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ORIGINAL ARTICLE

Claspin overexpression is associated with high-grade histology and poor prognosis in renal cell carcinoma

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Abstract

Renal cell carcinoma (RCC) is one of the most common human cancers. We previously reported that claspin is a key regulator in the progression of gastric cancer, and it likely plays an important role in cancer stem cells of gastric cancer. However, the significance of claspin in RCC has not been examined. First, we analyzed the expression and distribution of claspin in 95 RCC cases by immunohistochemistry. In the nonneoplastic kidney, the staining of claspin was either weak or absent, whereas RCC tissue showed nuclear staining. In total, claspin expression was detected in 45 (47%) of 95 RCC cases. The claspin staining appeared relatively stronger in high nuclear grade RCC than in low nuclear grade RCC. Claspin-positive RCC cases were associated with higher T grade, tumor stage, nuclear grade, vein invasion, and poorer prognosis. *CLSPN* siRNA treatment decreased RCC cell proliferation. The levels of phosphorylated Erk and Akt were lower in *CLSPN* siRNA-transfected RCC cells than in control cells. In addition, claspin was coexpressed with CD44, epidermal growth factor receptor, p53, and programmed death ligand-1. These results suggest that claspin plays an important role in tumor progression in RCC and might be a prognostic marker and novel therapeutic target molecule.

KEYWORDS

cancer stem cell, claspin, EGFR, PD-L1, renal cell carcinoma

1 | INTRODUCTION

Renal cell carcinoma is a common type of human cancer that accounts for 3% of adult malignancies worldwide.[1] Although therapeutic outcomes have recently improved for surgical treatment of early RCC, it remains one of the world's leading causes of cancer-related death.[2] The prognosis of advanced RCC is poor in part because RCC is often resistant to traditional therapies such as radiation therapy and chemotherapy. Epidermal growth factor receptor-tyrosine kinase inhibitors and immunocheckpoint inhibitors targeting PD-1 or its ligand PD-L1 showed promising therapeutic efficacy in advanced RCC.[3-7] Recent reports showed that the expression of EGFR and PD-L1 is a poor prognostic factor in RCC.[6-8] Prognostic biomarkers are important in the guidance of therapeutic options and

Abbreviations: ALDH1, antialdehyde dehydrogenase isoform 1; ATR, ataxia-telangiectasia-mutated and Rad3-related kinase; Chk1, checkpoint kinase 1; CSC, cancer stem cell; EGFR, epidermal growth factor receptor; GC, gastric cancer; HER2, antihuman EGFR type 2; PD-1, programmed death-1; PD-L1, PD-1 ligand; RCC, renal cell carcinoma; RNAi, RNA interference; TCGA, The Cancer Genome Atlas.

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surveillance strategies.[9] Tumor-node-metastasis staging, nuclear grade, and vein invasion have been the most reliable prognostic factors in RCC.[9,10] However, their predictive accuracy remains limited due to individual variations. Therefore, there is a requirement for identifying new prognostic markers and new potential therapeutic targets in advanced RCC. Cancer stem cells are closely associated with chemotherapy resistance, recurrence, and metastasis. In RCC, CSCs have been reported as useful prognostic markers.[9]

Claspin is a nuclear protein related to DNA replication and damage response and is an important regulator for the S-phase checkpoint.[11] Phosphorylated claspin interacts with Chk1 to promote its activation by ATR-dependent phosphorylation.[11-13] Downregulation of claspin, and ATR and Chk1, greatly reduces cell survival and promotes alterations in cell cycle checkpoints and DNA repair systems.[12] These alterations could lead to genomic instability that triggers cancer development.[14-16] However, it has been reported that overexpression of claspin contributes to tumor proliferation in several human solid tumors, such as colon, lung, bladder, breast, ovarian, and cervical cancers.[16-19] Previously, we identified upregulation of the *CLSPN* gene in spheroid body-forming GC cell lines.[20] We also reported that overexpression of claspin is found in 47% of GC cases and is associated with tumor progression in GC.[20] Therefore, depending on the circumstances, claspin plays an ambivalent function of tumor suppression and promotion. To our knowledge, however, detailed function and expression profiles of the *CLSPN* gene in RCC have not previously been analyzed.

