Prognostic Value of *SOX9*, *E-Cadherin*, and *KLF4* Expressions in Clear Cell Renal Carcinoma

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ABSTRACT

Objective: To analyse the immunohistochemical staining of stem cell-related factors *SOX9* and *KLF4* in radical and partial nephrectomy specimens and to determine their relationship to grade, metastasis, stage, other prognostic parameters, and their predictive value.

Study Design: Descriptive study.

Place and Duration of the Study: Department of Pathology, Bolu Abant Izzet Baysal University, Faculty of Medicine, Bolu, Turkiye, from 2015 to 2022.

Methodology: The study included 92 cases diagnosed with clear cell renal cell carcinoma (ccRCC) from sections and blocks of partial and radical nephrectomies. Formalin-fixed and paraffin-embedded blocks were sliced. A Leica Bond Max staining device stained *SOX9*, *E-cadherin*, and *KLF4* onto the slides. Nuclear staining for *SOX9* and *KLF4* and membranous staining for *E-cadherin* were evaluated. Since *E-cadherin* loss has an important role in the development of malignancy, the *E-cadherin* staining pattern was considered when evaluating the staining patterns of *SOX9* and *KLF4*. The Mann-Whitney U and Kruskal-Wallis tests were applied to compare immunohistochemical data with prognostic factors such as gender, tumour size, grade, metastasis, and stage. The statistical analyses were conducted using the IBM SPSS version 20, with a p-value of less than 0.05 considered as statistically significant.

Results: Significant correlations were observed between *SOX9*, *KLF4*, and *E-cadherin* expression levels and high nuclear grade, with p-values of 0.005, <0.001, and 0.002, respectively. In addition, the overexpression of *SOX9* was significantly linked to distant metastasis and stage, with p-values of 0.010 and 0.025, respectively.

Conclusion: *SOX9*, *E-cadherin*, and *KLF4* expressions are linked to unfavourable prognostic factors in ccRCC. Thus, these immunohistochemical stains may serve as potential biomarkers in this cancer and aid in identifying prognosis and treatment response.

Key Words: *Clear cell renal carcinoma, SOX9 transcription factor, E-cadherin, Kruppel-like transcription factors, Prognosis*.

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INTRODUCTION

Renal cell carcinoma (RCC) is the most common malignant kidney tumour in adults, representing about 2% of all adult cancers. The incidence of renal cancer is typically reported as 6.1 per 100,000 in males and 3.2 per 100,000 in females.[1] The most common and aggressive histological type of RCC is clear cell RCC (ccRCC), developing in 60-80% of cases.[2,3]

Recent research has shown that cancer stem cells (CSCs) are now recognised as the key factor in tumour formation and metastasis. CSCs have various cell properties, including differentiation capacity, self-renewal, and resistance to apoptosis. Stem cell regulatory proteins are considered potential oncogenes due to their ability to regulate the CSC phenotypes.[4] CSCs have previously been shown to exist in RCC, contributing to resistance against radiotherapy and chemotherapy.[5,6]

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SOX9 (Sex-determining region Y (SRY)-related HMG box 9) belongs to the *SOX* gene superfamily. Recent studies have suggested that *SOX9* continues to be expressed in stem cell pools of ectoderm and endoderm-derived tissues, which may regulate CSCs.[4] *SOX9* functions as a transcription factor in the cell. *SOX9* gene is identified as an oncogene in lung adenocarcinoma, breast carcinoma, colorectal carcinoma, and prostate carcinoma.[7,8]

E-cadherin is a transmembrane glycoprotein involved in cell-cell adhesion found in epithelial tissues.[9] The loss of *E-cadherin* homophilic binding disrupts cell adhesion and intercellular connections, enabling cells to detach from the primary tumour, invade nearby tissues, and develop metastatic tumours.[10] *E-cadherin* has been previously demonstrated to exhibit tumour suppressor properties in various cancers, including hepatocellular carcinoma, head and neck cancers, squamous cell carcinoma of the skin and oesophagus, and melanoma.[11,12]

Kruppel-like factor 4 (*KLF4*) is part of the Kruppel-like factor family, which contains zinc fingers that play a role in various physiological processes, including cell cycle regulation, pluripotency, epidermal development, and the maintenance of tissue homeostasis. It acts as a transcription factor in the cell.

