Araştırma Makalesi (Research Article)

Beclin 1, P53 Mutasyonu, Ki-67 Proliferasyon İndeksi, Tümör Nekrozu ve Mikrovasküler İnvazyonun Böbrek Hücreli Karsinomlarda Prognoz Üzerindeki Etkisi ve Bunların Bilinen Prognostik Parametrelerle İlişkisi

The Effect Of Beclin 1, P53 Mutation, Ki-67 Proliferation İndex, Tumor Necrosis and Microvascular İnvasion On Prognosis İn Renal Cell Carcinomas and Their Relationship With Known Prognostic Parameters

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Özet

Amaç: Bu çalışmanın amacı böbrek hücreli karsinomlarda (BHK) yeni prognostik belirteçlerin belirlenmesine yardımcı olmak, otofajinin BHK patogenezindeki rolünü göstermek ve BHK'daki yeni tedavi yöntemlerine ışık tutmaktır.

Gereç ve Yöntemler: Klinikopatolojik evreler, tümör nekrozu ve mikrovasküler invazyon (MVI) retrospektif olarak belirlendi ve Beclin-1, Ki-67 ve p53 immünohistokimyasal olarak incelendi.

Bulgular: BHK'larında tümör nekrozu, MVI, Ki-67 ve p53; Fuhrman nükleer derece ve patolojik tümör evresi ile pozitif korelasyon gösterirken, Beclin-1 sadece nükleer derece ile ilişkiliydi. MVI ve Ki-67, uzak organ ve lenf nodu metastazı ile ilişkiliydi. Ki-67 ve nekroz, p53 ve Beclin-1 ekspresyonu ile pozitif koreleydi ancak MVI ile korele değildi. Beclin-1 nekroz ile koreleydi, ancak p53 ve MVI ile korele değildi. p53, Ki-67 ve MVI sağkalım ile negatif korelasyon gösterirken Beclin -1 ve nekrozun sağkalım üzerindeki etkisi gösterilemedi.

Sonuç: BHK'larda Ki-67, p53 ve MVI sağkalım üzerine etkileri olan prognostik parametrelerdir. Beclin-1, sağkalım ile ilişkili bir prognostik parametre olmamasına rağmen, tümör dokusunda ekspresyonunun arttığı bulundu. Aynı zamanda, tümör dışı böbrek parankiminde daha az boyama vardır. Beclin-1, otofajinin bir belirtecidir ve prognostik öneminden ziyade BHK patogenezinde rol oynadığı düşünülmektedir.

Anahtar kelimeler: Böbrek hücreli karsinom, Otofaji, Beclin 1, Ki-67, p53

Abstract

Objective: The aim of this study was to help identify new prognostic markers in renal cell carcinomas (RCC), to show the role of autophagy in the pathogenesis of RCC and to shed light on new treatment modalities in RCC.

Material and Methods: Clinicopathological stages, tumor necrosis and microvascular invasion (MVI) were determined retrospectively and Beclin-1, Ki-67 and p53 were studied immunohistochemically.

Results: Tumor necrosis, MVI, Ki-67, and p53 in RCCs were positively correlated with Fuhrman nuclear grade and pathologic tumor stage, while Beclin-1 was only associated with nuclear grade. MVI and Ki-67 were associated with distant organ and lymph node metastasis. Ki-67 and necrosis correlated positively with p53 and Beclin-1 expression, but not with MVI. Beclin-1 were positively correlated necrosis, but not with p53 and MVI. p53, Ki-67 and MVI were negatively correlated with survival, while the effect of Beclin -1 and necrosis on survival couldn't be demonstrated.

Conclusion: Ki-67, p53, and MVI in RCCs are prognostic parameters with effects on survival. Although Beclin-1 was not a prognostic parameter associated with survival, its expression in tumor tissue was found to be increased. At the same time, there is less staining in non-tumor renal parenchyma. Beclin-1 is a marker of autophagy and is thought to be involved in RCC pathogenesis rather than its prognostic significance.

Keywords: Renal cell carcinoma, Autophagy, Beclin 1, Ki-67, p53

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INTRODUCTION

Renal cell carcinomas (RCC), develop from renal tubular epithelial cells. More than 90% of kidney malignancies are renal cell carcinomas. RCCs are the 9th most common tumors in males and 14th in females (1).

The most important prognostic parameter in RCC is the tumor stage. The parameters used in tumor staging according to the criteria of the World Health Organization (WHO) 2016 have been determined as tumor diameter, perinephritic adipose tissue invasion, gerato fascia invasion, renal vein or vena cava inferior invasion, adrenal gland invasion or metastasis, lymph node metastasis, and distant organ metastasis. According to the 8th edition of the American Joint Committee on Cancer, published in 2017, other parameters that are not included in the TNM classification system, but which should be stated in reporting and which have prognostic significance are; the histological subtype of the tumor, the nuclear degree of the tumor (ISUP nuclear grading system or Fuhrman nuclear grade), microvascular invasion (MVI), presence of necrosis, sarcomatoid and rhabdoid differentiation (2). MVI has a prognostic significance independent of tumor type, diameter, and grade in RCC (3). Tumor necrosis has been shown to be a prognostic parameter independent of tumor stage and nuclear grade in clear cell and chromophobe RCC (4, 5). Although several prognostic parameters have been identified in RCC, immunohistochemically and molecularly, they have little clinical use (4).

