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Original Article

ERG and nestin: useful markers of immature vessels and novel prognostic markers in renal cell carcinoma

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Abstract: Objectives: Renal cell carcinoma (RCC) accounts for approximately 90% of all renal malignancy. Because a rich vasculature is an outstanding feature of RCC, information on the blood vessels of RCC might explain its tumor characteristics. Several researchers have noted the effects of tumor vessels on the clinicopathologic characteristics and prognosis of tumors; however, a clear association has not been established. We hypothesized that the immaturity of the neovasculature may be an important clinicopathologic characteristic forprognosis of RCC patients. ERG and nestin are new vascular markers that regulate vascular homeostasis and angiogenesis. Therefore, in the present study, we investigated how ERG and nestin were expressed with respect to tumor characteristics. Materials and Methods: IHC staining for ERG, nestin, CD31, and CD34 was performed for 217 renal tumors, including clear-cell RCC (ccRCC; n = 184), papillary RCC (pRCC; n = 14), chromophobe RCC (chRCC; n = 14), and oncocytoma (n = 5). Results: Vascular endothelial cells from normal kidney consistently showed strong nuclear expression of ERG and nestin. Conversely, a loss of ERG and nestin expression was observed in endothelial cells of some tumor blood vessels, which was associated with tumor progression. In particular, the loss of ERG expression was significantly associated with progression-free survival and overall survival (univariate analyses: $P = 0.027$ and $P = 0.004$, respectively; multivariate analyses: $P = 0.030$ and $P = 0.046$, respectively). Conclusion: A loss of ERG and nestin expression is associated with tumor progression, and loss of ERG is a powerful prognostic marker for ccRCC.

Keywords: Renal cell carcinoma, ERG, nestin, angiogenesis, immature vessel, prognostic factor

Introduction

Renal cell carcinoma (RCC) accounts for approximately 90% of all renal malignancy [1] and is characterized by rich neovascularization. RCC frequently shows a prominent vascular network. Because a rich vasculature is an outstanding feature of this tumor, information on the blood vessels of RCC might explain its tumor characteristics.

Angiogenesis, which refers to the proliferation and sprouting of existing blood vessels close to the tumor, is crucial for malignancy [2]. The degree of angiogenesis has previously been quantified using several measures, such as microvascular density (MVD), microvascular area (MVA), the expression of angiogenic molecules within the tumor, and the presence of angiogenic receptors within the tumor tissue. MVD, one of the best-known indicators of angiogenesis, is given as the number of small vessels per square millimeter of tumor area. The degree of tumor angiogenesis based on MVD has been proven a useful predictor of survival in several cancers. MVD has been applied to solid tumors of many organs, including prostate [3], breast [4], stomach [5], ovary [6], bladder [7], colorectum [8], lung [9], and melanoma [10]. Increased MVD was associated with a poor prognosis in these tumors. However, in a meta-analysis, MVD did not prove to be a reliable predictor of survival in RCCs [11]. Alternatively, other measures, such as endothelial cell proliferation fraction [12] and MVA [13, 14], have been used in RCCs. However, determining the endothelial cell proliferation fraction is complex and results for MVA have been equivocal.

Table 1. Composition of 216 kidney tumor cases

Tumor type	n
ccRCC	184
pRCC	14
chRCC	14
Oncocytoma	5
Total	217

ccRCC, clear-cell renal cell carcinoma; pRCC, papillary renal cell carcinoma; chRCC, chromophobe renal cell carcinoma.

Yao [12] has identified two types of microvessels in RCCs: undifferentiated (CD31+/CD34-) and differentiated (CD34+). The researchers found that a higher proportion of undifferentiated microvessels was associated with greater malignant potential and poorer prognosis, while a higher proportion of differentiated microvessels was associated with lower malignant potential and better prognosis. In addition, Sato [13] reported the MVA of immature vessels was associated with the aggressiveness of RCC, suggesting it might be a novel prognostic factor. The authors defined immature vessels as those positive only for CD34, and mature vessels as positive for both CD34 and α-smooth muscle actin (α-SMA). Therefore, the quality in terms of maturity or differentiation of tumor vessels within RCCs could be more important than their quantity.

