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New biomarkers for differentiating renal neoplasms with eosinophilic cytoplasm: DARS2, reelin, and enkurin

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Ethics Committee Approval
The study was approved by Fırat University Non-Interventional Health Research Ethics Committee (date 01.12.2022 and number 2022/14-14). All procedures in this study involving human participants were performed in accordance with the 1964 Helsinki Declaration and its later amendments.

Conflict of Interest
No conflict of interest was declared by the authors.

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Abstract

Background/Aim: Chromophobe renal cell carcinoma (CRCC), eosinophilic variant clear cell RCC, and oncocytomas are renal neoplasms with eosinophilic cytoplasm, and their differential diagnosis is challenging despite significant advances in molecular pathology. Although many biomarkers have been identified for the differential diagnosis of these neoplasms, specific markers have not yet been reported. No studies were found in the literature on the relationship between these tumors and the new molecules DARS2, reelin, and enkurin. This paper aims to determine the roles of these proteins in renal neoplasms with eosinophilic cytoplasm.

Methods: The study retrospectively analyzed 30 EC RCC, 30 CRCC, and 30 oncocytoma cases, evaluated among renal neoplasms with eosinophilic cytoplasm, independent of demographic characteristics, in the Fırat University Medical Pathology Laboratory between 2012 and 2022. The most representative samples of the tumor were selected for each group, and the expression of DARS2, reelin, and enkurin proteins was evaluated by the immunohistochemical method.

Results: The histoscore of DARS2 expression was highest in EC RCC and least in CRCC. DARS2 was seen to differentiate CRCC from oncocytoma and EC RCC. The histoscore of reelin and enkurin protein expression was highest in oncocytoma and lowest in ECRCC. The difference between the groups was statistically significant ($P<0.05$).

Conclusion: DARS2 can be a useful biomarker for differentiating CRCC from EC RCC and oncocytoma, and enkurin and reelin can differentiate among these three groups.

Keywords: chromophobe renal cell carcinoma, eosinophilic variant clear cell renal cell carcinoma, oncocytoma, DARS2, reelin, enkurin

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Introduction

Renal Cell Carcinoma (RCC) originates from the renal cortex epithelium, mostly the upper pole of the kidney, and is a common urological cancer with the highest mortality [1]. RCC constitutes approximately 85% of all parenchymal kidney tumors and 3% of adult solid tumors, with men aged 60–70 years being the most commonly affected group [2]. Several tumor-related prognostic factors, such as tumor stage, size, histological subtype, ISUP nuclear grading, lymphovascular invasion, and the presence of sarcomatoid differentiation, are present in RCC [1,2]. However, the most crucial prognostic factor is the pathological stage, and the 5-year survival rate of patients with Stage I or II cancer at the time of diagnosis ranges between 80% and 90% [1,2].

While tumors detected at an early stage have a high response to treatment, the treatment of advanced renal cancer is difficult, and the mortality rate is significantly high due to blood or lymphatic spread [3].

Eosinophilic Variant Clear Cell RCC (EC RCC) is a high-grade tumor with cells containing granular eosinophilic cytoplasm often seen around areas of hemorrhage and necrosis. EC RCCs commonly metastasize to the lungs, liver, soft tissue, and pleura, with an average of 45% of renal vein invasion [4].

Chromophobe RCCs (CRCCs) are malignant epithelial kidney tumors that originate from the intercalated cells of the collecting duct system and have a better prognosis compared to EC RCC but still have metastatic potential. Several ultrastructural studies have shown that numerous cytoplasmic microparticles characterize typical pale cells of CRCC due to defective mitochondrial development [5]. Conversely, oncocytomas are mitochondria-rich cells originating from intercalated similar to CRCC and are benign epithelial neoplasms consisting of large cells with large eosinophilic cytoplasm [6,7].

Oncocytomas are rare, predominantly benign neoplasms of the epithelium, causing respiratory defects and developing as a result of inactivating mutations in enzymes or control regions encoded by the mitochondrial genome, leading to the accumulation of defective mitochondria [8]. Despite their clinical differences and changes in their response to treatment, differential diagnosis of EC RCC, CRCC, and oncocytoma, which have similar histological structures, is one of pathology's most crucial and difficult aspects [9,10].