In early stage RCC, treatment is partial or radical nephrectomy. However, 30% of partial or radical nephrectomy cases can progress to advanced stage RCC (6). Advanced stage RCCs are more resistant to treatment. In addition to the treatment of chemotherapy and radiotherapy in advanced stage RCC, immunotherapy and antiangiogenic treatments are applied as new treatment modalities (7). However, in order to overcome nutritional deficiency due to antiangiogenic treatment in RCC, tumor cells have been shown to improve resistance by activating the mechanisms of autophagy (7). Most tumors use autophagy to survive during multiple stress situations, cytotoxic therapy and target chemotherapy treatment (8).

Autophagy is an adaptation mechanism that is involved in the physiological process to provide cellular homeostasis in eukaryotic cells to meet the energy requirement of the cell in starvation, hypoxia and metabolic stress situations and to keep them alive(8,9). It has also been reported in many pathological processes such as tumors, neurodegenerative diseases, infectious diseases and inflammatory diseases (9). Several genes have been identified with autophagy, which are called Autophagy-Related Genes (ATGs). One of these genes is the BECN (the gene encoding the Beclin 1 protein) which is the mammalian homolog of the yeast gene ATG6 (10). Beclin 1 is essential in autophagy regulation (11). The monoallelic deletion of Beclin 1 gene causes breast, prostate and ovarian cancer (12). Studies in mice have shown that heterozygous loss of Beclin 1 increases tumorigenesis and monoallelic deletion has led to the spontaneous tumor formation, such as B-cell lymphoma, hepatocellular carcinoma and lung carcinoma(11). This shows us that Beclin 1 acts as a tumor suppressor gene.

Beclin-1 was first described as bcl-2-associated protein (14). Beclin 1 is a novel Bcl-2 homolog domain only protein (BH3) (13). BH3 is located at the N-terminal fragment of Beclin- 1 and allows it to interact with bcl-2. Bcl-2 (localized in the endoplasmic reticulum) is an anti-apoptotic protein that inhibits autophagy via Beclin-1 (10). Bcl-2 does not lose its anti-apoptotic properties when interacting with Beclin-1 (14). Beclin-1 is not essential for apoptosis, but interacts with anti-apoptotic proteins because of it is BH3 only protein. Also, proapoptotic BH3 only proteins Bad, Bid, Bim, Puma, and Noxa have apoptosis regulating and autophagy enhancing effects (15). In other words, autophagy and apoptosis are in constant interaction with each other.

p53 is a tumor suppressor gene that increases DNA damage, oncological stress and hypoxia. p53 in mammalian cells is localized in the mitochondria and between nucleus and cytoplasm. When p53 is activated, it translocates to the nucleus and increases the target gene expression. As a result, the cell cycle arrests, either DNA damage is repaired or apoptosis occurs (8). The apoptosis-enhancing effect of p53 is well known. p53 enhances apoptosis with Bax a member of the bcl-2 family, Puma and Bid that's are BH3 only proteins. However, p53 is associated with autophagic pathways, although not as direct as apoptosis. p53 plays a role in autophagy regulation in subcellular localization (8). p53 has the function of suppressing direct autophagy paradoxically, regardless of its transcriptional function in the nucleus (16). Autophagy is activated in p53 deletion and reduction (17). In other words, p53 has both inhibitory effects on autophagy regulation and activating action on different pathways.

Ki-67 is a nuclear non-histone protein associated with cell proliferation. The Ki-67 protein is an indicator that exists throughout all the active phases of the cell cycle (G1, S, G2, M) and is not detected in the resting phase (G0 phase). Ki-67 proliferation index is mostly associated with the clinical course of the neoplastic disease (18).

Despite intensive research, the role of autophagy in human malignancies is not well understood. There are also controversial results in RCC studies. In this study, we have studied immunohistochemically on Beclin 1 (autophagy marker), tumor suppressor gene p53 and Ki-67 antibodies in RCCs. Our aim is to compare these markers with necrosis and microvascular invasion with tumor stage and nuclear grade, which are prognostic parameters for the tumor, and to compare the effects of these markers on their correlation and survival.

MATERIAL and METHODS

Patient selection: In this study, we retrospectively evaluated 94 patients with RCC who underwent 72 radical and 22 partial nephrectomies at Ankara Ataturk Training and Research Hospital Department of Pathology between 2004-2008 (Ethical document no: 2009/01/01c). The study was planned according to the principles of the Helsinki Declaration.

