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Prognostic significance of immunohistochemical staining for myoferlin in clear cell renal cell carcinoma and its association with epidermal growth factor receptor expression

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Abstract

Objectives: To reveal the prognostic significance of myoferlin expression in clear cell renal cell carcinoma (ccRCC) and its association with epidermal growth factor receptor (EGFR) expression and cytoplasmic features based on lipogenic metabolism.

Materials and methods: Immunohistochemical (IHC) staining for myoferlin and EGFR was conducted on 450 resected ccRCC specimens using tissue microarray, which was measured semiquantitatively. MYOF and EGFR mRNA data were acquired from the TCGA database. The cytoplasmic characteristics of ccRCC based on lipogenic metabolism were retrieved from previous study. Survival analyses were carried out for progression-free, overall, and cancer-specific survival using Kaplan-Meier with the log-rank tests and Cox regression models. Discrimination of prognostic prediction was analyzed by Harrell's C-index. In addition, cross-correlation analyses of myoferlin expression with clinicopathological characteristics and EGFR expression were performed using Pearson's χ^2, Mann-Whitney U, and Pearson's r tests.

Results: High expression of myoferlin, which was related to high WHO grade ($P< 0.001$) and high TNM stage ($P= 0.074$), was significantly associated with short progression-free ($P< 0.001$), overall ($P< 0.001$), and cancer-specific survival ($P< 0.001$) of ccRCC patients. In multivariate analysis, IHC staining for myoferlin was independently associated with progression-free (hazard ratio [HR] = 1.734, $P= 0.021$), overall (HR = 1.750, $P= 0.004$), and cancer-specific (HR = 1.723, $P= 0.044$) survival when adjusted for TNM stage and WHO grade, with significantly improved C-indices. Furthermore, the expression of myoferlin and EGFR showed positive relationship at the protein ($P< 0.001$) and at the mRNA levels ($r = 0.478$, $P< 0.001$). In addition, clear to light-granular cytoplasm, representative of low-risk lipogenic metabolism, was predominantly observed in myoferlin-low ccRCC ($P= 0.004$).

Conclusions: IHC staining for myoferlin was a useful prognostic biomarker of ccRCC. We hypothesize that EGFR overexpression and lipogenic metabolism alteration underlie the protumorous functions of myoferlin on ccRCC.

Keywords: Myoferlin Protein; Clear Cell Renal Cell Carcinoma; Immunohistochemistry; Prognosis; ErbB Receptors

1. Introduction

Myoferlin is one of the ferlin family proteins initially identified in the human muscle [1]. The ferlin family of proteins that consists of dysferlin, otoferlin, myoferlin, and Fel1L4-6 shares conserved multiple C2-domains, which mediate vesicle fusion under physiological conditions [1]. Mutations in dysferlin and otoferlin impair exocytosis at the injured myotubes, leading to limb-girdle muscular dystrophy 2B, and at the auditory hair cell, which is linked to deafness, respectively [1]. In line with this, myoferlin is enriched in the caveolae/lipid-rich microdomain of the plasma membrane and late endosome, participating in vesicle transportation and endo/exocytosis [1−3].

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Recently, myoferlin was revealed to be overexpressed in various types of cancer and was implicated in tumor biology, including epithelial-to-mesenchymal transition [4,5], migration or invasion [4,6–8], proliferation [7–10], angiogenesis [11], lipid metabolism [12], and metastasis [7,12]. In addition, the prognostic implication of myoferlin has been demonstrated in patients with pancreas adenocarcinoma [9], head and neck squamous cell carcinoma [13], melanoma [5], and clear cell renal cell carcinoma (ccRCC) [14], where the elevated expression of myoferlin was associated with poor prognosis. Although the functional implication of myoferlin has been investigated extensively in breast and pancreatic cancer cells [4,6,9,11,12], it is still enigmatic how myoferlin promotes the aggressive behavior of ccRCC.