Although many immunohistochemical markers are used in differential diagnosis along with morphological findings, the inadequacy of these markers increases the need for an ideal single immunohistochemical marker or panel [10].

DARS2 is a mitochondrial protein with effects on tumorigenesis, and studies conducted on the relationships between mitochondrial dysfunctions and tumorigenesis made it valuable to examine mitochondrial proteins for many tumors [11]. Reelin is a protein that plays a significant role in regulating neuronal migration, dendritic growth/branching, dendritic spine formation, synaptogenesis, and synaptic plasticity in the brain, and it also affects the development of signaling pathways of lymphatic vessels, mammary glands, submaxillary glands, small intestine, cartilage, bone, and the immune system, liver fibrosis, and multiple cancers in adults [12]. Enkurin (canonical transient receptor potential) is a calcium-permeable cationic plasma membrane channel and was the subject of treatment-targeted studies for various cancer types. Enkurin binds to the oncogenic transcription factor (C-Jun) promoter, modulating many genes, and exerts anti-metastatic effects [13].

Here we examine the roles of DARS2, Reelin, and Enkurin proteins in the differential diagnosis of EC RCC, CRCC, and oncocytoma.

Materials and methods

Participants

We retrospectively re-evaluated all resection materials diagnosed as renal cell carcinoma at the Medical Pathology Laboratory of Fırat University between 2012 and 2022. Ethics approval was obtained from the Fırat University Non-Interventional Health Research Ethics Committee on 01.12.2022 (2022/14-14). We included 90 cases of renal cell carcinoma diagnosed with a renal cell carcinoma subtype and had their tumor tissue removed by total or partial nephrectomy. Cases diagnosed with needle biopsy and those in which the tumor subtype could not be determined excluded from the study. We studied 30 diagnosed cases of EC RCC, 30 Chromophobe RCC, and 30 oncocytoma cases. The patient's age, gender, type of surgery (total/subtotal resection), and pathological diagnosis were obtained from patient files and pathology reports.

Immunohistochemistry

For each disease group, up to ten Hematoxylin-Eosin stained sections were examined, and an immunohistochemical examination was performed by selecting the samples that best represented the tumor areas. The tissue samples of the groups were evaluated by a pathologist and a histologist blinded to the study.

Immunohistochemistry

Immunohistochemical procedures were performed as described by Kocaman and Artas [14]. Tissue microarray slides 3 µm thick were used for immunohistochemistry (IHC). We used the following antibodies: Anti-AspRS antibody (Sc-166535; Santa Cruz Biotechnology, Oregon, USA), anti-Reelin antibody (Sc; MyBioSource, Santa Cruz Biotechnology, Oregon, USA), and polyclonal anti-Enkurin Antibody (PA5-58028; ThermoFisher Waltham, Massachusetts, USA). Using indirect immunohistochemical staining, we calculated a histoscore to measure tissue levels of DARS2, Reelin, and Enkurin.

Microscopic evaluation of staining intensity

We used a scoring system to assess the distribution and intensity of staining, where the distribution was scored as 0.1 for <25%, 0.4 for 26-50%, 0.6 for 51-75%, and 0.9 for 76-100%. The intensity of staining was scored as 0 for no staining, 0.5 for very little staining, 1 for little staining, 2 for moderate staining, and 3 for very strong staining. We calculated a histoscore by multiplying the distribution and intensity scores [14].

Statistical analysis

We analyzed the data using the Statistical Package for Social Sciences for Windows version 22.0 (SPSS, Chicago, IL) program. Descriptive data were expressed as mean (standard error) and numbers. We evaluated the distribution of the data using the Shapiro-Wilk Test. We used the One-Way Analysis of Variance (ANOVA) Test and the Post-Hoc Dunn Test to

compare the data showing normal distribution. The significance level was set at $P<0.05$.

