging. However, the levels of CA9 gene expression in the blood were higher in the response group. In another study, a significant correlation was found between the increasing serum CA9 after neoadjuvant treatment chemotherapy and histological tumor stage. But the increase in serum CA9 were not correlated with histologic tumor regression score [23]. In our study, the high levels of CA9 gene expression in the blood before neoadjuvant treatment suggested that CA9 might be a marker for positive response to treatment.

The pre-treatment status of HIF-1α has been found to predict pathological response and outcomes in clinical stage II/III RC [24]. Another study, which showed an independent relationship between HIF-1α gene expression and poor response in advanced TNM stage RC, argued that HIF-1α can supplement the currently used prognostic methods to better manage the disease treatment process [25]. It showed that HIF-1α tissue gene expression in the response group was

Table 2: Beclin 1, Survivin, HIF-1α and CA9 gene expression and protein levels before and after neoadjuvant therapy.

n=25	Before neoadjuvan therapy			After neoadjuvan therapy			
Gene expression	**25%**	**Median**	**75%**	**25%**	**Median**	**75%**	**p-Value**
Tissue Survivin (FC)	49.21	180.39	8,404.31	9.98	88.44	421.18	0.244
Tissue Beclin 1 (FC)	102.89	21,920.61	1,715,264.94	0.75	27.89	873.10	**0.004***
Tissue HIF-1α (FC)	20.81	279.17	6454.48	145.99	1584.71	4,182.47	0.498
Tissue CA9 (FC)	3.21	25.68	956.83	5.22	86.07	429.84	0.639
Blood Survivin (FC)	0.002	0.021	0.948	0.001	0.002	0.019	**0.017***
Blood Beclin 1 (FC)	0.23	19.97	107.78	0.02	0.30	39.35	0.189
Blood HIF-1α (FC)	0.001	0.009	0.973	0.001	0.003	0.010	0.055
Blood CA9 (FC)	0.10	10.62	75.14	0.12	9.58	40.33	0.627
Protein levels	**25%**	**Median**	**75%**	**25%**	**Median**	**75%**	**p-Value**
Tissue Survivin	0.67	0.89	1.02	0.91	0.94	1.06	0.543
Tissue Beclin 1	1.09	1.49	1.73	0.81	0.98	1.08	0.357
Tissue HIF-1α	UD	UD	UD	UD	UD	UD	UD
Tissue CA9	0.98	1.33	1.68	0.15	0.36	0.91	**0.021***
Blood Survivin, ng/L	63.83	80.24	125.85	23.55	49.80	139.49	0.181
Blood Beclin 1, ng/mL	2.75	6.13	9.05	3.23	3.68	5.93	**0.030***
Blood HIF-1α, ng/mL	0.39	0.74	1.22	0.40	0.53	0.87	**0.040***
Blood CA9, ng/mL	0.02	0.14	0.84	0.08	0.50	1.22	0.616

FC, Fold change; UD, Undetermined. *p-Value <0.05 was considered to be statistically significant. Tissue protein levels given as Beclin/β-Actin, Survivin/β-Actin, HIF-1α /β-Actin, CA9/β-Actin ratio.