Worldwide, approximately 270,000 patients are diagnosed with renal cell carcinoma annually, and ccRCC accounts for 65% to 70% of renal cell carcinoma [15,16] ccRCC is enriched with the activation of several receptor tyrosine kinases, including epidermal growth factor (EGF) receptor (EGFR) and vascular endothelial growth factor (VEGF) receptor (VEGFR) [17]. Myoferlin is a key regulator of the stability and/or function of receptor tyrosine kinases [4]. For example, myoferlin was necessary for stabilization of VEGFR-2 and insulin-like growth factor 1 receptor in endothelial cell and myoblasts, respectively, by regulating their degradation [3,18]. Conversely, myoferlin was shown to decrease EGFR expression in breast cancer and hepatocellular carcinoma (HCC) cells [8,19]. In the human kidney cell line, some of the downstream molecules of EGFR showed inconsistent reaction to myoferlin: phosphorylated (p-) ERK was up-regulated but p-AKT was not induced by canonical myoferlin [20]. Activation of EGFR is known to initiate multiple signaling pathways, including the PI3K-AKT and Ras-Raf-MEK-ERK cascades, which may be important for the progression of ccRCC [17]. Moreover, the interaction between EGFR and VEGFR is one of the resistance mechanisms of ccRCC against VEGFR-targeting agents [17]. Although immunohistochemical (IHC) expression of myoferlin and EGFR in ccRCC was studied separately [14,21], their relationship was not analyzed in ccRCC.

Furthermore, the vesicle trafficking function of myoferlin was essential for lipid and glucose metabolism, as deeply investigated in breast and pancreas carcinoma [12]. The evolution of lipogenic metabolism appears to be related to ccRCC progression [15]. Molecular alterations involved in this oncologic shift in metabolism, the Warburg effect, were associated with poor survival of patients with ccRCC [22]. However, the association between lipogenic metabolism and myoferlin expression is still unknown in ccRCC.

In this study, we aimed to reveal the prognostic significance of myoferlin expression in ccRCC and its association with EGFR expression and cytoplasmic features based on lipogenic metabolism.

2. Materials and methods

2.1. Patients

Overall, 450 patients with ccRCC who received radical ($n = 287$) or partial ($n = 163$) nephrectomy between 2003 and 2008 at the Seoul National University Hospital were assembled. Any patients with bilateral disease or a history of Von Hippel-Lindau syndrome were excluded. Every sample was reviewed by regarding the histologic type, TNM stage, and WHO grade of the tumor. The TNM stage was reclassified according to the American Joint Committee on Cancer—Cancer Staging Manual, eighth edition [23]. Clinical data were obtained from medical records. This study was in accordance with the ethical standards of the institutional review board of Seoul National University Hospital (H-1803-119-932). Informed consent of the patients was waived by the institutional review board.

2.2. Tissue microarray

Two cores (2 mm in diameter) per patient were embedded in the new recipient paraffin blocks using a trephine apparatus (Superbiochips Laboratories, Seoul, Republic of Korea) for tissue microarray (TMA) construction. Each core was from a different area to overcome the issue of intratumor heterogeneity.

2.3. Immunohistochemistry

IHC staining was conducted on 4-μm-thick TMA sections using the Benchmark autostainer (Ventana, Tucson, AZ) according to the manufacturer's instructions. Rabbit monoclonal antibodies against myoferlin (1:200; primary incubation time, 30 minutes; HPA014245, Sigma-Aldrich, St. Louis, MO) and EGFR (RTU; primary incubation time, 8 minutes; 3C6, Ventana) were used. This antimyoferlin antibody, HPA01425, was widely used throughout studies on breast and pancreas cancer [11,19,24]. Myoferlin and EGFR were stained in ccRCC cells mainly in a membranous manner. The non-neoplastic vascular smooth muscle, endothelial cells, distal tubules, and collecting ducts were also reactive to myoferlin. The expression levels of myoferlin (Fig. 1A,B) and EGFR (Fig. 1C,D) were measured semiquantitatively, by multiplying the staining proportion score (0, 0%; 1, <33%; 2, 33%–66%; 3, >66%) and the intensity score (0, absent; 1, weak; 2, strong). For the cases with discordant IHC levels between the 2 TMA cores, the mean value of both scores was used. Regarding myoferlin, the multiplied expression score ≥2 was set as the cutoff between high and low expression because it was more significantly related to patient survival than any other value. As a negative control of IHC staining, a TMA slide was stained without primary antibody to myoferlin or EGFR that produced absolutely negative result. The association of