Results

General characteristics of the subjects

The demographic characteristics of the patients are given in Table 1. Thirty EC RCC, CRCC, and oncocytoma group patients were evaluated. In these cases, CRCC was more common in women, and EC CRCC and oncocytoma were more common in men. Among the tumor groups, the mean patient age in eosinophilic clear cell renal cell carcinoma cases was 59.00 years (min–max: 32–85); the mean age in chromophobe renal cell carcinoma cases was 60.60 years (min–max: 27–80); the mean age in oncocytoma was 63.70 years (min-max: 34–82); and no significant difference was detected between the groups in terms of age ($P=0.303$). When the groups were evaluated in terms of gender, a significant difference was detected ($P=0.009$).

Table 1: Summary of patients' clinical data

	Oncocytoma	CRCC	EC RCC	*P*-value
N (F/M)	30 (13/17)	30 (18/12)	30 (8/22)	0.009
Age	63.7 (34–82)	60.6 (27–80)	59 (32–85)	0.303

CRCC: Chromophobe renal cell carcinoma, EC RCC: Eosinophilic variant clear cell. Descriptives are expressed as median (min-max).

Histochemical findings

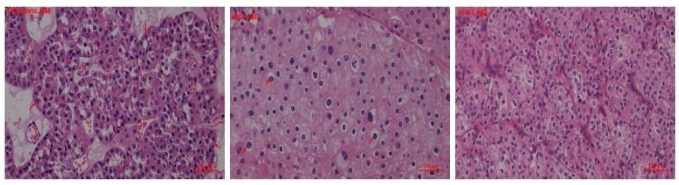
In the histopathological examination, oncocytoma sections showed tumors that consisted of solid cell nests in the loose edematous stroma, large granular eosinophilic cytoplasm, central nucleus, and uniform image. The CRCC sections showed tumors of cell layers with large eosinophilic reticular cytoplasm, significant cytoplasmic borders, clear perinuclear halo, and irregular hyperchromatic nuclei. For the EC RCC sections, we observed tumors with large pale eosinophilic cytoplasm, hyperchromatic nuclei in places, and slightly pleomorphic cell nests (Table 2, Figure 1).

Table 2: Histoscore of DARS2, reelin and enkurin for eosinophilic variant clear cell RCC, chromophobe renal cell carcinoma, oncocytoma

	EC RCC	CRCC	Oncocytoma
DARS2	2.40 (0.43)	1.06 (0.15)[a]	2.46 (0.40)[b]
Reelin	0.01 (0.02)	0.73 (0.17)[a]	1.15 (0.28)[ab]
Enkurin	0.02 (0.09)	0.33 (0.18)[a]	1.03 (0.17)[ab]

CRCC: Chromophobe renal cell carcinoma, EC RCC: Eosinophilic variant clear cell, a: compared with the EC RCC group, b: compared with the CRCC group

Figure 1: Hematoxylin-eosin image in eosinophilic variant clear cell RCC, chromophobe renal cell carcinoma, oncocytoma lesion areas



Immunohistochemical findings

Using immunohistochemistry, we stained the tissue samples of EC RCC, CRCC, and oncocytoma with DARS2, Reelin, and Enkurin. We formed a histoscore based on the extent and intensity of the staining. We compared the groups regarding DARS2, Reelin, and Enkurin expression. We evaluated and photographed the slides under a Zeiss Axio (Scope A1 Berlin, Germany) microscope ($P<0.05$) (Figures 1–4, Table 2).

DARS2, reelin, and enkurin immunoreactivity

DARS2, reelin, and enkurin cytoplasmic staining were performed in CRCC, EC RCC, and oncocytoma samples. We examined the immunohistochemical staining for DARS2 immunoreactivity under light microscopy and obtained the following findings. As shown in Table 2, DARS2 expression was detected in the EC RCC, CRCC, and oncocytoma groups. DARS2 expression was mostly observed in EC RCC and least in CRCC and oncocytoma. When we compared DARS2 expression between the groups, we found that it differentiated CRCC from oncocytoma and EC RCC, and the difference was statistically significant ($P<0.05$) (Figure 2, Table 2).