Therefore, we hypothesized that immaturity of the neovasculature may be more important than MVD in RCCs. The present study investigated how vascular markers, including erythroblast transformation-specific-related gene (ERG), nestin, CD34, and CD31, were expressed based on tumor characteristics. First, we focused on ERG because this protein is a highly sensitive immunohistochemical marker for vascular differentiation. However, due to its recent introduction on the commercial market, minimal research has been performed. In addition, because ERG is expressed in the nucleus, the evaluation is objective and easy. ERG is expressed throughout the life of the endothelium and is a regulator of vascular homeostasis and angiogenesis [15, 16]. ERG is essential for postnatal vascular development and is involved in tumor angiogenesis and growth [17]. ERG has been described in several tumors, such as prostatic carcinoma [18], acute myeloid leukemia [19], and Ewing sarcoma [20]. Haber [21] suggested that ERG could be a novel, reliable, and specific marker for endothelial cells in central nervous system (CNS) tumors. However, ERG expression in RCCs has not been studied to date and its value compared with other vascular endothelial (VE) markers has not been reported.

Another marker of interest was nestin, which is closely associated with angiogenesis [22]. In a previous study, nestin expression was found in hemangioblastomas associated with von Hippel-Lindau (VHL) mutations, such as clear-cell RCCs (ccRCCs) [22]. We hypothesized that nestin was also expressed in neoplastic vessels in ccRCCs because they share a similar mechanism of angiogenesis with hemangioblastomas. However, the association of nestin expression with patient prognosis in RCC has not been reported in previous studies. Thus, ERG and nestin expression in RCCs were evaluated in the present study using immunohistochemistry (IHC). The expression of other VE markers (CD31 and CD34) was also assessed and compared. In the present study, the degree of ERG and nestin expression in endothelial cells in RCCs, the association with clinicopathologic factors, and prognostic significance were evaluated.

Materials and methods

Case selection

We retrospectively reviewed 216 surgically resected kidney tumor specimens at Soonchunhyang University Bucheon Hospital (2001-2012). We ensured that specimens before the year 2012 were from patients who were followed up for at least 5 years. The selected 217 cases consisted of ccRCC (n = 184), papillary RCC (pRCC; n = 14), chromophobe RCC (chRCC; n = 14), and oncocytoma (n = 5; **Table 1**). All cases were independently reviewed by two pathologists (A. Moon and M. Jung), who confirmed the histologic diagnoses. Clinicopathologic measures of the tumor, including histologic subtype, size, the Tumor, Node, Metastasis (TNM) staging system, the International Society of Urological Pathology (ISUP) nucleolar grade, presence of metastasis, and lymphovascular invasion were also assessed. Clinical information was retrieved from medical records.

Table 2. Antibodies used in the study

Antibody	Clone	Company
ERG	EPR3864	Ventana, Tucson, AZ, USA
CD31	1A10	Leica Biosystems, Newcastle, UK
CD34	QBEnd/10	Leica Biosystems, Newcastle, UK
Nestin	10c2	Santa Cruz Biotechnology, Dallas, TX, USA

ERG, erythroblast transformation-specific-related gene; CD31, cluster of differentiation 31; CD34, cluster of differentiation 34.

Tissue microarray (TMA) construction

Formalin-fixed (10%), paraffin-embedded tissue blocks were obtained for the selected 216 cases. Tissue microarrays (TMAs) were constructed from the blocks. A representative area of each tumor, including the area with the highest grade was carefully selected from a hematoxylin and eosin (H&E)-stained section. For each case, two cores 3 mm in diameter were obtained. Cores were also taken from areas of normal kidney tissue if present in the block. Normal kidney TMAs were used as controls.

IHC

To overcome intratumoral heterogeneity, 10 cases of ccRCC, 3 cases of chRCC, 3 cases of pRCC, and all cases of oncocytoma were stained as whole sections. ERG expression was mostly decreased in the area of highest ccRCC grade. TMA sections were subjected to IHC staining for CD31, CD34, nestin, and ERG using antibodies listed in **Table 2**.