Clinicopathological characteristics of the patients are shown in **Table 1.** Resection specimens were reevaluated for histopathological parameters including histological subtype, histological grade, pathological stage, tumor diameter, capsule and gerato fascia invasion, perirenal fat tissue invasion, pelvic fat tissue invasion, ureter invasion, renal vein and microvascular invasion, presence and degree of necrosis, lymph node and distant organ metastases, tumor differentiation (sarcomatoid and rhabdoid), tumor circumference changes in renal parenchyma **(Table 2).** The median postoperative follow-up duration of patients was 20.7 months (range 3-53 months). Pathological examinations were carried out by a single pathologist based on the 2017 8th edition American Joint Committee on Cancer TNM classification system and WHO 2016.

Immunohistochemical evaluation: Immunohistochemical studies were performed on 4 micron-thick sections made of formalin fixed paraffin embedded blocks. The sections were incubated with Ki-67 (Santa Cruz Biotechnology; Ki-67 (H-300): sc-15402 rabbit IgG, 200µg / ml) 1/200 dilution for 120 minutes, p53 (NeoMarkers company: p53 Ab-8 (Do-7): mouse IgG2b, 200µg / ml) 1/100 dilution for 60 minutes, Beclin 1 (Santa Cruz Biotechnology: sc-48381 BECN 1 (G-11): mouse IgG2b, 200µg / ml) 1/100 dilution for 90 minutes. DAB (3,3'Diaminobenzidine) chromogen was applied to observe immunoreaction. In our study as positive control tissue; tonsil for Ki-67, epididymis for Beclin 1 and high-grade clear cell RCC tissue for p53 were used. Nuclear

Table 1. Clinicopathological characteristics of the patients

Characteristics	No (%)
Mean±SD Age(range)	58.4±12.1(19-82)
Gender	
Female	26(27.7)
Male	68(72.3)
Localization	
Right	52(55.3)
Left	42(44.7)
Histological subtypes	
Clear cell RCC	74 (78.9)
Type I papillary RCC	7 (7.4)
Type II papillary RCC	5(5.3)
Chromophobe cell RCC	3(3.2)
Multiloculated cystic RCC	1(1.1)
Collector ductus carcinoma	2 (2.1)
Mucinous tubular and spindle cell carcinoma	2 (2.1)
Furhman nuclear grade	
I	10 (10.6)
II	40 (42.6)
III	24 (25.5)
IV	20 (21.3)
Pathological T stages	
pT1a	32(34)
pT1b	14 (14.9)
pT2	8 (8.5)
pT3a	30 (31.9)
pT3b	5 (5.3)
pT3c	1 (1.1)
pT4	4 (4.3)
Other pathologic features	
Chronic pyelonephritis	84 (89.4)
Simple cyst	15 (15.9)
Adenoma	2 (2.2)
Dysplasia	1 (1.1)

brown staining was positive for Ki 67 and p53 antibodies. Cytoplasmic brown staining was positive for the Beclin 1 antibody. For Ki-67 and p53, the ratio of nuclei showing positive staining was determined by counting 500 cells at 10 different fields. For Beclin 1, the ratio of cells showing positive staining was determined by counting 500 cells at 10 different fields. The immunoreactivity score was determined for the percentage of Beclin 1 staining. The percent positivity was scored as '0' (no immunoreactivity detected), '1' (1–10%), '2' (>11–50%), '3' (>51– 90%), or '4' (>90%) **(Figure 1).**

Statistical Analysis

Differences between groups were tested using Pearson chi-square and Fisher's exact tests. The relationship of Ki-67 and p53 with binary groups was compared with the Mann-Whitney-U test and Kruskal-Wallis test was used for comparison with 3 and more groups. Spearman's rank correlation test was used for correlation analysis. Kaplan Meier log-rank test was applied for survival analysis. All statistical analysis were performed using SPSS 15.0 software and p value <0.05 was considered significant.

Table 2. Distribution of cases, according to histological findings

Histological findings	Number of cases (%)	
	Present	Absent
Capsule invasion	59 (62.8%)	35 (37.2%)
Gerota fascia invasion	4 (4.3%)	90 (95.7%)
Perinephric fat tissue invasion	35 (37.2%)	59 (62.8%)
Renal pelvis invasion	21 (22.3%)	73 (77.7%)
Ureteral invasion	4 (4.3%)	90 (95.7%)
Renal vein invasion	11 (11.7%)	83 (88.3%)
Microvascular invasion	21 (22.3%)	73 (77.7%)
Adrenal gland invasion	11 (11.7%)	83 (88.3%)
Lymph node metastasis	7 (7.4%)	87 (92.6%)
Distant metastasis	7 (7.4%)	87 (92,6%)
Sarcomatoid differentiation	7 (7.4%)	87(92.6%)
Rabdoid differentiation	7 (7.4%)	87(92.6%)
Tumor necrosis	51 (54.3%)	43 (45.7%)