The expression of reelin and enkurin proteins was highest in oncocytoma, less in CRCC, and least in EC RCC. We found the difference in expression between the groups to be statistically significant ($P<0.05$) (Figures 3 and 4, Table 2).

Figure 2: Immunohistochemical reactivity (red arrow) of DARS2 protein at lesion sites in eosinophilic variant clear cell RCC, chromophobe renal cell carcinoma, oncocytoma.

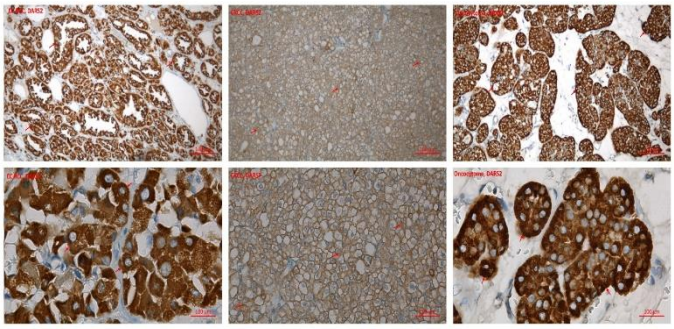


Figure 3: Immunohistochemical reactivity (red arrow) of reelin protein at lesion sites in eosinophilic variant clear cell RCC, chromophobe renal cell carcinoma, oncocytoma.

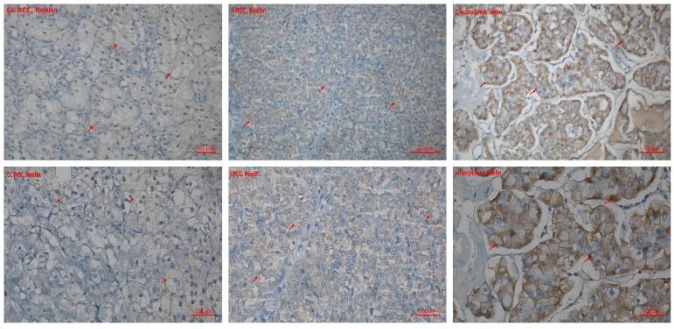
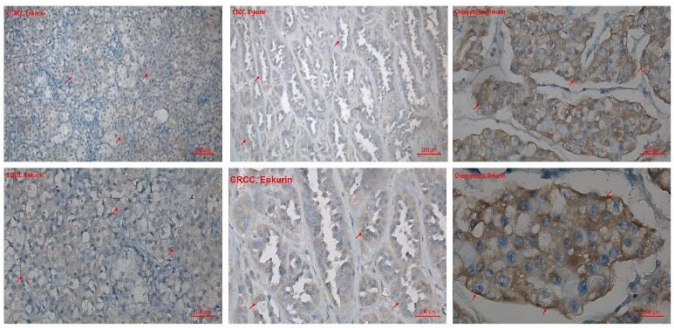


Figure 4: Immunohistochemical reactivity (red arrow) of enkurin protein at lesion sites in eosinophilic variant clear cell RCC, chromophobe renal cell carcinoma, oncocytoma.



Discussion

Immunohistochemistry is a valuable diagnostic tool in cases where RCCs with eosinophilic cytoplasm cannot be diagnosed based on morphological results, a current differential diagnosis problem [7]. The histopathological distinction of oncocytomas, Chromophobe RCC, and eosinophilic variant clear cell RCC is an important and frequently encountered challenge for pathologists. Tumoral structures with granular eosinophilic cytoplasm, hyperchromatic nuclei, and slightly pleomorphic cells are generally observed, making it difficult to diagnose histopathologically. Many molecules, such as kidney-specific cadherin, parvalbumin, claudin-7, and claudin-8, are sensitive

biomarkers for renal neoplasms, including Chromophobe RCC and oncocytoma [10]. However, previous studies have reported that many of the markers used are insufficient in the differential diagnosis, and it has become imperative to develop tumor-specific biomarkers. For example, CD117 is secreted from normal adult kidney parenchyma and can be used to differentiate classical RCC cases from other RCCs when it is negative. However, it is useless in differentiating oncocytoma from Chromophobe RCC because this marker is positive in both tumors [15].