Depending on the target gene, it can activate or suppress transcription through different mechanisms.[13] *KLF4* downregulation is often observed in gastric, colorectal, hepatic, and prostate cancers.[14-16]

The aim of this study was to examine the expressions of *SOX9*, *KLF4*, and *E-cadherin* in ccRCC cases, explore the relationships between these markers and among ccRCC patients, and identify the associations between these markers and clinicopathological parameters with prognostic relevance.

METHODOLOGY

The study included partial and radical nephrectomy specimens from 92 ccRCC patients who underwent surgery at the Department of Pathology, Bolu Abant Izzet Baysal University, Faculty of Medicine, Bolu, Turkiye, from 2015 to 2022. Nephrectomy materials diagnosed with non-clear cell renal cell carcinoma (ccRCC), non-tumour resection materials, and needle biopsies were excluded. The selected blocks were those that best represented the tumour and were most appropriate for immunohistochemical analysis.

Formalin-fixed paraffin-embedded ccRCC tissue blocks were retrieved from the pathology department archives for retrospective analysis. All gross specimens were meticulously examined according to the guidelines established by the College of American Pathologists. Histological diagnosis was made through H&E staining of sections obtained from these blocks. Data on various parameters, such as age, gender, tumour size, grade, stage, perirenal fat invasion, renal sinus invasion, sarcomatoid and rhabdoid differentiation, lymphatic, and vascular invasion, and distant metastasis, were recorded.

Four-micron sections were obtained from the formalin-fixed paraffin-embedded blocks. These sections were stained with primary antibodies against *SOX9*, *E-cadherin*, and *KLF4* using a Leica Bond-Max fully automated immunohistochemistry system. Immunohistochemical staining was carried out with a standard compact polymer technology kit. Two pathologists independently evaluated the immunohistochemically stained slides.

The sections were stained using the *SOX9* antibody (Epitomics, Burlingame, USA, clone: EP317, diluted at 1/150). Colon adenocarcinoma sections were positive controls for *SOX9*, with nuclear staining considered a positive result. The positive cells' total percentage (1: 1-10%, 2: 11-40%, 3:41-70%, 4: 71-100 %) and staining intensities (0-3) were determined.

Sections were stained with *E-cadherin* antibody (Leica, Newcastle, UK, clone: 36B5, diluted: 1/25). Membranous staining for *E-cadherin* was considered positive, and metastatic invasive ductal carcinoma in the axillary lymph node was used as a positive control. The total percentage of positive cells (0: No staining, 1: 1-24%, 2: 25-49%, 3: 50-74%, 4: 75-100%) and their staining intensities (0-3) were assessed.

Sections were stained with *KLF4* antibody (Millipore, Temecula, USA, clone: 1E6, diluted: 1/250). Normal colon tissue sections served as positive controls. Nuclear staining was regarded as a positive indicator for *KLF4*. The total percentage of positive cells (0: No staining, 1: ≤1%, 2: >1%) and their staining intensities (0-3) were evaluated.

Finally, a combined immune score for each immunohistochemical staining was calculated by summing the staining intensity and percentage scores. The combined immune scores were then analysed.

Considering the previous studies, taking at least 93 observations with 80% power and 5% margin of error was sufficient, hypothesising that the effect size between the groups would be 0.31. The number of samples was calculated with the PASS 11.0 programme. In descriptive statistics, mean and standard deviation or median and 25^{th} – 75^{th} percentile values will be given for numerical variables, and number and percentage values will be given for categorical variables. Kolmogorov-Smirnov test was used to make the normality assumption. Since the assumptions were not met, the Mann-Whitney U test was used to determine the difference between the two groups. The Kruskal-Wallis test was used to determine whether there were differences between three or more groups because the assumptions were unmet. In case of a difference, a pairwise comparison test determined the groups that created the difference. Statistical analysis was performed using the Kruskal-Wallis test to determine if prognostic factor measurements such as tumour grade and stage were based on immunohistochemical staining positivity. A paired samples t-test was performed to reveal whether there was any significant difference between immunohistochemical staining positivity in the tumour and peritumoural kidney parenchyma. All statistical analyses were conducted using the IBM SPSS version 20, with a p-value of less than 0.05 considered as statistically significant.