The present study is the first detailed analysis of claspin expression in RCC including its clinicopathological significance and biological function. To clarify the pattern of expression and localization of claspin in RCC, we undertook immunohistochemical analysis of surgically resected RCC samples and investigated the association between claspin expression and various clinicopathological characteristics. We also analyzed the effect of inhibiting claspin expression on cell growth of RCC cells. In addition, we investigated the relationship between claspin expression and representative cancer-related molecules, including CSC markers, in RCC.

2 | MATERIALS AND METHODS

2.1 | Tissue samples and cell lines

In this retrospective study, 95 primary tumors were collected from patients diagnosed as having RCC who underwent curative resection surgery at Hiroshima University Hospital (Hiroshima, Japan). All samples were obtained with patient consent, and the present study was approved by the Ethical Committee for Human Genome Research of Hiroshima University. However, written informed consent was not obtained; thus, for strict privacy protection, all identifying information associated with the samples was removed before the analysis. Only patients without preoperative radiotherapy or chemotherapy were enrolled in the study. The study cohort included 70 men and 25 women. Postoperative follow-up was scheduled every 1, 2, or 3 months during the first 2 years after surgery and every 6 months thereafter, unless more frequent follow-up was deemed necessary. Chest X-rays, chest computed tomography scans, and serum chemistry analyses were undertaken at every follow-up visit. Recurrence was evaluated from the patient records at Hiroshima University Hospital. Patients were followed by their physician until the patient's death or date of the last documented contact. Tumor staging was evaluated according to the TNM stage grouping system.

Human RCC-derived cell lines ACHN, Caki-1, and 786 were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). All cell lines were maintained in RPMI-1640 (Nissui Pharmaceutical) containing 10% FBS (Whittaker) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

2.2 | Immunohistochemistry

We used archival formalin-fixed, paraffin-embedded tissues from 95 patients who had undergone surgical excision of RCC between 2002 and 2012 at Hiroshima University Hospital. Immunohistochemical analysis was carried out with a Dako Envision+ Mouse Peroxidase Detection System (Dako Cytomation). Antigen retrieval was carried out by microwave heating in citrate buffer (pH 8.0) for 60 minutes. Peroxidase activity was blocked with 3% H_2O_2-methanol for 5 minutes, and the sections were incubated with normal goat serum (Dako Cytomation) for 20 minutes to block nonspecific Ab binding sites. Sections were incubated with a rabbit polyclonal anti-claspin Ab (1:20 000, clone ab3720; Abcam), anti-CD44 Ab (H-CAM) (1:00, clone DF1485; Novocastra), ALDH1 Ab (1:400, clone 44/ALDH1; BD Biosciences), anti-CD133 Ab (1:00, clone AC133, Miltenyi Biotec), anti-L-EGFR Ab (1:20, clone EGFR 113; Leica Biosystems), anti-HER2 Ab (1:300, clone PN2A; Dako Cytomation), anti-p53 Ab (1:50, clone DO-7; Dako Cytomation) and anti-PD-L1 Ab (ab205921, 1:300, clone 28-8; Abcam) for 1 hour at room temperature, followed by incubation with Envision+ anti-mouse peroxidase for 1 hour. The sections were incubated with DAB Substrate-Chromogen Solution (Dako Cytomation) for 5 minutes for color reaction and then counterstained with 0.1% hematoxylin. Negative controls were created by omission of the primary Ab.

When more than 10% of tumor cells were stained, immunostaining was considered positive for claspin (according to the median cut-off values rounded to the nearest 10%). Using these definitions, 2 observers (GK and KS) without knowledge of the clinical and pathologic parameters or patient outcomes, independently reviewed immunoreactivity in each specimen. If there were either slight discrepancies between 2 sections or interobserver differences, they were resolved by consensus review at a double-headed microscope after independent review. The expressions of CD44, ALDH1, CD133, EGFR, HER2, and PD-L1 were scored in all tumors as positive or negative. When more than 10% of tumor cells showed membranous staining, the immunostaining was considered positive. Scoring of ALDH1 was according to the intensity of Ab staining (0, no staining; 1+, weak staining; 2+, moderate staining; 3+, intense staining). All cases with Ab staining of 3+ or 2+ were defined as ALDH1 positive. The expression of PD-L1 in RCC has been reported in several studies, and various criteria have already existed. Thus, we

decided that the immunostaining of PD-L1 was considered positive according to median cut-off values rounded off to the nearest 5%.

