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DYSF expression in clear cell renal cell carcinoma: A retrospective study of 2 independent cohorts

Mihyang Ha, M.S.[a,1], Hoim Jeong, M.S.[b,1], Jong Seong Roh, Ph.D.[b], Beomgu Lee, B.S.[b], Myoung-Eun Han, Ph.D.[a], Sae-Ock Oh, M.D., Ph.D.[a], Dong Hyun Sohn, Ph.D.[b,*], Yun Hak Kim, M.D., Ph.D.[c,d,*]

[a] *Department of Anatomy, Pusan National University School of Medicine, Yangsan, Republic of Korea*
[b] *Department of Microbiology and Immunology, Pusan National University School of Medicine, Yangsan, Republic of Korea*
[c] *Department of Anatomy and Department of Biomedical Informatics, Pusan National University School of Medicine, Yangsan, Republic of Korea*
[d] *Biomedical Research Institute, Pusan National University Hospital, Busan, Republic of Korea*

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Abstract

Objective: Renal cell carcinoma (RCC) is the most typical type of kidney cancer in adults. Hypercalcemia is a well known paraneoplastic syndrome associated with RCC and recent studies have reported that hypercalcemia is closely related to the poor prognosis of RCC patients. Clear cell RCC (ccRCC) is the most common and aggressive subtype of RCC. Although the histological classification of RCC is important for determination of appropriate treatment strategies, effective biomarkers for predicting prognosis of ccRCC have not yet been identified. Since calcium levels affect the prognosis of RCC patients, we evaluated whether the calcium-sensing genes on the plasma membrane, including those encoding calcium channels, CaSR, GPRC6a, and *DYSF*, could be used as biomarkers to predict the prognosis of ccRCC patients.

Methods: Information from 537 patients from The Cancer Genome Atlas (TCGA; $n = 446$) and International Cancer Genome Consortium (ICGC; $n = 91$) was used in this study. Among these genes, *DYSF* was the only gene whose expression correlated with overall survival of both TGCA and ICGC patients.

Results: Although *DYSF* gene expression was higher in ccRCC tissue than in normal kidney tissue, Kaplan-Meier curves showed that the survival rate of ccRCC patients with high *DYSF* expression was significantly higher than that of patients with low *DYSF* expression (TCGA, $P < 0.0001$; ICGC, $P = 0.0011$). We also validated the potential of *DYSF* as a prognostic biomarker for ccRCC by conducting a time-dependent area under the curve (AUC) analysis and 5-years receiver operating characteristic curve analysis. Finally, multivariate regression analysis revealed that the expression of *DYSF* is independent of other prognostic parameters (TCGA, $P = 0.017$; ICGC, $P = 0.006$).

Conclusions: These results suggested that *DYSF* may play a suppressive role in the progression of ccRCC and could act as a promising prognostic biomarker for predicting the survival of ccRCC patients.

Keywords: *DYSF*; Clear cell renal cell carcinoma; TCGA; ICGC; Prognostic marker

1. Introduction

Renal cell carcinoma (RCC) is the most common renal malignancy in adults and originates from the lining of the proximal convoluted tubule [1,2]. Clear cell RCC (ccRCC), the most common subtype of RCC, accounting for 70% to 80% of RCC cases [3,4]. This subtype tends to be diagnosed at an advanced stage and has a higher rate of metastasis compared with other subtypes, such as papillary RCC and chromophobe RCC [5]. As RCC is resistant to radiotherapy

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*Corresponding authors. Tel.: 82-51-510-8046; fax: 82-51-510-8049.
E-mail addresses: dhsohn@pusan.ac.kr (D.H. Sohn), yunhak10510@pusan.ac.kr (Y.H. Kim).

[1]Mihyang Ha and Hoim Jeong contributed equally to this work.

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and chemotherapy, surgical resection is the main curative intervention for patients with localized RCC [6−8]. To date, patients with advanced RCC can be treated but not cured completely [8,9], and RCC patients with similar morphology and tumor stages are often known to exhibit different clinical course. Therefore, accurate prediction of prognosis and determination of appropriate treatment strategy is essential for prolonging the life of RCC patients [10].