Immunostains for ERG, nestin, CD31, and CD34 were performed using a Bond Polymer Intense Detection System (Vision BioSystems, Mount Waverley, Victoria, Australia) according to the manufacturer's instructions with minor modifications. Briefly, 4-μm-thick sections of formalin-fixed, paraffin-embedded tissues were deparaffinized with Bond Dewax Solution (Vision BioSystems). An antigen retrieval procedure was then performed using Bond ER Solution (Vision BioSystems) for 30 minutes at 100°C. Endogenous peroxidases were quenched by incubation with hydrogen peroxide for 5 minutes at room temperature. These sections were then incubated for 15 minutes at room temperature with antibodies against ERG, nestin, CD31, and CD34 using a biotin-free polymeric horseradish peroxidase-linker antibody conjugate system in a Bond-max autostainer (Vision BioSystems). Nuclei were counterstained with hematoxylin. Endothelial cells in normal kidney served as positive controls. The same normal tissues incubated without antibodies served as negative controls.

Stained sections were evaluated under a light microscope at 200× magnification. In the case of ERG, nuclear staining was considered to indicate positive expression. In the case of nestin, cytoplasmic staining was considered to indicate positive expression. Due to lack of specific studies on ERG expression, a semi-quantitative scoring model was used to objectively evaluate the immune pattern. Briefly, the score was the sum of the percentage of positive endothelial cells (0, none; 1, ≤25%; 2, 25-50%; or 3, >50%) and staining intensity (0, none; 1, weak; 2, moderate; 3, strong) [23]. Tumors were divided into three groups based on ERG expression: negative (final scores, 0-2), equivocal (final scores, 3-4), and positive (final scores, 5-6). In the case of nestin expression, tumors were divided into three groups based on a previous study [22]. In that study, the expression of nestin in endothelial cells was evaluated based on positive cells found in a single microscopic field: negative, no staining; equivocal, positive cells found with 200× magnification; and positive, positive cells found with 100× magnification [22]. All slides were examined and scored by two independent pathologists (A. Moon and E. Han) who were blinded to clinicopathological data and patient identity. Disagreements between the two pathologists were resolved by consensus.

Statistical analysis

A statistical analysis was performed using SPSS Statistics (ver. 20.0; IBM Corp., Armonk, NY, USA). The *t*-test was used to compare between the protein expression groups and the clinicopathologic measures. Survival curves were plotted using the Kaplan-Meier method and significance was determined using the log-rank test. Multivariate relationships between