2.3 | Western blot analysis

Tumor cells were lysed for western blotting as described previously.[21] The lysates (40 μg) were solubilized in Laemmli sample buffer by boiling and then subjected to 10% SDS-PAGE and electrotransferred onto a nitrocellulose filter. The filter was incubated with the primary Ab against claspin. The claspin Ab was a polyclonal Ab that had been raised in our laboratory, and its specificity has been characterized in detail. Peroxidase-conjugated anti-mouse IgG was used in the secondary reaction. Immunocomplexes were visualized with an ECL Western Blot Detection System (Amersham Biosciences). β-Actin antibody (Sigma Chemical) was also used as a loading control.

2.4 | RNA interference

To knock down endogenous claspin, RNAi was carried out as described previously.[22] Small interference oligonucleotides for claspin and a negative control were purchased from Invitrogen. Transfection was carried out using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. Briefly, 60 pmol siRNA and 10 μL Lipofectamine RNAiMAX were mixed in 1 mL RPMI medium (10 nmol/L final siRNA concentration). After 20 minutes of incubation, the mixture was added to the cells, and these were plated on dishes for each assay. The cells were analyzed at 48 hours after transfection in all experiments.

2.5 | Cell growth assays

An MTT assay was carried out to examine cell growth. The cells were seeded at a density of 4000 cells/well in 96-well plates. Cell growth was monitored after 1, 2, and 4 days. We undertook 3 different experiments and calculated the mean and SD in each of the MTT assays and the modified Boyden chamber assays.

2.6 | Statistical analysis

Correlations between the clinicopathological parameters and claspin expression were analyzed using Fisher's exact test. Kaplan-Meier survival curves were constructed for claspin-positive and claspin-negative patients, and the survival rates of the 2 groups were compared. Differences between the survival curves were tested for statistical significance by log-rank test. Univariate and multivariate Cox regression analyses were used to evaluate the associations between clinical covariates and survival as described previously.[23] A P value of less than 0.05 was considered to indicate statistical significance. The SPSS software program (SPSS Inc.) was used for all statistical analyses. Another gene expression profile and survival data are available on a total of 843 RCC patients from TCGA database.[24]