Plasma calcium levels are strictly regulated by many hormones, including parathyroid hormone (PTH), 1,25-dihydroxy vitamin D, calcitonin, and ionized calcium [11−13]. PTH is produced by the parathyroid glands, which can both increase serum calcium and decrease serum phosphorus via direct and indirect stimuli of osteoclasts [14]. Hypercalcemia is a condition in which the calcium level in blood is higher than normal and is usually a result of overactive parathyroid glands. However, hypercalcemia is also associated with malignancy, typically occurring in up to 44% of the patients with cancer [15]. Especially, hypercalcemia is common in advanced stage cancer and is associated with a poor prognosis [16,17].

Approximately 10% to 40% of patients with RCC have been reported to develop a paraneoplastic syndrome (PNS) during the course of the disease [18]. Among the PNS associated with RCC, hypercalcemia is the most common PNS in RCC patients, affecting 13% to 20% of them [19−21]. PTH-related peptide (PTHrP) increases renal calcium reabsorption by binding to the common PTH/PTHrP receptor [22,23] and PTHrP levels were reported to be elevated in up to 15% of all patients with RCC [24]. Hypercalcemia in RCC patients frequently mimics primary hyperparathyroidism and has been attributed to tumor secretion of PTH-related protein [25]. More importantly, recent studies have reported that elevated corrected calcium levels are closely related to the poor prognosis of RCC patients [26,27]. Therefore, we evaluated the potential of calcium-sensing genes on the plasma membrane, including those encoding calcium channels, CaSR, GPRC6a, and *DYSF* [28,29], as prognostic biomarkers of ccRCC patients. In this study, we analyzed the expression of these genes in ccRCC patients from The Cancer Genome Atlas (TCGA) [30,31] and International Cancer Genome Consortium (ICGC) cohorts [32]. We found that the expression of *DYSF* is correlated with overall survival (OS) of patients in both TGCA and ICGC cohorts, suggesting that *DYSF* might act as a prognostic biomarker for ccRCC.

2. Materials and methods

2.1. Data accession

Patients' data from TCGA and ICGC cohorts was obtained from ICGC data portal (dcc.icgc.org) in March 2018 [30−32]. Patients with "Not available" expression values of target genes or insufficient clinical data (stage, grade, and survival) were excluded. All analyses in this study were performed with R software version 3.5.0 (The R foundation for Statistical Computing, 2018).

2.2. Statistical analyses

The differences in *DYSF* expression between patients at low stage (I and II) and high stage (III and IV) were analyzed by Wilcoxon signed rank sum test. We also analyzed the differences in *DYSF* expression between paired normal and ccRCC tissues using Wilcoxon signed rank sum test. We conducted the test using "*coin*" package in R software. We used several survival analysis methods to predict the OS as described in our previous studies [33,34]. To identify the accuracy of discriminatory power, we used Uno's *C*-index, area under the curve (AUC) values at 5 years, Kaplan-Meier curve with optimal cut-off values, and Cox regression analyses. The optimal cut-off value had maximal *C*-index with 5-fold cross-validation in each dataset as we defined in the previous studies [35,36]. Patients were divided into 2 groups based on the optimal cut-off value. The patients with lower *DYSF* expression than the cutoff were classified into the low expression group and patients with higher *DYSF* expression than the cutoff were classified into the high expression group.

3. Results

3.1. Patient information from TCGA and ICGC

We analyzed 537 patients from TCGA ($n = 446$) and ICGC ($n = 91$) to determine whether *DYSF* could act as a prognostic biomarker of patients with ccRCC (Table 1). Among the patients from TCGA, 48.4% were in stage I, 10.3% in stage II, 24.9% in stage III, and 15.9% in stage IV. Among TCGA patients, 2% of the patients had grade I, 42.4% had grade II, 39.2% had grade III, and 15.2% had grade IV ccRCC. More than half (52.7%) of the ICGC patients were in stage I, and the percentages of patients in stage II, stage III, and stage IV were 13.2%, 14.3%, and 9.9%, respectively. The TNM stage distribution of TCGA and ICGC patients is described in Table 1. The TCGA patients consisted of White ($n = 413$, 92.6%), Black or African American ($n = 20$, 4.5%), and Asian ($n = 7$, 1.6%) background. However, the ICGC data did not provide grade and race information of the patients. In both cohorts, the mean age of the patients was approximately 60 years.