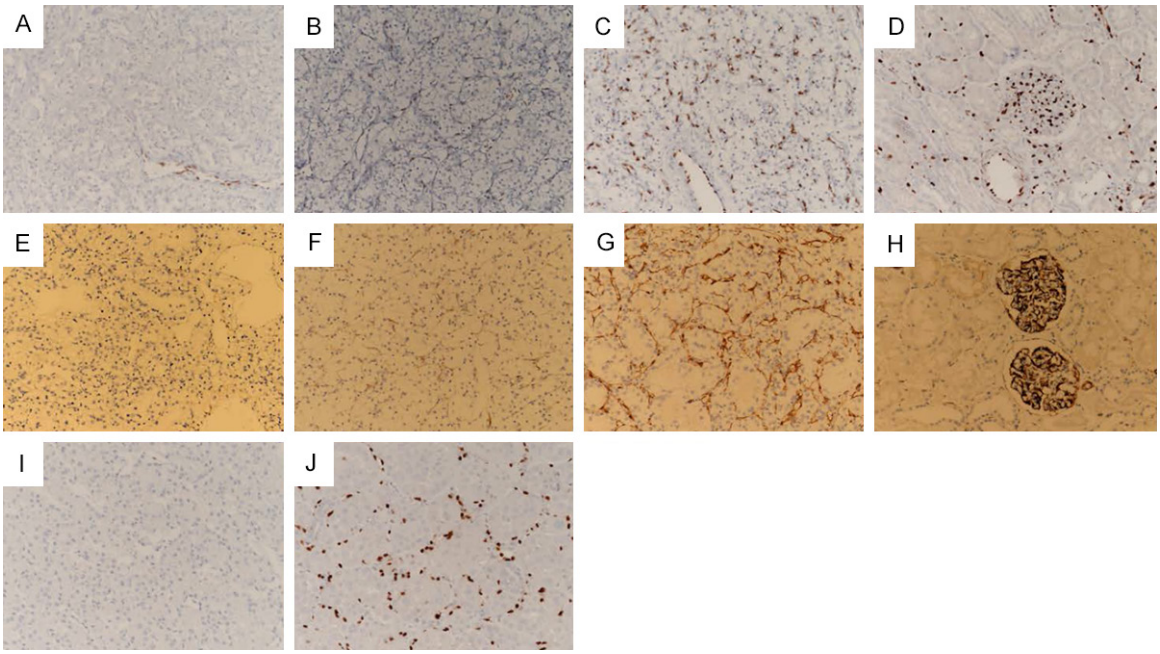

Figure 1. Erythroblast transformation-specific-related gene (ERG) expression (magnification 200×). (A) Negative expression of ERG in clear-cell renal cell carcinoma (ccRCC); normal vessel shows positive ERG expression in the lower right corner, (B) equivocal expression of ERG in ccRCC, (C) positive expression of ERG in ccRCC, (D) expression of ERG in normal kidney cortex, (E) negative expression of nestin in ccRCC, (F) equivocal expression of nestin in ccRCC, (G) positive expression of nestin in ccRCC, (H) expression of nestin in normal kidney cortex, (I) negative expression of ERG in chromophobe RCC, (J) no loss of the expression of ERG in oncocytoma.

patient survival and biomarkers, along with other clinical parameters, were investigated using a Cox proportional hazards regression model. A *P*-value <0.05 was considered significant.

Results

Vascular marker expression, including ERG and nestin, in ccRCC

Under a light microscope at 200× magnification, IHC staining of intratumoral endothelial cells was evaluated. A total of 217 cases were divided into three groups based on the degree of ERG and nestin expression as mentioned above: negative, equivocal, and positive. Among 184 ccRCCs, 31.5% (n = 58) were negative, 15.2% (n = 28) equivocal, and 53.2% (n = 98) positive for ERG based on IHC staining (**Figure 1A-C**). Vascular endothelial cells of the normal kidney in the control group consistently showed strong nuclear immunoreactivity for ERG (**Figure 1D**). Loss of ERG expression was associated with a higher ccRCC stage (P = 0.006). In addition, 11.4% (n = 21) were negative, 63% (n = 116) equivocal, and 25.5% (n = 47) positive for nestin based on IHC staining (**Figure 1E-G**). Nestin was strongly expressed in the glomerular endothelial cells in non-neoplastic renal tissue (**Figure 1H**). Similar to ERG, loss of nestin expression was associated with higher ccRCC stage (P = 0.036). Furthermore, a significant correlation was observed between ERG and nestin expression (P<0.001). The correlation of ERG and nestin immunoreactivities with clinicopathologic variables in 184 patients with ccRCC is shown in **Table 3**. With CD31 and CD34, differences in expression were not observed between mature and immature blood vessels, and the expression was similar in all blood vessels. Therefore, it was used for comparison as a control group.

Survival analysis and clinicopathologic measures in ccRCC

The results of a univariate survival analysis based on clinicopathologic measures in ccRCC are shown in **Table 4**. ERG expression level, age, ISUP nucleolar grade, and stage group were all significantly associated with progression-free survival (PFS) and overall survival (OS). In contrast, nestin expression level and

Table 3. Correlation of erythroblast transformation-specific-related gene and nestin immunoreactivity with clinicopathologic variables in 184 patients with clear-cell renal cell carcinoma

	n (n = 184)	P of ERG†	P of nestin†
Age		0.985	0.350
≤63	90		
>63	94		
Gender		0.157	0.565
Male	123		
Female	61		
Grade		0.082	0.303
G1/2	93		
G3	70		
G4	21		
Stage group		0.006*	0.036*
I	111		
II	31		
III/IV	42		
Distant metastasis			
Absent	176	0.209	0.261
Present	8		
Nestin		<0.001*	

ERG, erythroblast transformation-specific-related gene. *Statistically significant (P<0.05). †Calculated using the chi square test.

sex were not significantly associated with PFS and OS. Univariate analysis showed that loss of ERG expression was significantly associated with poor PFS and OS (P = 0.027 and P = 0.004, respectively; **Figure 2**). Nestin expression was not significantly associated with poor PFS or OS in ccRCCs (P = 0.608 and P = 0.301, respectively; **Table 4**). Multivariate analysis also showed that loss of ERG expression was a significant prognostic factor for both PFS and OS (P = 0.030 and P = 0.046, respectively; **Tables 5** and **6**).