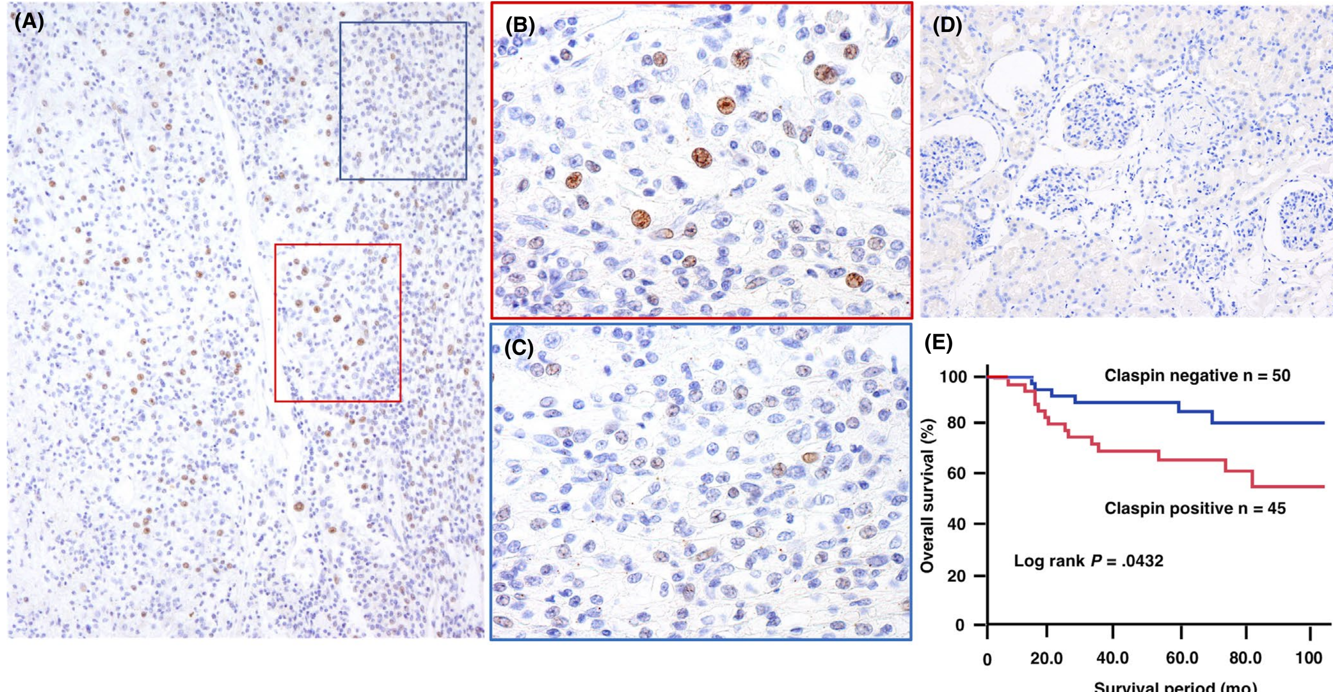

FIGURE 1 Immunohistochemical analysis of claspin. A, Claspin expression in renal cell carcinoma (RCC) tissue (anticlaspin Ab immunohistochemical staining; magnification, ×100). B, High-magnification image of claspin expression in high nuclear grade tumors (anticlaspin Ab immunohistochemical staining, ×400). C, High-magnification image of claspin expression in low nuclear grade tumors (anticlaspin Ab immunohistochemical staining, ×400). D, Claspin expression in corresponding nonneoplastic kidney (anticlaspin Ab immunohistochemical staining, ×100). E, Kaplan-Meier plot of survival for patients with RCC by tumor claspin expression

3 | RESULTS

3.1 | Expression analysis of claspin in RCC

Claspin expression was detected in 45 (47%) of 95 RCCs by immunohistochemistry, and it showed nuclear staining in tumor cells (Figure 1A). In high-power fields, claspin staining in the high nuclear grade tumors appeared relatively stronger than that in the low nuclear grade tumors (Figure 1B,C). In the nonneoplastic kidney, the staining of claspin was either weak or absent in epithelial and stromal cells (Figure 1D). Next, we analyzed the relationship between claspin expression and various clinicopathological characteristics. Claspin expression was associated with higher T grade (*P* = .0251), tumor stage (*P* = .0211), nuclear grade (*P* = .0105), and vein invasion (*P* = .0347) in claspin-positive than in claspin-negative RCC cases (Table 1). Claspin expression was not associated with age, sex, N grade, M grade, or histology. We next examined CLSPN expression in TCGA kidney RCC RNA sequencing database that included 488 clear cell RCCs, 160 papillary type 1 RCCs, 70 papillary type 2 RCCs, and 81 chromophobe RCCs. Through bioinformatics analysis, we found that expression of CLSPN was significantly higher in all 4 of these types of RCC tissues than in adjacent normal tissues.

TABLE 1 Relationship between claspin expression and clinicopathologic characteristics in the 95 renal cell carcinoma cases

	Claspin expression		
	Positive (%)	Negative	*P* value
Age, years			
≤65 (n = 48)	24 (50)	24	NS
>66 (n = 47)	21 (45)	26	
Sex			
Female (n = 25)	11 (44)	14	NS
Male (n = 70)	34 (49)	36	
T grade			
T1 (n = 60)	23 (38)	37	.0209
T2/T3/T4 (n = 35)	22 (62)	13	
N grade			
N0 (n = 82)	38 (46)	44	NS
N1/2/3 (n = 13)	7 (54)	6	
M grade			
M0 (n = 78)	34 (44)	44	NS
M1 (n = 17)	11 (65)	6	
Stage			
Stage I (n = 58)	22 (38)	36	.0211
Stage II/III/IV (n = 37)	23 (62)	14	
Histology			
Clear (n = 68)	33 (42)	35	NS
Non-clear (n = 27)	12 (44)	15	
Nuclear grade			
Grade 1/2 (n = 65)	25 (38)	40	.0105
Grade 3 (n = 30)	20 (67)	10	
Vein invasion			
v0 (n = 69)	28 (41)	41	.0301
v1 (n = 26)	17 (65)	9	

Note: P values were calculated with Fisher's exact test.
Abbreviation: NS, not significant.