3.2. Evaluating prognostic significance of calcium channels, CaSR, GPRC6a, and DYSF

To assess target genes as prognostic biomarkers of ccRCC, we conducted Kaplan-Meier curve analysis that compared the relationship between expression levels of target genes and patient survival. Although calcium channels, *CaSR* and *GPRC6a*, were not associated with survival (data

Table 1
Patients' characteristics in TCGA and ICGC cohorts.

		TCGA (%)	ICGC (%)
Stage	I	216 (48.4%)	48 (52.7%)
	II	46 (10.3%)	12 (13.2%)
	III	111 (24.9%)	13 (14.3%)
	IV	71 (15.9%)	9 (9.9%)
	NA	2 (0.4%)	9 (9.9%)
Grade	I	9 (2.0%)	–
	II	189 (42.4%)	–
	III	175 (39.2%)	–
	IV	68 (15.2%)	–
	NA	5 (1.1%)	–
T Stage	T1	221 (49.6%)	54 (59.3%)
	T2	57 (12.8%)	13 (14.3%)
	T3	161 (36.9%)	22 (24.2%)
	T4	7 (1.6%)	2 (2.2%)
N Stage	N0	205 (46.0%)	79 (86.8%)
	N1	14 (3.1%)	2 (2.2%)
	NX	227 (50.9%)	10 (11.0%)
M Stage	M0	376 (84.3%)	81 (89.0%)
	M1	70 (15.7%)	9 (9.9%)
	MX	–	1 (1.1%)
Race	White	413 (92.6%)	–
	Black (or African American)	20 (4.5%)	–
	Asian	7 (1.6%)	–
	NA	6 (1.3%)	–
Sex	Male	290 (65.0%)	52 (57.1%)
	Female	156 (35.0%)	39 (42.9%)
Age (mean ± standard deviation)		60.62 ± 12.80	60.47 ± 10.03
Number of patients		446	91

not shown), *DYSF* was significantly associated with patient survival in both cohorts (Fig. 1). The survival rate of patients with high *DYSF* expression was higher than that of patients with low *DYSF* expression, in both TCGA ($P< 0.0001$; Fig. 1A) and ICGC ($P = 0.0011$; Fig. 1F) cohorts. The survival rates were higher in TCGA patients with high *DYSF* expression in stages I and II ($P = 0.04$; Fig. 1B) and stages III and IV ($P = 0.05$; Fig. 1C). In addition, TCGA patients with high *DYSF* expression exhibited a better prognosis in both grades I to II ($P = 0.014$; Fig. 1D) and III to IV ($P = 0.014$; Fig. 1E). However, the survival rates were high in ICGC patients with high *DYSF* expression only in stages III and IV ($P = 0.035$; Fig. 1H), but not in stages I and II ($P = 0.11$; Fig. 1G).

3.3. *DYSF* gene expression in ccRCC

Using the Wilcoxon signed rank test on TCGA and ICGC patients' information, we evaluated *DYSF* expression level between early and late stages of ccRCC. *DYSF* expression was higher in stages I and II than in stages III and IV in TCGA cohort ($P< 0.001$) (Fig. 2A). However, in the ICGC patients, the difference in *DYSF* expression in stages I and II and in stages III and IV was not statistically significant (Fig. 2B). We also compared *DYSF* expression between ccRCC tissue and paired normal kidney tissue in TCGA patients using the Wilcoxon signed rank test. The *DYSF* expression levels in ccRCC tissues were significantly higher than those in the paired normal kidney tissue ($P < 0.001$) (Fig. 2C).

3.4. Multivariate analysis shows prognostic significance of *DYSF* expression

To verify whether *DYSF* expression level is an independent predictor of ccRCC patients' survival, we performed Cox regression analyses. In univariate analysis, *DYSF* expression, age, stage, and grade were associated with OS of TCGA patients, while *DYSF* expression and stage were associated with OS of ICGC patients. After adjustment for the significant parameters, multivariate regression analyses showed that *DYSF* expression is independent of age, stage, and grade in both TCGA and ICGC cohorts (Table 2).

3.5. Evaluation of the performance of *DYSF* expression as a ccRCC biomarker

To evaluate the performance of *DYSF* expression as a prognostic biomarker in ccRCC, we examined Uno's *C*-index in the time-dependent AUC analysis and the AUC values in the receiver operating characteristic (ROC) curves (Fig. 3). In the time-dependent AUC analysis, Uno's *C*-indices were 0.611 and 0.615 for the TCGA and ICGC cohorts, respectively (Fig. 3A). The 5-year ROC curves showed that AUC values for the TCGA and ICGC cohorts were 0.585 and 0.696, respectively (Fig. 3B). The results of the TCGA and ICGC cohorts were consistent with each other in both the time-dependent AUC analysis and the 5-year ROC analysis.