RESULTS

A total of 92 cases were included in the study, comprising 69 (75%) males and 23 (25%) females, with a mean age of 59.5 ± 10.6 years ranging from 29 to 85 years. The average tumour diameter was 5.46 cm, and the median tumour diameter was 4.50 cm. Based on tumour size, patients were categorised into two groups, ≤4 cm and >4 cm, according to median tumour diameter. Tumour grading was assessed according to the WHO/ISUP classification. Histological grades were distributed with 9 (9.8%) patients in grade 1, 53 (57.6%) patients in grade 2, 23 (25%) patients in grade 3, and 7 (7.6%) patients in grade 4 (Figure 1A-D). Distant metastasis was observed in 14 patients (20%). Regarding tumour stage, 57 (62%) of patients were classified as stage 1, 4 (4.3%) as stage 2, 15 (16.3%) as stage 3, and 16 (17.4%) as stage 4. The clinicopathological characteristics of the cases are presented in Table I.

SOX9 nuclear staining was observed in tumour cells in all cases. Weak staining was detected in 17 (18.5%) cases, moderate staining in 32 (34.8%) cases, and intense staining in 43 (46.7%) cases (Figure 2A-C). The combined immune scores from the staining extent and intensity ranged between 2 and 7. *SOX9*

staining was found to be more intense at the invasive margins, where it merged with the normal renal parenchyma. Higher *SOX9* expression was observed in tumour tissue compared to peritumoural kidney parenchyma ($p < 0.001$).

Table I: The clinicopathological features of the patients.

Parameters		Total n = 92 n (%)
Age	≤60	47 (51.1%)
	>60	45 (48.9%)
Gender	Female	23 (25%)
	Male	69 (75%)
Tumour diameter	≤4 cm	40 (43.5%)
	>4 cm	52 (56.5%)
Grade	G1	9 (9.8%)
	G2	53 (57.6%)
	G3	23 (25%)
	G4	7 (7.6%)
Lymphatic and vascular invasion	Present	14 (15.2%)
	Absent	78 (84.8%)
Metastasis	M0	56 (80%)
	M1	14 (20%)
Stage	I	57 (62%)
	II	4 (4, 3%)
	III	15 (16, 3%)
	IV	16 (17, 4%)

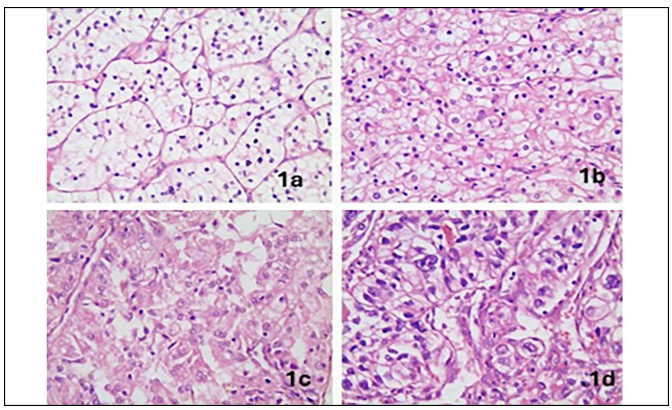

Figure 1: (A) Grade 1, cells with abundant clear cytoplasm and small hyperchromatic nuclei are seen. Nucleoli are indistinct at 40x magnification. (B) In grade 2, eosinophilic nucleoli are evident at 40x magnification. (C) Grade 3 cells with eosinophilic cytoplasm with prominent eosinophilic nucleoli at 40x magnification. (D) Grade 4, large pleomorphic cells are seen at 40x magnification.