3.2 | Relationship between claspin expression and prognosis in RCC

We undertook a Kaplan-Meier analysis to investigate the association between claspin expression and patient prognosis. Claspin expression was significantly associated with a poorer prognosis (*P* = .0432, log-rank test; Figure 1E). We also undertook univariate

TABLE 2 Univariate and multivariate analysis of factors influencing survival in 95 renal cell carcinoma patients

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age, years				
≤65	1 (reference)	.0875		
>65	2.16 (0.92-5.46)			
Sex				
Female	1 (reference)	.2111		
Male	2.04 (0.70-8.66)			
T grade				
T1	1 (reference)	.0002		
T2/T3/T4	5.01 (2.16-12.53)			
N grade				
N0	1 (reference)	<.0001		
N1/N2/N3	8.17 (3.11-20.29)			
M grade				
M0	1 (reference)	<.0001		
M1	16.00 (6.31-42.05)			
Stage				
Stage I	1 (reference)	<.0001	1 (reference)	.0042
Stage II/III/IV	6.01 (2.53-15.72)		4.03 (1.54-11.42)	
Nuclear grade				
Grade 1/2	1 (reference)	.0003	1 (reference)	.0434
Grade 3	4.78 (2.08-11.89)		2.59 (1.03-6.87)	
Claspin expression				
Negative	1 (reference)	.0437	1 (reference)	.1896
Positive	2.36 (1.02-5.89)		1.77 (0.75-4.29)	

Note: P values were calculated with Fisher's exact test.

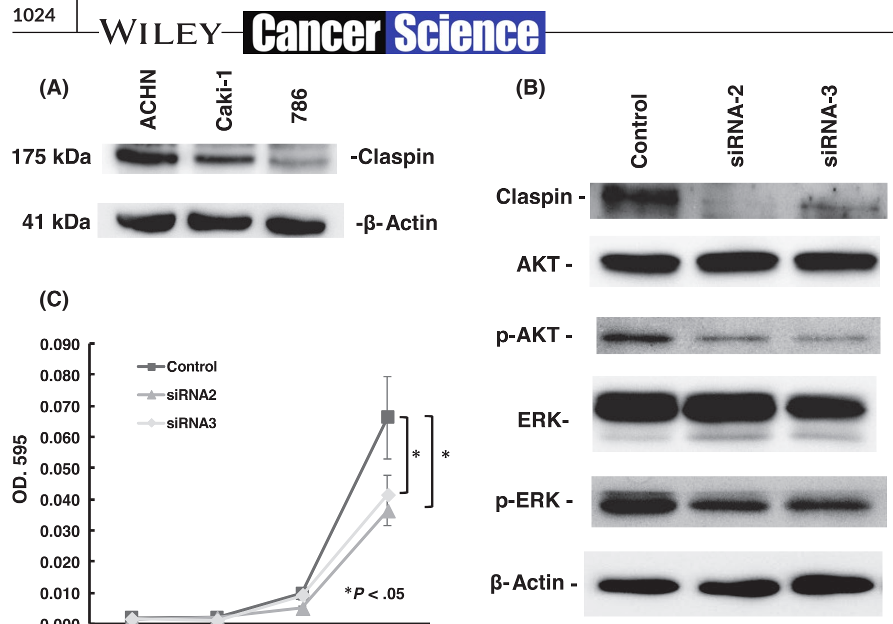

FIGURE 2 Effect of claspin downregulation. A, Anticlaspin Ab was detected in the band at approximately 175 kDa on western blot analysis of 3 renal cell carcinoma cell lines. β-Actin was used as a loading control. B, Western blot analysis of claspin, Akt, phosphor-Akt (pAkt), ERK1/2 and phosphor ERK1/2 (pERK1/2) in ACHN cells transfected with the claspin siRNA and negative control siRNA. C, Effects of claspin knockdown on the growth of ACHN cells. Cell growth was assessed by an MTT assay at 1, 2 and 4 days after seeding on 96-well plates. Means (bars) and standard deviation (SD; error bars) of 3 different experiments are shown

and multivariate Cox proportional hazards analyses but did not find claspin expression to be an independent prognostic predictor (Table 2). Furthermore, the RNA sequencing data from TCGA provided the survival analysis between RCC cases and *CLSPN* mRNA. The result showed a tendency for claspin expression to be associated with poor prognosis in RCC patients.