4. Discussion

The human *DYSF* gene encodes for dysferlin protein, which is a type II transmembrane protein with several calcium-sensing C2 domains [28,37]. Dysferlin is highly expressed in skeletal muscle and participates in muscle cell membrane repair by facilitating injury-triggered acid sphingomyelinase secretion [38]. Mutations in *DYSF* result in the development of muscular dystrophies and mice with *Dysf* deficiency exhibit impaired muscle membrane repair [39,40]. Although dysferlin is known to be expressed in the kidney [41], the normal function of dysferlin in the kidney and the prognostic significance of *DYSF* in ccRCC remain unknown. In this study, we identified that *DYSF* expression is correlated with overall patient survival in both TGCA and ICGC cohorts and that high expression of *DYSF* is associated with good prognosis of ccRCC patients.

It has previously been reported that dysferlin is expressed in tubular and glomerular epithelial cells in the kidney, and its deficiency causes glomerular proteinuria, which suggested that the expression of dysferlin in glomeruli might be

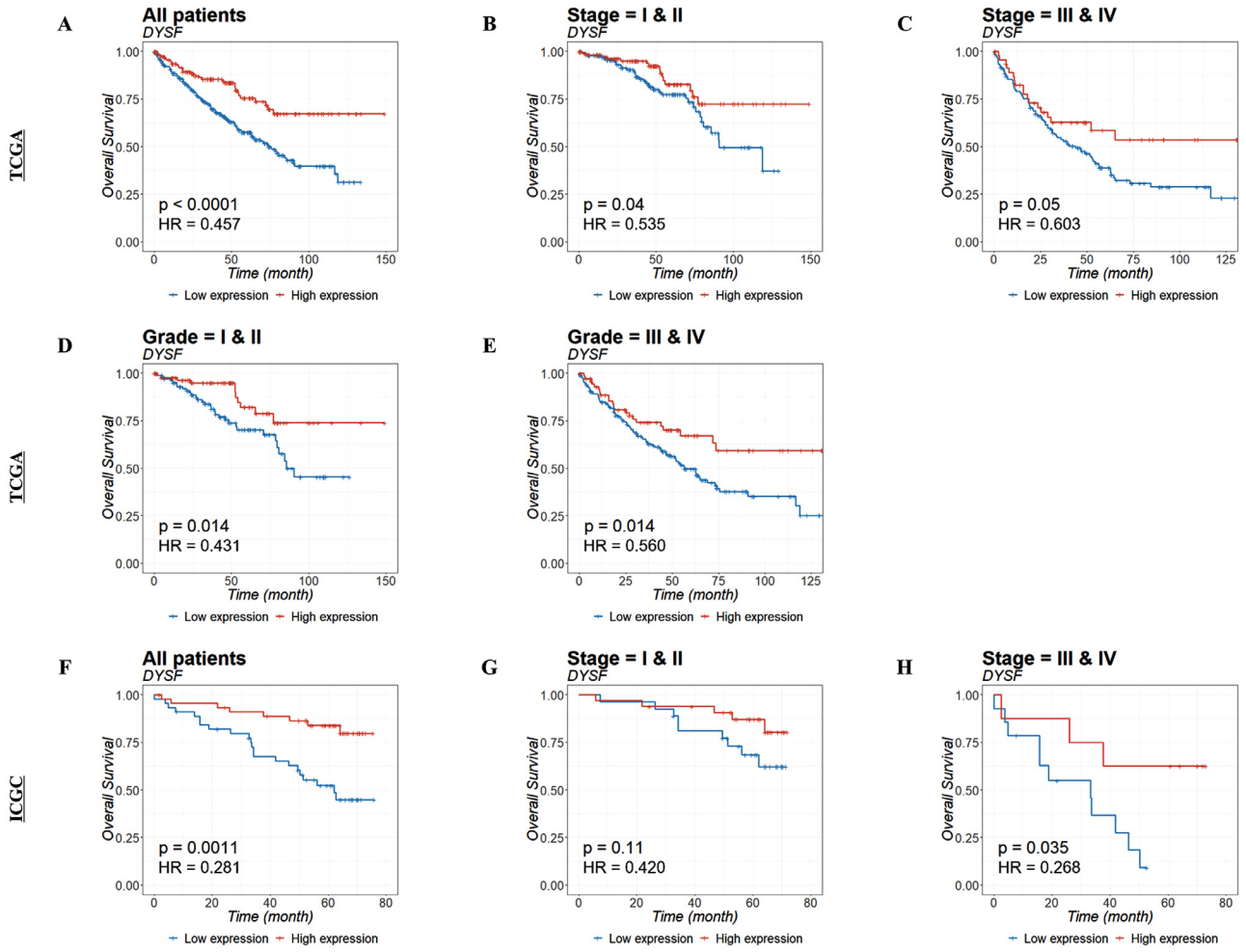

Fig. 1. Kaplan-Meier survival analysis of ccRCC patients with respect to *DYSF* gene expression. Survival analysis was performed in ccRCC patients from TCGA (A, B, C, D, and E) and ICGC (F, G, and H) cohorts. Survival was compared based on the following subgroups: all patients (A and