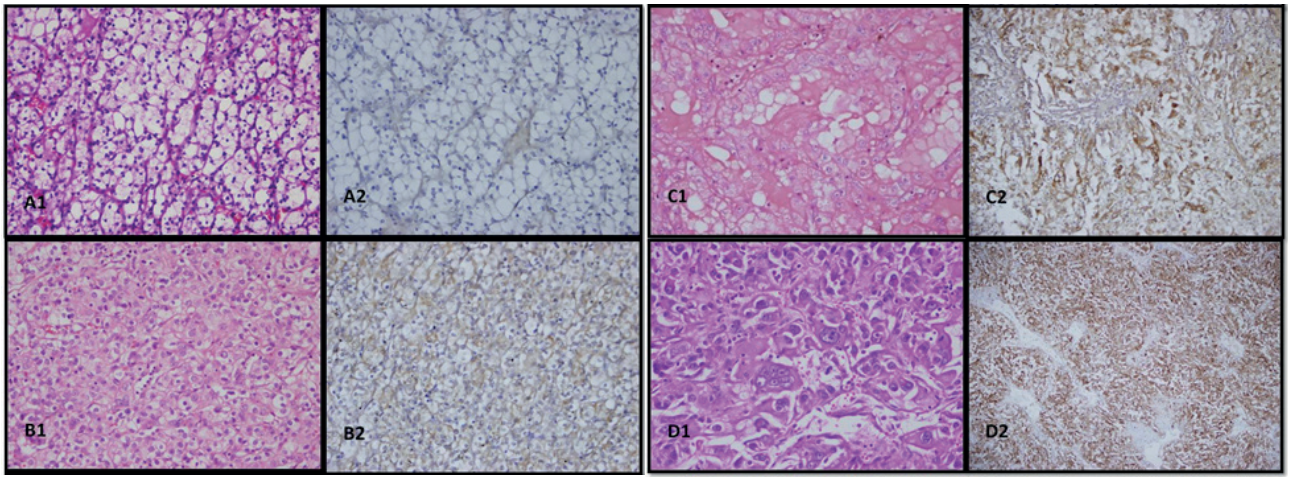

Figure 1. Histomorphology by HE staining of Fuhrman nuclear grade I clear cell RCC (A1), No immunoreactivity with Beclin 1 antibody (A2) (400× objective). Histomorphology by HE staining of Fuhrman nuclear grade II clear cell RCC (B1), Beclin 1 antibody with 1-10% immunoreactivity (B2) (400× objective). Histomorphology by HE staining of Fuhrman nuclear grade III clear cell RCC (C1), Beclin 1 antibody with 51-90% immunoreactivity (C2) (200× objective). Histomorphology by HE staining of Fuhrman nuclear grade IV clear cell RCC (D1), over 90% immunoreactivity with Beclin 1 antibody (D2) (100× objective). HE: hematoxylin and eosin.

RESULTS

Necrosis: Tumor necrosis was present in 51 (54.3%) of 94 RCC cases. A statistically significant relationship was found between the presence of necrosis, Fuhrman nuclear grades and pathological tumor stage (p=0.001) **(Table 3).** Necrosis was observed in 100% of tumors with stage pT4, while necrosis was observed in only 25% of stage pT1a tumors.

There was a statistically significant correlation between the presence of necrosis, tumor size (x^2 = 20.13 p = 0.000) and sarcomatoid and rhabdoid differentiation (x^2= 10.16 p = 0.006). No significant correlation was found between the presence of necrosis and distant organ metastasis (x^2 = 3.38 p = 0.066) and lymph node metastasis (x^2 = 3.38 p = 0.066). But necrosis was observed in 12 of 14 cases with distant organ or lymph node metastasis.

Microvascular invasion: MVI was present in 21 cases (22.3%). MVI was positively correlated with tumor stage, tumor grade, tumor size, necrosis, lymph node metastasis, and distant organ metastasis **(Table 4).** MVI was not significantly associated with p53, Beclin 1 and Ki-67 (p> 0.05).

Ki 67: Ki-67 is a proliferative index indicator and staining the cell nucleus. The mean Ki-67 staining percentage of tumor cells in our study was 17.03 (± 18.4) (min.: 0%, max: 70%, median value 10%) **(Figure 2A and B).** The Fuhrman nuclear grade, pathological stage and tumor diameter of RCC cases increased linearly with Ki-67 immunoreactivity (respectively, rho = 0.730 p<0.001; rho = 0.256 p = 0.013; rho = 0.332 p = 0.002).

There was a statistically significant difference between the histological subtypes and Ki-67 immunoreactivity avera-

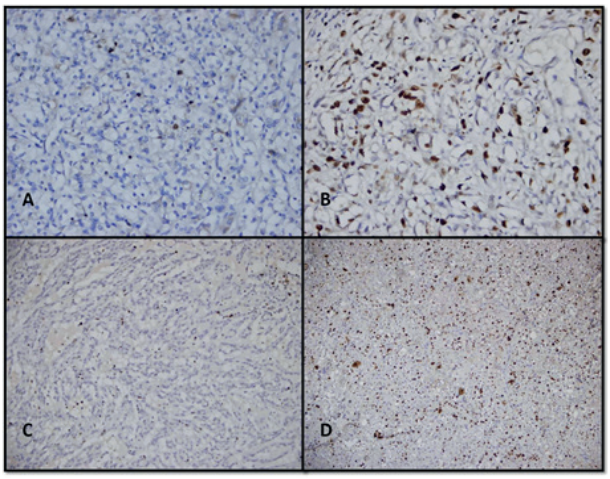

Figure 2. Low Ki-67 proliferation index in clear cell RCC (A), High Ki-67 proliferation index in clear cell RCC (B) (400× objective). Nuclear staining with p53 in a small number of cells in clear cell RCC (C) (200× objective), high p53 immunoreactivity (D) (100× objective).