3.3 | Effect of claspin downregulation on cell growth activity of RCC cells

We next investigated the effect of claspin inhibition on cell growth on RCC cells using siRNA. Western blot analysis of claspin in 3 RCC cell lines (ACHN, Caki-1, and 786) showed that expression of claspin was detected in all 3 lines (Figure 2A). We selected ACHN cells for knockdown analysis because the highest claspin expression was detected in these cells. Claspin expression was suppressed by treatment with siRNA2 and siRNA3, as confirmed by western blot analysis (Figure 2B). To investigate the possible antiproliferative effects of *CLSPN* knockdown, we carried out an MTT assay at 4 days after the transfection of siRNA. Cell proliferative ability was significantly reduced in *CLSPN* knockdown RCC cells compared with negative control siRNA-transfected RCC cells (Figure 2C).

3.4 | Effect of claspin inhibition on Akt and Erk pathways

To clarify the molecular signaling pathways associated with proliferation activities, we elaborated the phosphorylation of EGFR downstream molecules. Because EGFR activates the RAS-MAPK-Erk and Akt-phosphatidylinositol-4,5-bisphosphate 3-kinase signaling pathways, thus leading to cancer cell proliferation and survival, the effect

TABLE 3 Relationship between claspin expression and various molecules including cancer stem cell markers in the 95 renal cell carcinoma cases

	Claspin expression		
	Positive (%)	Negative	*P* value
CD44			
Positive (n = 27)	21 (78)	6	.0002
Negative (n = 68)	24 (35)	44	
ALDH1			
Positive (n = 64)	33 (52)	31	NS
Negative (n = 31)	12 (38)	19	
CD133			
Positive (n = 14)	5 (36)	9	NS
Negative (n = 81)	40 (49)	41	
EGFR			
Positive (n = 32)	22 (69)	10	.0029
Negative (n = 63)	23 (37)	40	
HER2			
Positive (n = 20)	10 (50)	10	NS
Negative (n = 75)	35 (47)	40	
p53			
Positive (n = 37)	24 (65)	13	.0064
Negative (n = 58)	21 (36)	37	
PD-L1			
Positive (n = 33)	24 (73)	9	.0003
Negative (n = 62)	21 (33)	41	

Note: P values were calculated with Fisher's exact test.

Abbreviations: ALDH1, aldehyde dehydrogenase isoform 1; EGFR, epidermal growth factor receptor; HER2, antihuman EGFR type 2; NS, not significant; PD-L1, programmed death ligand-1.

of CLSPN inhibition on EGFR signaling was analyzed in the present study. The results indicated that the levels of phosphorylated Akt and Erk were lower in claspin siRNA2- and siRNA3-transfected ACHN cells than in control cells (Figure 2B).

3.5 | Analysis of the correlation between claspin expression and various cancer-related molecules

We revealed that claspin could contribute to tumor progression in RCC and that it was associated with the Akt and Erk pathways in EGFR downstream molecules. We thus next performed immunohistochemical analysis of various cancer-related molecules in 95 RCC cases. Of these 95 RCC cases, 27 (28%) were positive for CD44, 64 (67%) were positive for ALDH1, 12 (15%) were positive for CD133, 32 (34%) were positive for EGFR, 20 (21%) were positive for HER2, 35 (37%) were positive for p53, and 33 (35%) were positive for PD-L1. Claspin expression was significantly associated with the expression of CD44 (P = .0002), EGFR (P = .0029), p53 (P = .0062), and PD-L1 (P = .0003; Table 3; Figure 3).

4 | DISCUSSION

In the present study, we investigated the clinicopathological significance of claspin expression in RCC. The analysis of data in TCGA showed that claspin expression was upregulated in RCC tissues. Our immunohistochemical analysis showed claspin expression was detected in 45 (47%) of 95 RCCs. Claspin staining appeared to be relatively stronger in high nuclear grade tumors than in low nuclear grade tumors. Moreover, claspin expression was associated with T grade, tumor stage, nuclear grade, vein invasion, and poor prognosis. In addition, knockdown of *CLSPN* by RNAi was found to inhibit cancer cell proliferation and the levels of phosphorylated Akt and Erk. Taken together, these results suggest that claspin serves an important role in the progression of RCC.