F), stage I and II (B and G), and stage III and IV (C and H). For the TCGA cohort, survival was also compared in grade I and II (D) and grade III and IV (E). *P* value was calculated using log-rank test and is provided at the bottom left corner of each dataset.

associated with glomerular permeability [41]. Although dysferlin function has been extensively studied in muscle cell membrane repair, it is also expressed in innate immune cells, including neutrophils and monocytes [42–44], regulates monocyte phagocytosis [45], and complements monocyte activation [46–48]. These findings suggested that dysferlin might play unique roles in the immune system.

Contrary to our expectations, *DYSF* expression was higher in ccRCC tissue than in normal kidney tissue. High expression levels of several genes lead to a better prognosis

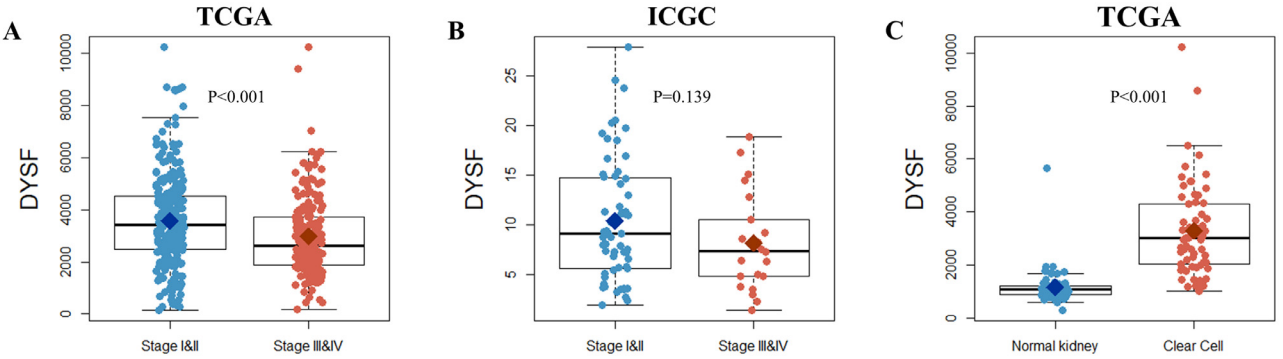

Fig. 2. Comparison of *DYSF* gene expression in TCGA and ICGC. The expression levels of *DYSF* gene were compared between stages I–II and III–IV in ccRCC patients from TCGA (A) and ICGC (B) cohorts. The *DYSF* expression levels between ccRCC and paired normal kidney from TCGA cohort were also compared (C). ICGC = International Cancer Genome Consortium; TCGA = The Cancer Genome Atlas; .

Table 2
Univariate and multivariate analyses of the overall survival in each cohort (*, **, and *** indicates significance at $P< 0.05$, $P< 0.01$, and $P< 0.001$, respectively).