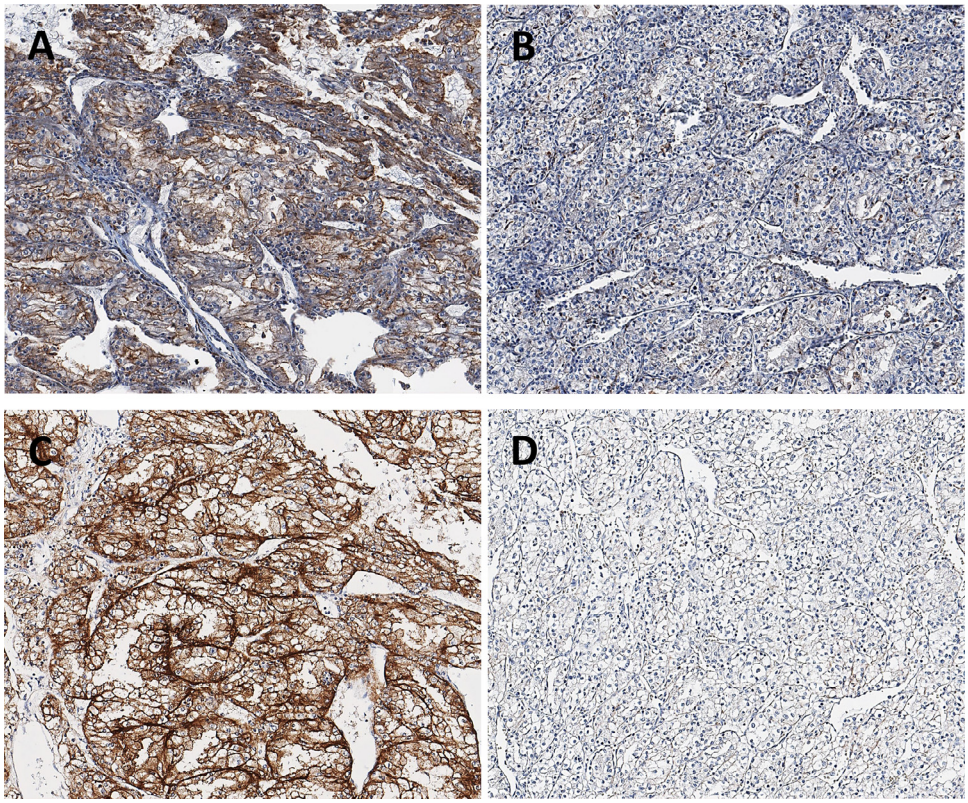

Fig. 1. Representative images of IHC staining for myoferlin and EGFR in ccRCC. (A) High expression of myoferlin (proportion score 3, >66%; intensity score 2, strong). (B) Low expression of myoferlin (proportion score 1, >0 and <33%; intensity score 1, weak). (C) EGFR expression level 6 (proportion score 3, >66%; intensity score 2, strong). (D) EGFR expression level 1 (proportion score 1, >0 and <33%; intensity score 1, weak). (C) and (D) correspond to the same field of (A) and (B), respectively (magnification, ×200).

the expression levels of myoferlin and EGFR was assessed in 435 tumors in which IHC staining for both proteins were evaluable. Furthermore, whole-section staining for myoferlin was performed on 10 randomly selected cases.