Cancer stem cells are thought to be responsible for recurrence or distant metastasis and for chemotherapy and radiotherapy resistance in many malignancies.[25] Cancer stem cells are frequently identified by surface protein markers such as CD44, CD105, CD133, ALDH1, and CXCR4 in RCC.[9,26,27] Among these surface markers, CD44 is the most frequently reported, and a positive correlation has been suggested with aggressive behavior in RCC.[28,29] In fact, many studies have shown

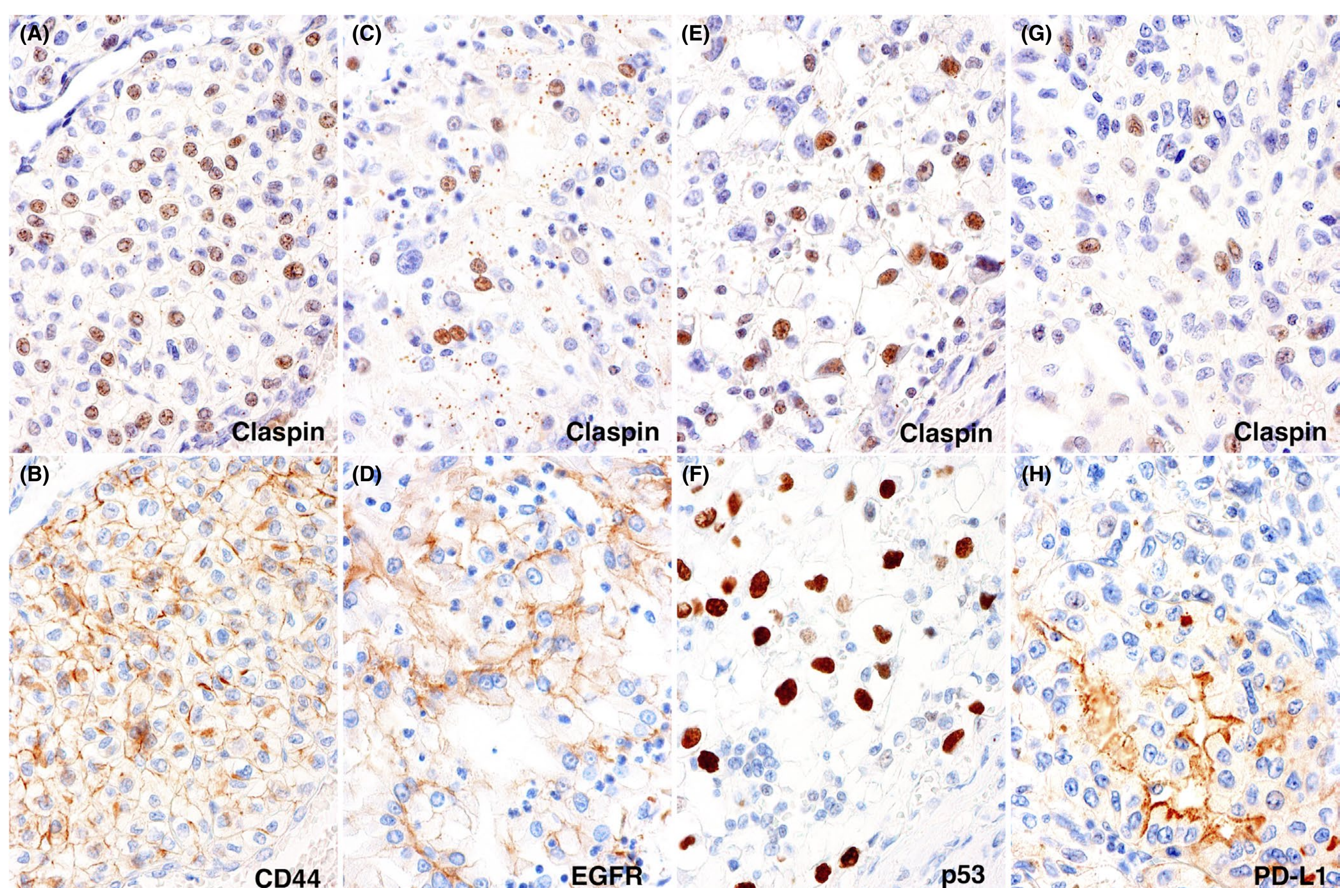

FIGURE 3 Immunohistochemical analysis of claspin and various molecules in consecutive tumor sections of renal cell carcinoma. A, C, E, G, Nuclear expression of claspin (anticlaspin Ab immunohistochemical staining; magnification, ×400). B, Membrane expression of CD44 (anti-CD44 Ab immunohistochemical staining, ×400). D, Membrane expression of epidermal growth factor receptor (EGFR) (EGFR Ab immunohistochemical staining, ×400). F, Nuclear expression of p53 (p53 Ab immunohistochemical staining, ×400. H, Membrane expression of programmed death ligand-1 (PD-L1) (PD-L1 Ab immunohistochemical staining, ×400)

that CD44 expression was reported to significantly correlate with high nuclear grade, vein invasion, recurrence, and poor prognosis, and it has been suggested to be a useful prognostic marker.[29-32] Moreover, CD44 promotes EGFR-mediated pathways, consequently leading to tumor cell growth, tumor cell migration, and chemotherapy resistance in solid cancers.[33] Therefore, as claspin expression in RCC was closely associated with CD44 expression in the present study, claspin might play an indispensable role in CSCs of RCC and might contribute to higher tumor stage, nuclear grade, and vein invasion in collaboration with CD44. In fact, overexpression of claspin increases resistance to radiotherapy and chemotherapy in lung and ovarian cancers.[19,34,35] Furthermore, we revealed that claspin expression was associated with p53 and poor prognosis. Similar to claspin, the tumor suppressor p53 is one of the key regulators in DNA damage response and the cell cycle checkpoint, and mutations of p53 have also been shown to lead to the generation of CSCs.[36,37] In addition, several reports have shown that p53 expression in RCC predicts a poorer outcome.[38] A recent study reported that claspin is also a marker of prognosis in non-small-cell lung cancer.[35] Thus, these results indicate that claspin might be one of the potential prognostic factors in RCC.