ERG expression in other renal tumors

Among 14 chRCC cases, 13 (93%) showed reduced ERG expression (negative: 78.6%, equivocal: 14.3%; **Figure 1I** and **Table 7**). Only 1 chRCC case was positive for ERG expression. Conversely, all oncocytoma cases (n = 5) were positive for ERG expression (**Figure 1F**). The difference in ERG expression between chRCC and oncocytoma was significant (P = 0.002) despite the small number of cases. Among pRCC cases,

Table 4. Univariate survival analysis according to clinicopathologic measures in clear-cell renal cell carcinoma (n = 184)

Finding	n	P of PFS	P of OS
ERG expression		0.027*	0.004*
Positive	98		
Equivocal	28		
Negative	58		
Nestin expression		0.608	0.301
Positive	47		
Equivocal	116		
Negative	21		
Age		0.006*	0.036*
≤63	90		
>63	94		
Sex		0.293	0.871
Male	120		
Female	64		
ISUP nucleolar grade		<0.001*	<0.001*
1	7		
2	86		
3	70		
4	21		
Stage group		<0.001*	<0.001*
1	111		
2	31		
3-4	42		

ERG, erythroblast transformation-specific-related gene; ISUP, International Society of Urological Pathology; PFS, progression-free survival; OS, overall survival. *Statistically significant (P<0.05).

ERG expression in vascular endothelial cells could not be sufficiently evaluated due to a marked decrease in MVD.

Discussion

Renal cell carcinoma (RCC) accounts for approximately 90% of all renal malignancies [1] and in order of frequency includes ccRCC, pRCC, chRCC, and others. Sporadic and hereditary ccRCCs are characterized by inactivation of the VHL tumor suppressor gene on chromosome 3p [24] which results in hyperactivity of hypoxia-inducible factor-α (HIF-α). VHL targets HIF-α for proteolysis. Inactivation of VHL results in the accumulation of HIF transcription factors rather than their ubiquitination. Consequently, HIF translocates to the nucleus and induces the transcription of vascular endothelial (VE) growth factor (VEGF) and platelet-derived