Table 3. The relationship between Fuhrman nuclear grade, pathological tumor stage, and necrosis

NECROSIS	Fuhrman nuclear grade		X2	P
	Grade 1-2	Grade 3-4		
Absent	33 (66%)	10 (23%)	18.75	0
Present	17 (34%)	34 (77%)		

	PATHOLOGICAL TUMOR STAGE					
	pT1a	pT1b	pT2	pT3a	pT3b	pT4
Absent	24 (75%)	7 (50%)	2 (25%)	7 (23%)	3(60%)	0
Present	8 (25%)	7 (50%)	6 (75%)	23 (77%)	2(40%)	4(100%)

Table 4. Tumor stage, Fuhrman nuclear grade, lymph node and distant organ metastasis and necrosis correlation with MVI

Spearman's rank correlation test	Nuclear grade	Tumor stage	LN met.	Distant met.	Necrosis
MVI	rho=0.361 p=0.000	rho=0.221 p=0.033	rho=0.529 p=0.000	rho=0.432 p=0.000	rho=0.236 p=0.022

LN: Lymph node , met: metastasis

ges (p = 0.01). Ki-67 immunoreactivity is markedly higher in papillary type 2 and collector duct carcinoma, which are known to have a poor prognosis, whereas chromophobe cell type, multiloculated cystic type, and mucinous tubular spindle cell type, which are known to have a good prognosis were low. Perinephritic adipose tissue invasion (p = 0.012), pelvic adipose tissue invasion (p<0.001), gerato fascia invasion (p = 0.01), lymph node metastasis (p = 0.011), adrenal gland invasion (p<0.001) distant organ metastasis (p = 0.002) and tumor necrosis (p = 0.017) were positively associated with Ki-67 immunoreactivity. There were no statistically significant effects of capsular invasion, renal vein invasion, ureter invasion and microvascular invasion on Ki-67 immunoreactivity (p> 0.05).

p53: 72 of our cases were stained with p53 and 22 of them had no staining. In all cases, p53 immunoreactivity was found to be 10.04% ± 14.038% (min. 0%, max. 60%, median value: 5). The number of cases with no staining was 22 (23.4%), the number of cases with ≤ 5% immunoreactivity was 38 (40.4%) and the number of cases with > 5% immunoreactivity was 34 (36.2%) **(Figure 2C and D)**. There was a statistically significant difference in p53 immunoreactivity with pathological stage, Fuhrman nuclear grade and adrenal gland invasion (p = 0.016; p = 0.006; p =0.02). p53 immunoreactivity was not statistically significantly correlated with capsular invasion, gerota fascia invasion, pelvic adipose tissue invasion, perinephric adipose tissue invasion, ureter invasion, renal vein invasion, microvascular invasion, necrosis, tumor size, histological subtype, lymph node and distant organ metastasis (p>0.05). Correlation analysis showed that the p53 immunoreactivity and Fuhrman nuclear grades were correlated (p = 0.002 rho = 0.310). According to the Spearman's rank correlation test, Ki-67 and p53 correlated positively (p<0.001 rho = 0.337).

Beclin 1: 26 cases (27.7%) had no staining with Beclin 1. In 16 cases (17%) between 1-10%, in 18 cases (19.1%) between 11-50%, in 21 cases (22.3%) between 51-90% and in 13 cases (13.8%) between 91-100% Beclin 1 staining was observed. In 73 cases, the immunoreactivity of Beclin 1 in non-tumor normal renal tubules was investigated. No staining was observed in 12.5% of the cases. Over 68% of cases showed more than 50% staining. While 27.7% of tumor tissues were not stained, 36.1% had over 50% staining. There was a statistically significant relationship between the Beclin 1 immunoreactivity groups and Fuhrman nuclear grades (x = 30.16 p=0.003) **(Table 5).** There was a positive correlation between Beclin

Tablo 5. Beclin 1 immunoreactivity according to Fuhrman nuclear grade

Fuhrman nuclear grade		Beclin 1 immunoreactivity percentages				
		0	1-10%	11-50%	51-90%	91-100%
Grade I	Number of cases % Grade % Beclin 1	4 40% 15.4%	4 40% 25%	1 10% 5.6%	0 0% 0%	1 10% 7.7%
Grade II	Number of cases % Grade % Beclin 1	17 42.5% 65.4%	5 12.5% 31.3%	7 17.5% 38.9%	9 22.5% 42.9%	2 5% 15.4%
Grade III	Number of cases % Grade % Beclin 1	5 20.8% 19.2%	5 20.8% 31.3%	3 12.5% 16.7%	8 33.3% 38.1%	3 12.5% 23.1%
Grade IV	Number of cases % Grade % Beclin 1	0 0% 0%	2 10% 12.5%	7 35% 38.9%	4 20% 19%	7 35% 53.8%
X^2	30.16					
P	0.003					