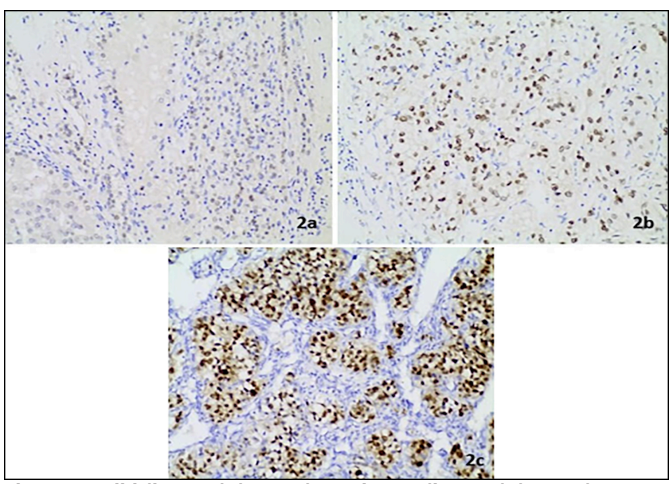

Figure 2: Mild (image (A), x200), moderate (image (B), x200), strong (image (C), x200) *SOX9* expressions in tumour cells.

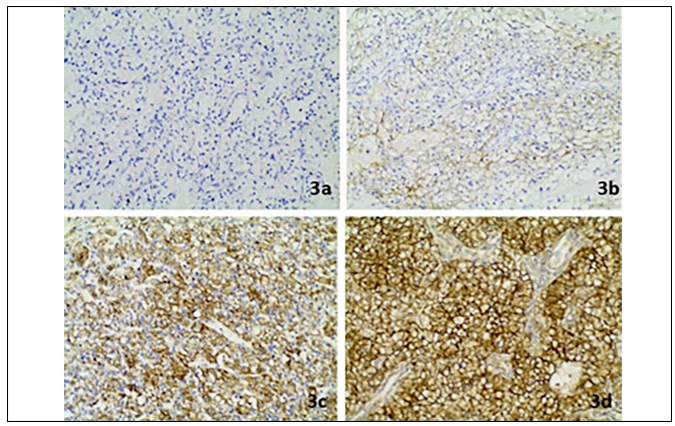

Figure 3: Negative (image (A), x200), mild (image (B), x200), moderate (image (C), x200), strong (image (D), x200) *E-cadherin* expressions in tumour cells.

Significant associations were found between *SOX9* expression and high nuclear grade ($p = 0.005$), sarcomatoid and rhabdoid differentiation ($p = 0.010$), distant metastasis ($p = 0.025$), and stage ($p = 0.020$). However, no significant association was observed between *SOX9* expression and age, gender, tumour size, necrosis, lymphatic, and vascular invasion, perirenal fat invasion, or renal sinus invasion.

E-cadherin exhibited positive membrane-associated staining in 88 (95.7%) cases. No staining was observed in 4 (4.3%) cases, weak staining in 28 (30.4%) cases, moderate staining in 44 (47.8%) cases, and intense staining in 16 (17.4%) cases (Figure 3A-D). The combined immune score from the staining extent and intensity ranged between 0 and 7. Higher *E-cadherin* expression was seen in peritumoural kidney parenchyma compared to tumour tissue ($p < 0.001$).

Significant correlations were found between decreased *E-cadherin* expression and high nuclear grade ($p = 0.003$), sarcomatoid and rhabdoid differentiation ($p = 0.036$), and distant metastasis ($p = 0.033$).

Considering combined immune scores by clinical staging, *E-cadherin* expression was lowest in Stage 4 tumours and highest in Stage 3 tumours. No significant differences were found between the *E-cadherin* combined immune scores and stage groups ($p = 0.304$).

E-cadherin expression did not differ significantly in age, gender, tumour diameter, necrosis, lymphatic and vascular invasion, perirenal fat invasion, and renal sinus invasion.