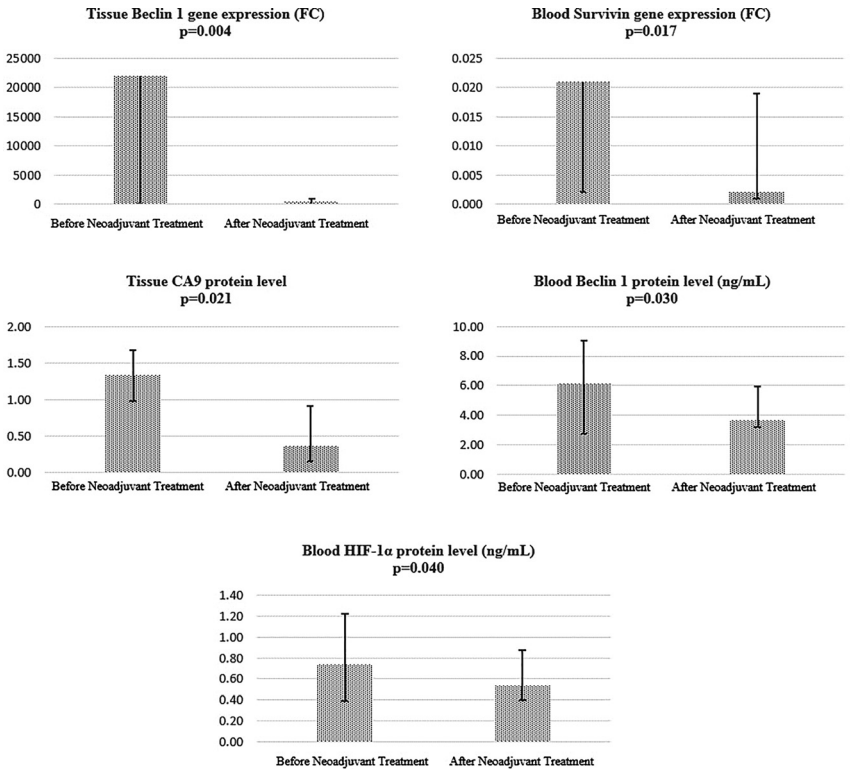


Figure 2: Examined gene mRNA expression and protein level in tissue and blood before and after neoadjuvant treatment. The bars and error lines are presented as median and interquartile values.

significantly lower than in the non-response group, and it was thought that HIF-1α gene expression might predict response to chemoradiation [26]. However, there are studies that do not find any significant difference between the HIF-1α–positive group and the HIF-1α–negative group in terms of pathological staging [27]. In our study, low HIF-1α protein levels in the response group suggested that blood HIF-1α may play a role as a prognostic marker.

Table 3: The relation among Beclin 1, Survivin, HIF-1α and CA9 in tissue and blood (correlation coefficient and p value).

Tissue gene expression	**Survivin**	**HIF-1α**	**CA9**	**Beclin 1**
Survivin	1	−0.156 (0.456)	−0.101 (0.639)	0.021 (0.935)
HIF-1α		1	0.480 **(0.018)***	0.416 (0.086)
CA9			1	0.664 **(0.003)***
Beclin 1				1
Blood gene expression	**Survivin**	**HIF-1α**	**CA9**	**Beclin 1**
Survivin	1	0.105 (0.616)	0.025 (0.907)	−0.089 (0.678)
HIF-1α		1	−0.356 (0.081)	−0.024 (0.913)
CA9			1	0.238 (0.262)
Beclin 1				1
Tissue protein levels	**Survivin**	**HIF-1α**	**CA9**	**Beclin 1**
Survivin	1	UD	0.419 (0.228)	−0.590 **(0.002)***
HIF-1α		UD	UD	UD
CA9			1	0.006 (0.987)
Beclin 1				1
Blood protein levels	**Survivin**	**HIF-1α**	**CA9**	**Beclin 1**
Survivin	1	0.722 **(<0.001)***	0.549 **(0.005)***	0.147 (0.493)
HIF-1α		1	0.753 **(<0.001)***	0.121 (0.572)
CA9			1	0.054 (0.802)
Beclin 1				1

UD, Undetermined. *p-Value <0.05 was considered to be statistically significant.

Similar to our findings, it has been shown that the gene expression levels of Survivin, which is another candidate marker in patients who underwent neoadjuvant therapy, did not correlate with downstaging [28]. However, some studies suggest that the stage of the tumor in tissue that had pre-treatment high Survivin gene expression levels regressed less after neoadjuvant therapy [29].

In a study that found a statistically significant relationship between the pre-treatment expression of Beclin 1 in cancer tissue and the tumor downstaging, Beclin 1 was thought to be a predictive biomarker for the efficacy of chemoradiotherapy in RC patients [11]. In our study, however, there was no significant change in Beclin 1 that could help predict the response to neoadjuvant therapy.

Our study determined that tissue Beclin 1 and blood Survivin gene expressions and tissue CA9, blood Beclin 1, and blood HIF-1α protein levels decreased significantly after neoadjuvant therapy. This is the first known clinical study showing that the levels of tissue Beclin 1 and blood Survivin gene expression and tissue CA9, blood Beclin 1, and blood HIF-1α protein levels have decreased with neoadjuvant treatment in locally advanced RC.

In a previous study, HIF-1α protein expression in RC tissues was shown to decrease after neoadjuvant therapy [30]. While HIF-1α gene expression was positive in most of the preoperative biopsies, Korkeila et al. determined that HIF-1α gene expression decreased in most of the postoperative samples [31]. In our study, it was remarkable that the levels of blood HIF-1α protein decreased significantly, although there was no change in tissue and blood gene expression after neoadjuvant treatment.