2.4. mRNA expression of MYOF and EGFR

From the cBioPortal [25], mRNA expression data of *MYOF* and *EGFR* generated by the TCGA Research Network were obtained, which contained 510 ccRCC samples. Raw TPM values were normalized with linear modeling using the limma-voom in R 3.5.1.

2.5. Cytoplasmic features of ccRCC relative to the state of lipogenesis

In our previous study, cytoplasmic features of ccRCC were suggested to represent the metabolic progression of lipogenesis [15]. From this prior publication [15], cytoplasmic characteristics of 329 samples were retrieved. Briefly, the color and texture of the cytoplasm observed in hematoxylin and eosin TMA slides were classified into 3 categories based on lipogenic metabolism progression: clear to light-granular cytoplasm, deep-granular or eosinophilic cytoplasm, and nonclear cytoplasm with high-grade features [15]. For statistical purposes, cases in the latter 2 categories were grouped together into "nonclear" group.

2.6. Statistics

Interrelation between myoferlin expression and clinicopathological characteristics or EGFR expression was analyzed by χ^2, Mann-Whitney, or Pearson's r tests. The progression-free survival (PFS) period was calculated from the interval between surgery and recurrence, progression, metastasis, or the last follow-up visit for patients who did not show any of these. The overall survival (OS) duration was defined by the period between surgery and death from any cause or the last follow-up. The cancer-specific survival (CSS) duration was determined by the interval between surgery and cancer-related death or the last follow-up visit. Kaplan-Meier analysis and the log-rank test were used to compare survival. A Cox proportional hazard regression model was used for univariate and multivariate survival analyses. Discrimination of prognostic prediction was analyzed by C-index. In all statistical analyses, a 2-tailed $P< 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS Statistics 23 (IBM Co., Armonk, NY) or Stata 13 (StataCorp, College Station, TX).

3. Results

3.1. Patient population

The clinicopathological characteristics of patients and their relationship with myoferlin expression are summarized in Table 1. Briefly, 332 men and 118 women were included. The mean age at diagnosis was 56.8 years (range, 20–81). Of the total cases, 90 (20%) and 223 (49.6%) presented with high TNM stage (III or IV) and WHO grade (3 or 4), respectively. Clinically and/or pathologically, 5 and 40 patients had lymph node involvement and distant metastasis at the diagnosis, respectively, 4 of whom had both.

3.2. High expression of myoferlin was associated with high WHO grade and TNM stage of ccRCC

IHC staining for myoferlin in the matched TMA and the full-section was concordant. Low and high expression of myoferlin was observed in 260 (57.8%) and in 190 (42.2%) tumors, respectively (Fig. 1A,B). High expression of myoferlin was significantly associated with high WHO grade ($P< 0.001$) and marginally with high TNM stage ($P= 0.074$) (Table 1).

3.3. High expression of myoferlin was predictive of adverse outcomes in patients with ccRCC

The median follow-up duration was 121 months (range, 1–177), during which 78 patients had progression of disease, 110 patients died, and 60 died of the disease. Median OS was not reached. High myoferlin expression was significantly associated with short PFS ($P< 0.001$), OS ($P< 0.001$), and CSS ($P< 0.001$) in patients with ccRCC (Fig. 2). In univariate Cox regression analyses (Table 2), patients with myoferlin-high ccRCC were more likely to experience disease progression (hazard ratio [HR] = 2.310, $P< 0.001$), to die (HR = 1.970, $P< 0.001$) and to die due to the disease (HR = 2.420, $P= 0.001$) than those with myoferlin-low tumors. Multivariate analyses revealed that myoferlin-high expression was an independent prognostic factor of shorter PFS (HR = 1.734, $P= 0.021$), OS (HR = 1.750, $P= 0.004$), and CSS (HR = 1.723, $P= 0.044$), when adjusted for both TNM stage and WHO grade (Table 2). The addition of myoferlin expression significantly improved the C-indices for the prediction of PFS (from 0.813 to 0.828; $P= 0.036$) and CSS (from 0.858 to 0.872, $P= 0.041$) with TNM stage and WHO grade as a baseline model, and of OS (from 0.674 to 0.709; $P= 0.020$) with TNM stage as a baseline model. In subgroup analyses, moreover, myoferlin-high ccRCC, either in low or high stage/grade, tend to show poor PFS, OS, and CSS (supplementary Figs. 1 and 2).