KLF4 demonstrated positive nuclear staining in 21 (22.8%) cases. No staining was observed in 71 (77.2%) cases, weak staining in 5 (5.4%) cases, moderate staining in 9 (9.8%) cases, and intense staining in 7 (7.6%) cases (Figure 4A-D). The combined immune score from the staining extent and intensity ranged from 0 to 5. In the peritumoural kidney parenchyma, nuclear staining was observed in the tubules, mainly in the distal and collecting tubules. Mild positive staining was observed in 10 (10.9%) of 92 cases. Higher *KLF4* expression was seen in peritumoural kidney parenchyma compared to tumour tissue ($p < 0.001$).

Table II: *SOX9*, *E-cadherin*, and *KLF4* expression by prognostic factors.

CCRCC		*SOX9*		*E-cadherin*		*KLF4*	
		Mean ± SD	p-value	Mean ± SD	p-value	Mean ± SD	p-value
Age	≤60	5.00 ± 1.69	0.392	4.13 ± 1.81	0.456	0.64 ± 1.49	0.234
	>60	4.69 ± 1.83		4.42 ± 1.43		0.93 ± 1.58	
Gender	Female	4.78 ± 1.85	0.727	4.09 ± 1.73	0.360	1.04 ± 1.69	0.317
	Male	4.87 ± 1.74		4.33 ± 1.61		0.70 ± 1.48	
Tumour diameter	≤4 cm	5.09 ± 1.63	0.198	4.17 ± 1.51	0.616	1.26 ± 1.84	0.004
	>4 cm	4.61 ± 1.86		4.37 ± 1.76		0.30 ± 0.96	
Grade	G1	5.56 ± 1.50	0.005	4.78 ± 1.30	0.002	3.00 ± 2.06	0.000
	G2	4.51 ± 1.76		4.68 ± 1.49		0.74 ± 1.44	
	G3	4.74 ± 1.68		3.78 ± 1.38		0.26 ± 0.91	
	G4	6.86 ± 0.37		2.14 ± 2.03		0.00 ± 0.00	
Lymphatic and vascular invasion	Present	5.14 ± 1.83	0.464	3.57 ± 2.10	0.178	0.36 ± 0.92	0.336
	Absent	4.79 ± 1.75		4.40 ± 1.52		0.86 ± 1.61	
Metastatis	M0	4.68 ± 1.71	0.025	4.44 ± 1.51	0.040	0.90 ± 1.63	0.107
	M1	5.79 ± 1.76		3.36 ± 2.02		0.14 ± 0.53	
Stage	I	4.98 ± 1.67	0.020	4.33 ± 1.36	0.200	1.18 ± 1.79	0.020
	II	3.50 ± 1.29		5.50 ± 1.29		0.00 ± 0.00	
	III	3.93 ± 1.79		4.40 ± 1.99		0.20 ± 0.77	
	IV	5.56 ± 1.75		3.63 ± 2.09		0.13 ± 0.50	

Significant associations existed between reduced *KLF4* expression and high nuclear grade ($p < 0.001$), high stage ($p = 0.020$), tumour diameter ($p = 0.004$), and renal sinus invasion ($p = 0.024$).

KLF4 expression did not significantly differ based on age, gender, necrosis, lymphatic and vascular invasion, perirenal fat invasion, or distant metastasis.

Table II shows the relationship between clinicopathological factors and combined immune scores of *SOX9*, *KLF4*, and *E-cadherin* staining.

DISCUSSION

ccRCC is the most prevalent subtype of RCC, and various prognostic factors have been described for it to date. The most prominent one may be the tumour's pathological stage. Among tumours of the same stage, poor prognostic parameters include tumour grade, presence of necrosis, and sarcomatoid and rhabdoid differentiation.[17]

Numerous immunohistochemical and molecular studies have been conducted on the clinical use of CSCs-related factors in determining the prognosis of RCC and ccRCC.[6] This study explored the roles of *SOX9* and *KLF4*, along with recognised factors, in regulating the CSC phenotype and influencing the development and prognosis of ccRCC.