Table 6. Correlation analysis of Beclin 1, p53 and Ki67 immunoreactivity

Spearman's Correlation Analysis		Ki-67	p53	Beclin 1
Ki-67	rho	1.000	0.337	0.359
	p	.	0.001	0.000
p53	rho	0.337	1.000	0.195
	p	0.001	.	0.060
Beclin1	rho	0.359	0.195	1.000
	p	0.000	0.06	.

1 immunoreactivity and Fuhrman nuclear grades in tumor cells (rho = 0.420 p<0.001). Beclin 1 immunoreactivity was not statistically significantly correlated with pathologic stage, capsular invasion, gerota fascia invasion, pelvic adipose tissue invasion, perinephric adipose tissue invasion, ureter invasion, renal vein invasion, adrenal gland invasion, microvascular invasion, lymph node and distant organ metastasis (p>0.05). A significant correlation was found between Beclin 1 immunoreactivity and necrosis (x = 12.2 p = 0.016).

There was a statistically significant difference between the Beclin 1 immunoreactivity groups and Ki-67 (p = 0.006) and p53 (p = 0.023). There was a positive correlation between Beclin 1 immunoreactivity and Ki-67 immunoreactivity, but no correlation with p53 immunoreactivity **(Table 6).** In addition, the immunoreactivity of p53 and Ki-67 also correlated.

A statistically significant negative correlation was found between Ki-67 immunoreactivity and overall survival (rho =-0.338 p = 0.002). While Ki-67 immunoreactivity increased, the survival time was decreased.

A statistically significant negative correlation was found between p53 immunoreactivity and overall survival (rho = -0.240 p = 0.03). While p53 immunoreactivity increased, the survival time was decreased.

There was a statistically significant effect of microvascular invasion on survival (x = 5.41 p = 0.02).

There was no statistically significant effect of the presence of necrosis on overall survival (p> 0.05), but necrosis was present in 8 of 9 cases who died.

DISCUSSION

It is important to determine prognostic parameters clinically, histopathologically and radiologically in RCC at the time of diagnosis. Histopathologically, the most important parameter is the stage of the tumor. In addition, histological subtype of tumor, tumor grade, microvascular invasion, and tumor necrosis are also important prognostic parameters. Cheville et al. study was shown that tumor necrosis was a prognostic parameter independent of tumor stage and grade in clear cell and chromophobe cell RCC but tumor necrosis in papillary RCC was not significant (5). In some studies, tumor necrosis is reported to be a prognostic factor related to survival regardless of tumor grade, TNM stage and tumor size(5, 19). Delahunt et al. determined that 10-year cancer-specific survival was 30% in patients with tumor necrosis, especially in nuclear grade 3 tumors, while this ratio was 62% in patients without tumor necrosis, and suggested that microscopic coagulative tumor necrosis should be added as an additional grading parameter in the ISUP nuclear rating system (20).

In our study, necrosis was detected in 51 cases (54.3%). A statistically significant relationship was found between necrosis and Fuhrman nuclear grade, tumor stage and tumor diameter. 34 (77.3%) of the 44 patients with Fuhrman nuclear grade 3 and 4 had necrosis, but necrosis was seen in only 17 (34%) of 50 cases with Fuhrman nuclear grade 1 and 2. The association of necrosis with sarcomatoid and rhabdoid differentiation was found to be significant. Tumor necrosis was not associated with p53, but was significantly associated with Ki-67 and Beclin 1 expression. 69.2% of RCC cases without Beclin 1 immunoreactivity did not have necrosis. Beclin 1 immunoreactivity was more than 50% in 49 of RCC cases with necrosis. Immunoreactivity was not observed in 41.9% of cases without necrosis. Briefly, the Beclin 1 immunoreactivity appears to be relatively related to the presence of necrosis. There was no statistically significant effect of necrosis on overall survival, but eight of nine patient who died has tumor necrosis. At the same time, tumor necrosis was found to be correlated with tumor stage, tumor size, Fuhrman nuclear grade and Ki-67 proliferation index.

Microvascular invasion and microvascular density have been shown to have a significant impact on metastasis, recurrence risk, and survival (21). In the study of Kwon et al., MVI and tumor necrosis in pT1b RCC were found to be independent prognostic factors from tumor stage (22). Bedke et al. study showed that MVI was found to be a weak prognostic factor correlating with tumor stage, Fuhrman nuclear grade and sarcomatoid differentiation in RCC (23). In our study, there were 21 (22.3%) cases with microvascular invasion. Similarly to other studies, a statistically significant relationship was found between MVI and tumor stage, Fuhrman nuclear grade, necrosis, lymph node and distant organ metastases in our study. MVI was not associated with Ki-67, p53, and Beclin 1 expression. In our study, there was a statistically significant relationship between microvascular invasion and survival. In other words, survival was reduced in RCC with microvascular invasion.