Sprenger et al. found that tissue Survivin expression significantly decreased after neoadjuvant therapy in RC. They highlighted that Survivin gene expression may be an optimal prognostic marker for individual response monitoring and bears the potential for anti-Survivin strategies in future randomized clinical trials [32]. In the present study, although the Survivin gene expression level did not decrease in the tissue, its decrease in the blood after neoadjuvant treatment suggests that it be considered to function as a potential prognostic marker.

The present findings are significant for understanding the regulative crosstalk that occurs between the pathways during different physiological conditions in RC. While the unmodified forms of Beclin 1 function in autophagy, the C-terminal fragment of Beclin 1 (Beclin 1-C) after cleavage by caspase plays an important role in apoptosis. Survivin suppresses caspase 3, thereby decreasing apoptosis in cancer cells. It has been determined that the Survivin protein expression decreased and the turnover of Survivin protein increased by the downregulation of Beclin 1 [33, 34]. This position may explain the linkage between

autophagy and apoptosis. HIF-1α directly binds to the promoter region of the CA9 and Survivin genes and functions as a transcriptional activator and upregulator [34–36]. Detecting a relationship between tissue HIF-1α and CA9 gene expression, between blood HIF-1α and CA9 protein levels, between tissue CA9 and Beclin 1 gene expression, and between blood Survivin with HIF-1α and CA9 protein levels can explain the mechanism linking apoptosis, autophagy, and the adaptation to hypoxia.

The most important limitation of the current study is the inability to reach a sufficient number of targeted patients who complied with the inclusion criteria and the reluctance of patients to participate in the study. The gender distribution of the patients included in the study seems to be a disadvantage (male and female proportions were 80 and 20%, respectively). However, other studies have also stated that the rates of female and male patients were 29.3–42.5 and 57.5–70.7% in RC, respectively [37, 38]. Due to the epidemiological nature of RC, our data was thought to reflect the distribution in the local population.

In addition, tissue HIF-1α protein levels could not be analyzed, despite many optimization studies. Previous studies have shown that HIF-1α can only be expressed in a hypoxic environment, whereas CA9 is expressed in normoxic environments under certain physiological and regulative conditions. However, the expression of CA9 in a hypoxic environment increased in parallel with HIF-1α [39, 40], and while tissue CA9 protein levels can be detected, the reason why tissue HIF-1α protein levels cannot be determined could be related to post-transcriptional regulations and the dynamic physiological condition of hypoxia within the targeted RC tissue that was reflected in the expression pattern of the RC tissue.

In this study, the gene expression and protein expression levels of the same molecules in the same samples (blood and tissue) were not consistent with each other. The reason for this may be that gene expression is regulated in various steps and that the various processes involved in this arrangement are integrated. For example, miRNAs and siRNAs in cells are complementary to the regulation of target mRNAs and protein levels. Furthermore, transcription factor binding sites and chromatin modifications are affected by transcriptional control in the different organisms and physiological conditions.

Conclusion

Our study found a statistically significant relationship between blood levels of CA9 gene expression and HIF-1α protein and response to treatment, and it also found that tissue Beclin 1 and blood Survivin gene expression and tissue CA9, blood Beclin 1, and blood HIF-1α protein levels were reduced after neoadjuvant therapy. Therefore, these markers may be useful in prognosis and in predicting the selection or efficacy of the applied treatment regimen. Unnecessary surgical indications of patients with complete response to treatment by gene expression or protein level analysis examined before and after neoadjuvant therapy may be prevented.

Thus, the optimization of treatment methods can be accomplished by the application or development of new treatment strategies that have fewer or no harmful side effects. This can be a preliminary study for further studies on distant metastasis and survival. Cost effectiveness can be increased by applying personalized treatment.

Due to the limitations of our study, further studies with larger numbers of patients and more homogeneous gender distribution might be necessary. Beclin 1, Survivin, HIF-1α, and CA9 candidate markers may also be promising in treatment management in other tumor types developing due to different physiopathological reasons.

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Ethical considerations: Detailed information was given to all participants about the study before their participation, and their signed consents were obtained. This original research study was performed in accordance with the Helsinki Declaration and with the approval of Izmir Dokuz Eylul University's Ethics Committee.

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