There are very few studies on *SOX9* in RCC.[2,8,18] Two of these studies examined the immunohistochemical expression of *SOX9* and its association with prognostic factors.[2] Two studies found a significant association between *SOX9* expression and high tumour grade.[8,18]

Li *et al*. found that 16 of 38 cases with metastasis were *SOX9* positive.[18] On the other hand, Wan *et al*. found *SOX9* expression to be significantly associated with increased stage.[8] Increased *SOX9* expression was detected in grade 4 tumours compared to grade 2 and 3 tumours. Additionally, elevated *SOX9* expression was observed in 14 cases with distant metastases.

Activation of epithelial-mesenchymal transition (EMT) results in the development of cancer stem cells and metastasis in malignant epithelial cells. The primary factor involved in EMT is *E-cadherin* downregulation.[3] Previous research has shown that *E-cadherin* expression strongly correlates with advanced pathological stages[9] and grades.[3,11,19,20] Therefore, decreased *E-cadherin* expression may be seen in the sarcomatoid component of the tumour.[21] This study identified a significant reduction in *E-cadherin* expression in high-grade tumours, with notably lower expression in the sarcomatoid and rhabdoid components.

Previous studies have demonstrated that the loss of *E-cadherin* expression is associated with tumour invasion, metastasis, and poor survival. Metastatic RCCs may also show significantly low *E-cadherin* expression.[3,9] The study discovered a significant relationship between distant metastasis and low *E-cadherin* expression. Loss of *E-cadherin* expression is often expected to affect EMT and lead to metastasis directly, and these findings align with those reported in the literature.[3]

Three studies have inquired about the role of *KLF4* in developing RCC and ccRCC. Their findings revealed that *KLF4* has a tumour suppressor role in developing RCC.[6,16,22,23] Song *et al*. showed that *KLF4* overexpression significantly inhibits proliferation in human ccRCC cell lines.[16]

Song *et al*. observed moderate-to-intense positive staining in tumour tissue in 40% of ccRCC cases, whereas Li *et al*. reported this rate as 13%.[16,22] Liu *et al*. investigated *KLF4* expression through molecular techniques.[6] They found *KLF4* downregulation in 16% of the cases. Three studies showed significantly lower *KLF4* expression in tumour tissue than in normal renal parenchyma.[6,16,22] In this study, *KLF4* stained positive in 22.8% of the cases, with positivity found in 10.9% of normal renal parenchyma.

Li *et al*. found significant associations between low *KLF4* expression, stage, and lymph node metastasis.[22] Significantly higher *KLF4* expression was found in Stage 1 tumours than Stage 3 and 4 tumours. Additionally, *KLF4* expression was lower in patients with distant metastasis.

Song *et al*. found significantly lower *KLF4* expression in cases with a high tumour diameter (above 7 cm).[16] Substantially lower *KLF4* expression was observed in cases with a tumour diameter over 4 cm. Similarly, when the tumour diameter was categorised as either less than or greater than 7 cm, significantly lower *KLF4* expression was found in the group with larger tumour diameters.

The present study has several limitations. Due to its single-centre design and small sample size, the findings could not be generalised. Additionally, extensive research on the expression of *SOX9* and *KLF4*, particularly with immunohistochemical data, is limited in ccRCC of the kidney. The limited availability of comparable data makes it challenging to interpret the existing information.

CONCLUSION

Analysis of CSC-related factors in cases with ccRCC suggests that changes in the expression of these factors may be one of the oncogenic mechanisms underlying the pathogenesis of ccRCC. Specifically, findings highlight that *SOX9* and *KLF4* expressions contribute to the carcinogenesis and progression of ccRCC, indicating that CSC-related factors are likely involved in its pathogenesis.

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ETHICAL APPROVAL:
This study was approved by the Bolu Abant Izzet Baysal University's Ethics Committee for Clinical Research (No: 2020/226).

PATIENTS' CONSENT:
Patients' consent was not required as the patients' identities were not disclosed or compromised.

COMPETING INTEREST:
The authors declared no conflict of interest.

AUTHORS' CONTRIBUTION:
SO: Designed the study, collected and analysed the data, and prepared the manuscript.
SED: Designed the study, reviewed and edited the manuscript.
Both authors approved the final version of the manuscript to be published.

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