No information was found in the literature regarding the relationship of DARS2, Reelin, and Enkurin proteins with EC RCC, CRCC, and oncocytoma. Aminoacyl-tRNA Synthetases (ARSs) are critical enzymes that synthesize proteins by catalyzing amino acids with tRNAs [16]. Aspartyl-tRNA Synthetase 2 (DARS2), encoded by the Class II aminoacyl-tRNA Synthetase family gene, is a mitochondrial enzyme specifically aminoacylates Aspartyl-tRNA and has been reported to be a novel biomarker for bladder cancer and acute leukemia [17-19]. Additionally, a previous study showed that DARS2 could be a biomarker to differentiate malignant mesothelioma from lung adenocarcinoma [20].

This study, DARS2 expression was detected in all groups, with the highest expression in EC RCC and oncocytoma. DARS2 expression differentiated CRCC from EC RCC but not from oncocytoma. These findings suggest that DARS2 may be associated with the tumorigenesis effect of EC RCC and oncocytoma. Overexpression of DARS2 has been previously shown to accelerate tumorigenesis in hepatocellular carcinoma [21]. In oncocytoma, the overexpression of DARS2 can be explained by the fact that this tumor is rich in mitochondria, and DARS2 is a mitochondrial protein [11]. The significantly lower expression of DARS2 in CRCC compared to oncocytoma may be due to more cells with pale eosinophilic cytoplasm, which are poorer in mitochondria [22]. Overall, DARS2 may be a potential biomarker for distinguishing between RCC subtypes.

The RELN gene encodes reelin, a large glycoprotein that functions in neuronal and non-neuronal tissues. Reelin is involved in developing various tissues, including the liver, kidney, and breast. Studies have shown that its expression is decreased in certain cancers such as breast, stomach, and pancreatic cancer [23]. In breast cancer, the RELN gene is epigenetically dysfunctional in the cancerous area, while normal tissues adjacent to the tumor continue to release reelin. Low reelin release has been linked to increased cancer cell migration, positive lymph node involvement, and poor prognosis. Conversely, increased reelin levels may have a suppressive effect on cancers [24,25].

Enkurin is a novel molecule with unresolved structure and function. It was first reported as an essential adapter in localizing a Ca2+ permeable ion channel in sperm [26]. Recent research has shown that Enkurin may act as a tumor suppressor in colorectal cancers and lung adenocarcinoma, inhibiting the proliferation, migration, and invasion of tumor cells. Epigenetic deficiency of Enkurin may accelerate tumor progression. These findings suggest that Enkurin could be an effective target for cancer therapy [27-29].

The expression of Reelin and Enkurin proteins was highest in oncocytoma, less in CRCC, and less in EC RCC, with statistically significant differences between the groups. Previous studies have shown that decreased secretion of these proteins is associated with increased cancer aggressiveness, possibly due to epigenetic deficiency. Conversely, higher levels in less aggressive and benign neoplasms may be due to their tumor suppressor roles [12,25]. Therefore, the low secretion of Reelin and Enkurin in EC RCC in this study may also contribute to the aggressiveness of this tumor, although further research is needed to confirm this finding.

When the cases were analyzed according to their demographic characteristics, no significant differences were found in age, but significant differences were observed in gender.

Limitations

The present study had some limitations, most notably its retrospective design and the absence of other prognostic parameters. More comprehensive studies incorporating clinical findings, pathological stage, and prognostic factors will greatly aid in understanding the relationship between these proteins and renal neoplasms with eosinophilic cytoplasm.

Conclusions

In conclusion, this study showed that DARS2, Reelin, and Enkurin proteins could be potentially effective and specific immunohistochemical markers for differentiating renal neoplasms with eosinophilic cytoplasm, which can be difficult to diagnose. Furthermore, the study suggests that Reelin and Enkurin proteins may hold promise in determining prognosis and developing targeted therapies for these neoplasms. However, further comprehensive studies are needed to explore the clinical implications of these findings and their potential for clinical application.

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