CLSPN knockdown affects the levels of EGFR downstream molecules. We showed that the levels of phosphorylated Akt and Erk were lower in claspin siRNA-transfected RCC cells than in control cells. As the phosphorylation of Akt and Erk results in the inhibition of apoptosis and contributes to tumor progression,[39] these results suggest that apoptosis could be induced in claspin-knockdown RCC cells. Moreover, our results showed that claspin expression was frequently observed in EGFR-positive RCC cells by immunohistochemistry. Epidermal growth factor receptor plays a significant role in promoting tumor progression, and its associated signaling pathways are well established targets in cancer therapy.[40] Overexpression of EGFR in RCC is correlated with cell proliferation, high tumor stage, high nuclear grade, tumor recurrence, and overall survival.[41] Thus, EGFR was considered a key factor in the prognosis of RCC and a potential target for the treatment of RCC. Li et al[42] reported the inhibition of EGFR-induced apoptosis by the suppression of claspin protein in drug-sensitive breast cancer cells. Taken together, these findings indicate that claspin could participate in the activation of EGFR, and the inhibition of claspin could suppress the EGFR signaling pathway and induce apoptosis in RCC cells.

The present study found that claspin expression was significantly associated with PD-L1 expression. The PD-1/PD-L1 pathway is one of the most important signaling pathways in immune checkpoint therapy. Recent studies have revealed that immune checkpoint inhibitors targeting PD-1/PD-L1 signaling have shown a promising response in patients with advanced RCC.[8,43] Also, PD-L1 expression was associated with adverse clinicopathological features, including higher nuclear grade, necrosis, and sarcomatoid transformation. Interestingly, positive correlations have been shown between PD-L1 and EGFR expression in RCC.[8] Therefore, the present study showed that claspin might be a promising molecule for treating RCC. However, further extensive study will be required to elucidate the detailed molecular mechanism of its activity in tumor cell biology, which might improve our understanding of RCC carcinogenesis and tumor progression.

CONFLICT OF INTEREST

The authors declare no conflicts of interest in association with the present study.

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