In some studies, a significant correlation was found between cancer-specific survival and Ki-67 score, and it was reported that it could be used as a strong marker to detect patients with poor prognosis (24). Rioux-Leclercq N. et al. found a significant correlation between cancer-specific survival and Ki-67 score. In their study, the Ki-67 index showed 20% score as a strong predictor of survival of patients (24). The mean survival was 67 months in patients with a Ki-67 score of <20%, and 42 months in patients with ≥ 20%(24). In some studies, there was a significant correlation between the diameter of the tumor and Ki-67 score (24) ,whereas the others were not correlated (25). Oda T. et al. study shows that there was no significant relationship between the tumor diameter and Ki-67 score in incidental RCC, but Ki-67 index / apoptotic index was found to be strongly associated with growth rate of carcinoma(26). In our study, unlike this study a significant relationship was found between Fuhrman nuclear grades and Ki-67 scores. As the Fuhrman nuclear grade increased, the Ki-67 score increased linearly and a strong relationship was found between them. In our study, a significant difference was found in the Ki-67 score between the tumors that showed sarcomatoid and rhabdoid differentiation and not showing tumors. The mean Ki-67 proliferation index was 35.8% in patients with sarcomatoid differentiation and 13.8% in patients without differentiation.

The mean Ki-67 proliferation index was 34.7% in patients with rhabdoid differentiation and 13.8% in patients without differentiation. In the study of Rioux-Leclercq et al and Haitel et al, a significant relationship was found between Ki-67 score and pathological tumor stage (pT) (24, 27). In the studies of Yuba et al., there was no significant relationship between Ki-67 score and tumor stage (25). In our study, when the pathological stages were classified as pT1, pT2, pT3, and pT4, a significant correlation was found between the tumor stage and the mean Ki-67 staining percentage. In our study, Ki-67 scores were found to be 10.8% in pT1, 16.2% in pT2, 23.5% in pT3 and 31.2% in pT4. Cheville et al. found a significant relationship between tumor necrosis and Ki-67 index (28). In our study, a significant correlation was found between necrosis and Ki-67 index, which supports this study. The mean Ki-67 index was 21.3% in necrosis cases and 11.8% in non-necrosis cases. In our study, the Ki-67 proliferation was found positively correlated with p53 and Beclin 1 and not correlated with MVI. When the Ki-67 immunoreactivity and survival were compared, a statistically significant negative correlation was found. In other words, while the Ki-67 immunoreactivity was increasing, the survival was decreased.

In some studies, it has been reported that p53 cannot have a prognostic value in RCC (29, 30). In a study by Rioux-Leclercq et al., p53 expression was found to be effective on survival in univariate analyses, but it was not an independent prognostic factor for survival in multivariate analyses (24). In a study by Noon et al. p53 was found to be an independent risk factor in RCC and correlates with lymph node metastasis and distant organ metastasis (31). In some studies, no relation was found between p53 mutation and tumor histologic subtype and tumor grade in RCC (32, 33). In our study, a statistically significant difference was found between Fuhrman nuclear grade and p53 immunoreactivity average of our cases. As Fuhrman nuclear grade increased, the percentage of p53 staining increased in direct proportion. P53 immunoreactivity was found in an average of 7.67% in Fuhrman nuclear grade 1 and 2 and 14.16% in Fuhrman nuclear grade 3 and 4 cases. In our study, a statistically significant difference was found between the pathological stages in terms of p53 immunoreactivity averages but no correlation was observed. The mean p53 immunoreactivity of our cases was 7.2%, 3.75%, 15.17% and 21.50% in pT1, pT2, pT3, and pT4, respectively.

There was no significant relationship between microvascular invasion, lymph node and distant organ metastasis with p53 immunoreactivity. In our study, p53 immunoreactivity was found to be 15.4% for rhabdoid differentiation, 9.14% for sarcomatoid differentiation, 9.65% for those without rhabdoid or sarcomatoid differentiation, and the difference between them was not statistically significant. There was no correlation between p53 immunoreactivity and tumor necrosis and Beclin 1 in RCC. When p53 immunoreactivity and survival were compared, it was found that there was a statistically significant negative correlation.

In the study by Warburton et al., 119 cases of RCC were investigated, and high-level staining of Ki-67 and p53 was found to be associated with decreased survival, but no correlation was found between Ki-67 and p53 expressions (34). In our study, a significant correlation was found between p53 and Ki-67. At the same time, both showed a negative correlation with survival.