3.4. The expression of myoferlin and EGFR in ccRCC was in strong positive association both in protein and mRNA levels

ccRCC with high myoferlin showed higher level of EGFR expression than that with low myoferlin ($P< 0.001$) (Figs. 1 and 3A). Consistent with this, *MYOF* and *EGFR* mRNA in the TCGA database showed positive relationship ($r = 0.478$, $P< 0.001$) (Fig. 3B). Furthermore, enrichment of EGFR independently predicted poor PFS, OS, and CSS of ccRCC when adjusted to TNM stage and WHO grade (supplementary Fig. 3 and supplementary Table 1).

3.5. Clear cytoplasmic features were predominantly observed in myoferlin-low ccRCC

Features of clear to light-granular and "nonclear" cytoplasm were observed in 193 (58.7%) and 136 (41.3%) cases, respectively, which was significantly associated with myoferlin expression ($P= 0.004$) (Table 3). Notably, clear to light-granular cytoplasm was predominantly observed in myoferlin-low tumors (Table 3).

Table 1
Clinicopathological variables with their association with myoferlin expression.

	Myoferlin-low	Myoferlin-high	Total	*P*
Number	260 (57.8%)	190 (42.2%)	450	
Sex				<0.001[a]
Male	172 (66.2%)	160 (84.2%)	332 (73.8%)	
Female	88 (33.8%)	30 (15.8%)	118 (26.2%)	
Age (y)[b]	55.6 ± 12.8	58.5 ± 10.5	56.8 ± 12.0	0.029[c]
TNM stage				0.074[a]
I or II	216 (83.1%)	144 (75.8%)	360 (80.0%)	
III or IV	44 (16.9%)	46 (24.2%)	90 (20.0%)	
WHO grade				<0.001[a]
1 or 2	151 (58.1%)	76 (40.0%)	227 (50.4%)	
3 or 4	109 (41.9%)	114 (60.0%)	223 (49.6%)	
Size (cm)[b]	4.3 ± 2.7	4.6 ± 3.2	4.4 ± 2.9	0.989[c]

[a] Pearson's χ^2 test with Yates' correction.
[b] Mean ± standard deviation.
[c] Mann-Whitney *U* test (Kolmogorov-Smirnov test *P* value < 0.001).