Parameters	Univariate analysis				Multivariate analysis			
	P	HR	95% CI		*P*	HR	95% CI	
TCGA								
DYSF (categorical)	<0.001***	0.457	0.311	0.673	0.017*	0.618	0.416	0.918
Age	<0.001***	1.033	1.018	1.047	<0.001***	1.029	1.013	1.045
Stage (I and II vs. III and IV)	<0.001***	3.478	2.474	4.888	<0.001***	2.745	1.910	3.956
Gender (female vs. male)	0.333	0.850	0.612	1.181	0.708	0.936	0.661	1.324
Grade (I and II vs. III and IV)	<0.001***	2.247	1.572	3.212	0.045*	1.472	1.009	2.149
ICGC								
DYSF (categorical)	0.002**	0.281	0.125	0.632	0.006**	0.300	0.127	0.707
Age	0.109	1.031	0.993	1.071	0.685	1.009	0.969	1.050
Stage (I and II vs. III and IV)	<0.001***	4.796	2.264	10.16	<0.001***	5.123	2.313	11.347
Gender (female vs. male)	0.863	1.066	0.517	2.194	0.612	0.821	0.383	1.761

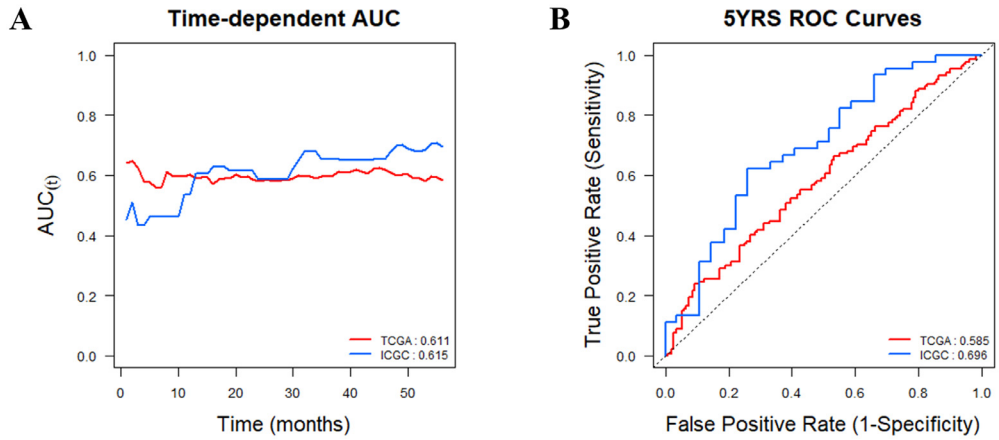

Fig. 3. Time-dependent area under the curve (AUC) analysis and receiver operating characteristic (ROC) curves at 5 years with respect to *DYSF* gene expression. Time-dependent AUC (A) and ROC curve at 5 years (B) in the TCGA and ICGC cohorts. *C*-index values and AUC values at 5 years are provided at the bottom right corner of each dataset.

in cancer patients, regardless of higher expression in cancer tissues compared to that in the normal. For example, the expression of breast cancer metastasis suppressor 1 (BRMS1), a well-known metastasis suppressor gene, was higher in breast cancer than in corresponding normal breast epithelium [49], while patients with high levels of BRMS1 expression showed a better prognosis than those with low BRMS1 expression [50]. In addition, Rho GDP dissociation inhibitor 2, another metastasis suppressor gene, showed prognostic patterns similar to those of *DYSF* [51]. Unlike tumor suppressors, these metastasis suppressors inhibit cancer metastasis without affecting the primary tumor when overexpressed or re-expressed [52]. The metastasis suppressor genes may be inactivated or have another function in the absence of cancer; however, when cancer develops, they might be overexpressed to prevent metastasis and inhibit cancer progression. Although the normal function of dysferlin in the kidney is still unclear, it is possible that dysferlin expression is induced by cancer development and that it plays an inhibitory function similar to metastasis suppressor genes in the progression of ccRCC. Taken together, our results suggest that *DYSF* might be a novel prognostic biomarker for ccRCC; further research will be necessary to elucidate the exact function of *DYSF* in cancer development and progression.

Conflict of interest

The authors declare no competing interests.

Data availability

The data used to support the findings of this study is available from the corresponding author upon request.

Author contributions

Conception and design: Y.H.K., D.H.S.; Acquisition of data: J.S.R., B.L., M.E.H., S.O.O.; Analysis and interpretation of data: M.H., H.J.; Writing and review of the

manuscript: M.H., H.J., D.H.S.; and Study supervision: Y.H.K., D.H.S. All authors read and approved the final version of this manuscript.

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