Autophagy is activated under normal conditions to protect the cell from hypoxia, endoplasmic reticulum stress, starvation and effects of cytotoxic drugs used in the treatment of cancer (8). In early-stage tumors, autophagy and apoptosis protect normal cells against the effects of oxidative stress and genomic instability (35), but in advanced-stage tumors, autophagy increases both tumor progression and tumor survival (35). Tumor cells need high levels of oxygen, nutrients, and energy to maintain their proliferation. Tumor cells use autophagy to escape from apoptosis and necrosis (36). Despite intensive research, the role of autophagy in tumor progression is not well understood and may vary depending on the tumor type. For example, the decreased expression of Beclin 1 in breast, esophagus, gastric, cervical and colon carcinoma was found to be a poor prognostic indicator (37). On the contrary, increased expression of Beclin 1 in pancreatic and nasopharyngeal carcinoma was found to be a poor prognostic indicator (38). High Beclin-1 expression was reported to be a good prognosis indicator in two separate studies with hepatocellular carcinoma and non-small cell lung carcinoma (39, 40). There are also controversial results in RCC studies. In some studies, it has been suggested that autophagy suppresses the progression of RCC, while in others it is suggested that autophagy contributes to tumor progression by providing an alternative source of energy to the tumor cells (37, 41). In the study of Wang et al., autophagy was found to be suppressed in clear cell RCC and low autophagy level was shown to accompany high-grade clear cell RCC (42). In a study by Deng et al., it was reported that autophagy was less observed in metastatic and advanced stage RCC than localized RCC and autophagy was negatively correlated with Fuhrman nuclear grade (43). In contrast to this study, Nishikawa et al.'s study shows that the Beclin 1 immunoreactivity was positively correlated with pT stage, Fuhrman nuclear grade and MVI (44). In a study by Mathew et al., they claimed that the tumor cells need a high rate of nutrients and energy to sustain high rates of proliferation, and therefore, according to their study, tumor cells use autophagy to escape from apoptosis and necrosis (45). Kai-Fu et al. reported that malignant cells exhibit low levels of autophagic activity, but that defective autophagy causes tumor cells to adapt to environmental conditions (46).

In our study, in 72.3% of RCCs, different degrees of expression of Beclin 1 were observed. In 73 cases, Beclin 1 immunoreactivity was observed in normal renal tubules adjacent to the tumor, and no staining was observed in 12.5% of the cases and some normal kidney tubule cells were stained in 87.5% of the cases. In our study, normal renal tubules were always tubules in adjacent areas of the tumor and some of them were damaged due to the compression effect of the tu-

mor. Almost all of the cases showed chronic pyelonephritis in the surrounding renal parenchyma, while less simple cyst, adenoma, and dysplasia were observed. Since autophagy was a mechanism involved in providing homeostasis in the normal physiological process, it was normal to have Beclin 1 expression in the damaged tubule epithelial cells. In our study, there was a statistically significant positive correlation between Beclin 1 immunoreactivity and Fuhrman nuclear grades. In 80% of tumors with Fuhrman nuclear grade 1, Beclin 1 immunoreactivity was negative or less than 10%. All tumors with Fuhrman nuclear grade 4 had varying rates of Beclin 1 immunoreactivity. There was also a positive correlation between Beclin 1 and Ki-67 immunoreactivity. The correlation of Beclin 1 with the Furhman nuclear grade and Ki67 immunoreactivity shows that as tumor differentiation decreases, in other words, as the Fuhrman nuclear grade increases, tumor cells vary from normal tissue to different cell forms and increase the use of autophagy. As tumor differentiation decreases, tumor cells tend to proliferate more rapidly, thus increasing nutrient and energy requirements. This means that the need to use autophagy is increased. Normally, autophagy prevents tumor growth as a tumor suppressor in the physiological process, whereas in tumor cells with decreased differentiation, it increases the adaptation of the tumor cells to tumor microenvironment and causes it to behave more aggressively. Therefore, there are differences between the tumor autophagy and the autophagy, we see around the tumor even if we cannot fully explain the mechanism differences. We think that autophagy in the tumor is a kind of defective autophagy, unlike physiological autophagy. Tumor cells use defective autophagy for their survival by reducing energy and oxygen requirements, while normal epithelial cells and damaged cells use autophagy to eliminate both damaged cells and to prevent tumor growth.

As a result, there are some limitations of our study. These are being a retrospective study, which was included only 94 cases, limited follow-up time, and investigating only a single marker associated with autophagy. Despite all the limitations, our study is the first study to investigate the relationship between histopathological prognostic factors, Ki-67, and p53 with autophagy in RCC and their effects on survival. It is still early to use of anti-autophagic or autophagy-inducing agents in the treatment of RCC is early, because we believe that in order to fully understand the autophagy mechanism in RCC, it is necessary to investigate the cancer-specific survival rates by conducting wider and longer follow-up studies. As studies on this issue increase, the role of autophagy, apoptosis, and necrosis in tumorigenesis will be better understood. Then, understanding how different forms of cell death are controlled and their role in cancer development, can shed light on the development of effective treatments.

Conflict of Interest and Financial Status

The authors declare that there is no conflict of interest to declare. The author(s) received no specific funding for this work

Research Contribution Rate Statement Summary

The authors declare that, they have contributed equally to the manuscript.

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