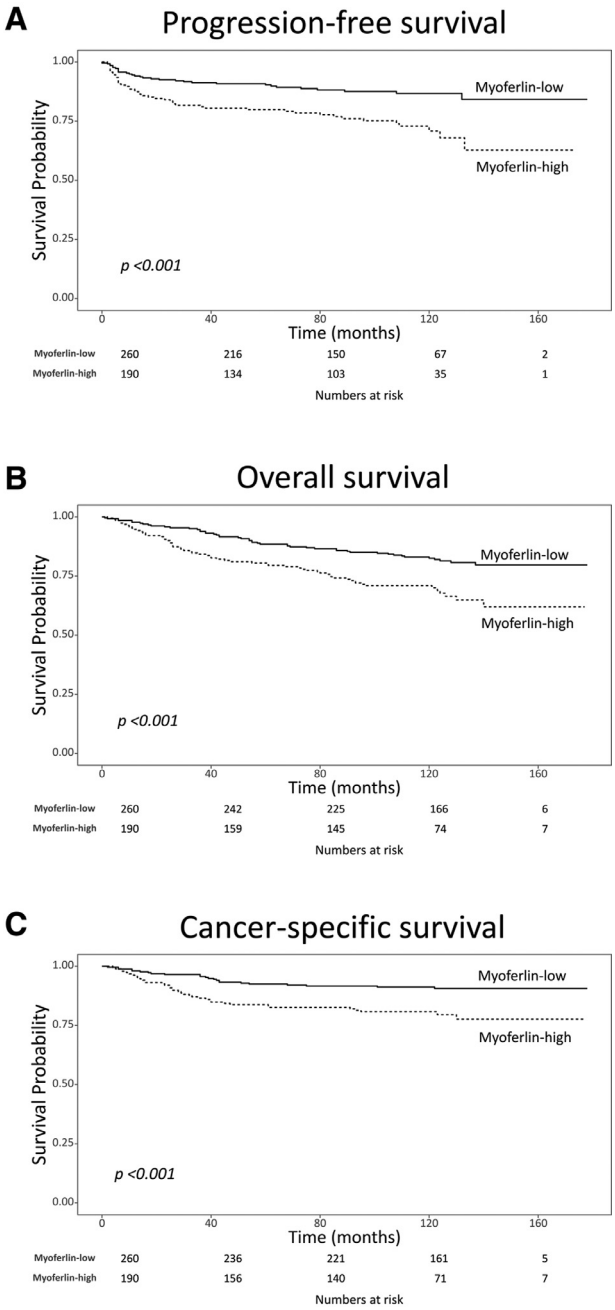

Fig. 2. Kaplan-Meier analyses of myoferlin expression in patients with ccRCC. (A) Progression-free survival. (B) Overall survival. (C) Cancer-specific survival.

4. Discussion

In this study, we investigated the prognostic significance of IHC staining for myoferlin in 450 patients with ccRCC. In accordance with a previous study [14], high expression of myoferlin was related to high WHO grade with marginal relation to high TNM stage. We identified that high myoferlin expression was significantly associated with the poor outcome of ccRCC. This was consistently demonstrated in the subgroups of stage and grade, even though statistical significance was limited by the small number of patients and survival events. Improvement of predictive accuracy with the addition of IHC staining for myoferlin, verified by C-statistics, reinforced the usefulness of IHC staining for myoferlin as a prognostic biomarker of ccRCC. Although myoferlin expression was reported to be related to PFS, but not to CSS of patients with ccRCC [14], we revealed that myoferlin-high expression was an independent prognostic factor of all prognostic endpoints. This may be ascribed to the number of patients, which is much larger in the current study than in the previous ($n = 152$) study [14]. The longer follow-up duration of the present study may also affect this difference [14].

Aside from ccRCC, a high reaction to myoferlin immunostaining was related to unfavorable outcomes in diverse malignancies [5,9,11–13] by engaging in pleiotropic protumorous machineries [4–7,9–12]. Recently, other oncogenic functions of myoferlin were described, including up-regulation of intranuclear translocation of signal transducer and activator of transcription 3 [7], maintenance of lipid metabolism [12], formation of vasculogenic mimicry [5], and transcription of transforming growth factor (TGF)-β1 [26]. However, there were some discrepancies in the detailed in vitro and in vivo effects of myoferlin alteration among studies. For example, loss of function of myoferlin decreased proliferation of hepatocellular, pancreas, and lung carcinoma cells but did not alter that of breast carcinoma cells [4,9–12]. High concentrations of myoferlin promoted vascularization in pancreas adenocarcinoma but not in lung carcinoma cells [10,11]. Third, the association of strong IHC staining for myoferlin with high-risk pathological characteristics and/or with poor survival was not the case in patients' samples of nonsmall cell lung carcinoma and endometrioid carcinoma; instead, high myoferlin expression was associated with low FIGO grade in endometrioid carcinoma [27,28]. These discordant results imply that the effects of myoferlin on cancer may be sensitive to the type of tumor or to the experimental condition (cell lines vs. human tissue), at least in part. Therefore, understanding the pathobiology how myoferlin leads to unfavorable prognosis of patients with ccRCC is important and it would aid in establishing myoferlin-targeted therapies [29].

We revealed that myoferlin and EGFR in ccRCC was positively associated both at the protein and at the mRNA levels. This is a novel finding of ccRCC that contradicts previous reports that myoferlin repression up-regulated EGFR expression [8,19]. Turtoi et al. found that targeted suppression of myoferlin in breast cancer cells increased the levels of EGFR, p-EGFR, and p-AKT, supposedly by impairing the degradation of p-EGFR dependent on caveolin-mediated endocytosis [19]. This prolonged activation of EGFR in myoferlin-deprived breast cancer was suggested to turn off EGF-EGFR signaling; hence, it in turn paradoxically down-regulated EGF-induced epithelial-to-mesenchymal transition and invasion [19]. In line with this observation, Hermanns et al. demonstrated that myoferlin down-regulation increased p-EGFR, Ras, and ERK

Table 2
Univariate and multivariate Cox regression analyses of prognoses of ccRCC.

	Univariate analysis Hazard ratio (95% CI)	*P*	Multivariate analysis[a] Hazard ratio (95% CI)	*P*
Progression-free survival				
Myoferlin (high vs. low)	2.310 (1.470–3.640)	<0.001	1.734 (1.085–2.771)	0.021
TNM stage (III or IV vs. I or II)	12.160 (7.600–19.450)	<0.001	9.636 (5.868–15.825)	<0.001
WHO grade (3 or 4 vs. 1 or 2)	4.000 (2.360–6.780)	<0.001	1.846 (1.038–3.281)	0.036
Overall survival				
Myoferlin (high vs. low)	1.970 (1.350–2.870)	<0.001	1.750 (1.190–2.573)	0.004
TNM stage (III or IV vs. I or II)	5.120 (3.510–7.470)	<0.001	4.489 (2.998–6.722)	<0.001
WHO grade (3 or 4 vs. 1 or 2)	2.240 (1.510–3.330)	<0.001	1.344 (0.875–2.065)	0.177
Cancer-specific survival				
Myoferlin (high vs. low)	2.420 (1.440–4.070)	0.001	1.723 (1.014–2.928)	0.044
TNM stage (III or IV vs. I or II)	17.540 (9.750–31.530)	<0.001	11.739 (6.365–21.652)	<0.001
WHO grade (3 or 4 vs. 1 or 2)	8.740 (3.970–19.230)	<0.001	3.531 (1.5348.129)	0.003

CI = confidence interval.
[a] adjusted to TNM stage and WHO grade.

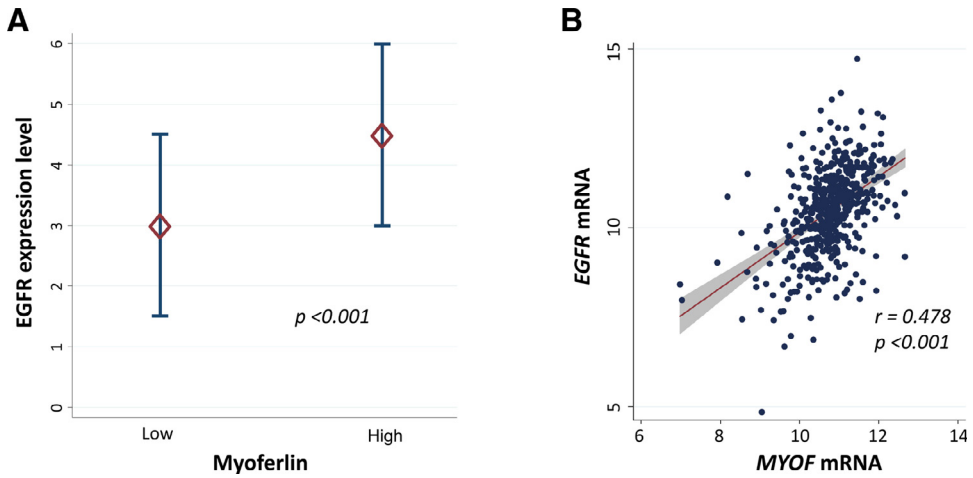

Fig. 3. Relationship between myoferlin and EGFR expression in ccRCC. (A) The median values (red square) with 25%–75% quartiles (blue line) of the levels of IHC staining for EGFR are 3.0 (1.5–4.5) and 4.5 (3.0–6.0) in myoferlin-low and -high ccRCC, respectively. Statistical analysis is based on Mann-Whitney *U* test. (B) *MYOF* and *EGFR* mRNA expression was positively associated. Values in each axis are normalized mRNA data derived from TCGA ccRCC database, using a linear model. Statistical analysis is based on Pearson's *r* test. Gray area indicates 95% confidence interval of the fitted line (red).

Table 3
Immunohistochemical staining for myoferlin and cytoplasmic features based on lipogenesis of clear cell renal cell carcinoma.

	Myoferlin-low (*n* = 217)	Myoferlin-high (*n* = 112)	Total (*n* = 329)	*P*[a]
Clear to light-granular	140 (64.5%)	53 (47.3%)	193 (58.7%)	0.004
"nonclear"categories[b]	77 (35.5%)	59 (52.7%)	136 (41.3%)	

[a] Pearson's χ^2 test with Yates' correction.
[b] Deep-granular or eosinophilic cytoplasm plus nonclear cytoplasm with high-grade features.

signaling machineries, whereas myoferlin re-expression decreased p-EGFR in HCC cells [8]. This EGFR up-regulation led myoferlin-silent HCC cells to oncogene-induced senescence [8]. On the other hand, resected nonsmall cell lung carcinoma tissue failed to show a significant interrelationship between IHC staining for myoferlin and EGFR [27]. Thus, myoferlin may interact with EGFR in patient's tissue in a way different from the inverse association demonstrated in cancer cell lines [8,19]. We hypothesize that myoferlin up-regulates EGFR in ccRCC by modulating exosomal signal transduction of autocrine TGF-β pathways which are known to induce and stimulate EGFR [17,30]. Exocytosis, including tumor exosome-associated signal transduction was impaired by myoferlin depletion [11]. In line with this, autocrine secretion of TGF-β1 was reduced in myoferlin-deficient breast cancer [26]. In addition, we suggest that the positive link

between myoferlin and EGFR may underlie the adverse outcome of ccRCC enriched with myoferlin or with EGFR [21]. Furthermore, modification of exosome by regulating myoferlin may aid in overcoming the resistance to tyrosine kinase inhibitors in ccRCC [17]. ccRCC is rich in cytoplasmic lipids and glycogen, which give rise to the typical clear color in microscopy. Our and other previous reports showed that the metabolic evolution of lipogenesis was an important phenomenon related to high-risk phenotypes of ccRCC [15,22]. Consistently, we identified that the clear to light-granular cytoplasm, representative of low-risk lipogenic metabolism [15], was observed mainly in myoferlin-low tumors. Given that the trafficking role of myoferlin was essential to the lipid and fatty acid metabolism of breast cancer [12], we speculated that myoferlin overexpression was connected to high-risk lipogenic metabolism of ccRCC.

5. Conclusions

IHC staining for myoferlin was significantly associated with the prognosis of ccRCC. In cross-correlation, ccRCC enriched with myoferlin was more likely to show high levels of EGFR expression and "nonclear" cytoplasmic characteristics based on lipogenic metabolism. Enrichment of EGFR as well as altered lipid metabolism would underlie the adverse outcome of myoferlin-high ccRCC, which needs further investigation for validation.

Conflict of interest

All authors declare that they have no conflict of interest.

Supplementary materials

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.urolonc.2019.07.002.

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