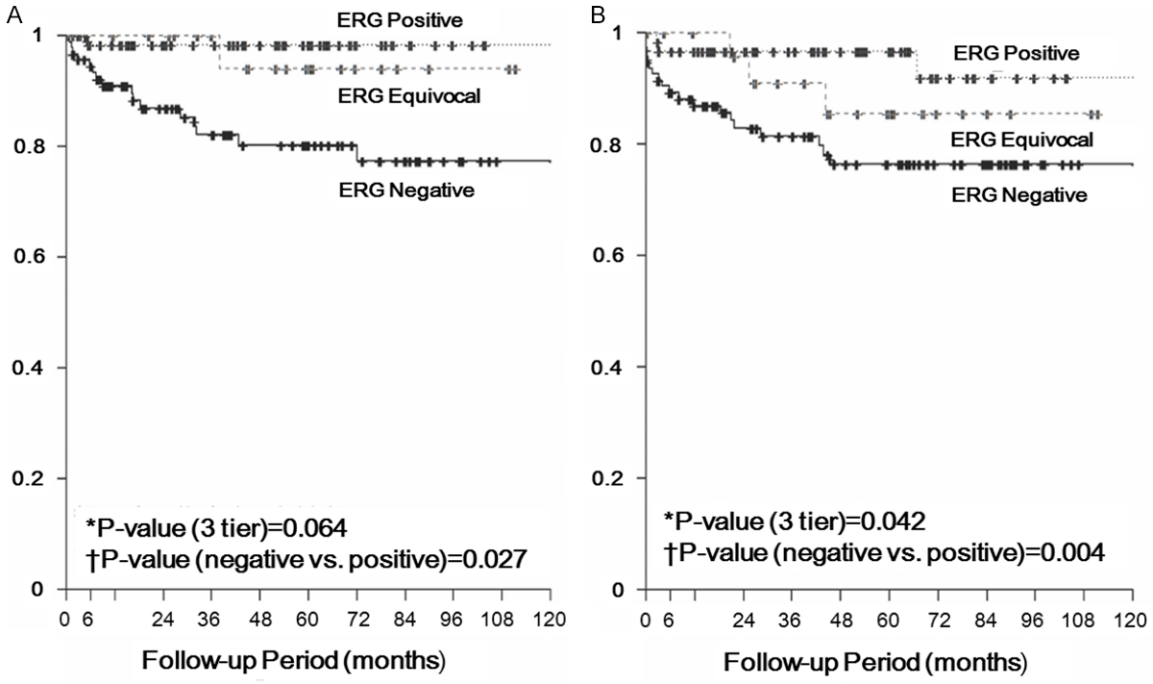

Figure 2. A. Kaplan Meier survival curve for progression-free survival. Kaplan Meier analysis shows decreased progression-free survival in the ERG negative group vs. ERG positive group. *Log rank test P = 0.064. †Negative vs. positive post hoc analysis, raw P = 0.027. B. Kaplan Meier survival curve for overall survival (OS). Kaplan Meier analysis shows decreased OS in the ERG negative group vs. ERG positive group. *Log rank test P = 0.0042. Negative vs. positive post hoc analysis, raw P = 0.004.

Table 5. Cox regression analysis for progression-free survival

	HR (95% CI)	P
ERG expression		
Positive	Reference	
Equivocal	3.209 (0.286-36.057)	0.345
Negative	10.575 (1.259-88.866)	0.030*
Nestin expression		
Negative	Reference	
Positive	2.252 (0.749-6.775)	0.149
Age	0.967 (0.931-1.005)	0.085
Sex		
Male	Reference	
Female	0.739 (0.225-2.432)	0.619
ISUP nucleolar grade		
1	Reference	
2	1.174 (0.059-23.383)	0.916
3	2.530 (0.132-48.396)	0.908
4	11.717 (0.606-2226.579)	0.892
Stage group		
1	Reference	
2	5.093 (1.133-22.888)	0.034*
3-4	10.199 (2.439-42.642)	0.001*

ERG, erythroblast transformation-specific-related gene; ISUP, International Society of Urological Pathology; HR, hazard ratio; CI, confidence interval. *Statistically significant (P<0.05).

growth factor among other proteins. The factors when released from tumor cells can bind to receptors on the surface of endothelial cells, promoting their migration and proliferation and increasing the permeability of the endothelium [25]. The activity of angiogenic factors is associated with oncogenesis, growth, and metastatic potential of RCC, indicating they hold promise as therapeutic targets. In patients with RCC, surgery can be curative if performed when the disease is at an early stage. However, inoperable or metastatic disease is not curable. Until recently, the only agents approved for the treatment of advanced RCC were the cytokines interferon and interleukin. However, only approximately 15% of patients benefited from these treatments, which are considerably toxic [26]. Recently, several efficacious biologic angiogenic inhibitors have been approved in the United States and Europe, including bevacizumab (anti-VEGF

Table 6. Cox regression analysis for overall survival

	HR (95% CI)	P
ERG expression		
Positive	Reference	
Equivocal	2.558 (0.225-29.066)	0.449
Negative	8.868 (1.039-75.663)	0.046*
Nestin expression		
Negative	Reference	
Positive	3.242 (1.137-9.247)	0.028*
Age	0.977 (0.941-1.014)	0.214
Sex		
Male	Reference	
Female	0.907 (0.272-3.022)	0.874
ISUP nucleolar grade		
1	Reference	
2	0.823 (0.038-17.924)	0.930
3	1.696 (0.083-34.717)	0.924
4	8.320 (0.405-170.795)	0.910
Stage group		
1	Reference	
2	5.438 (1.219-24.260)	0.026*
3-4	10.979 (2.726-44.222)	0.001*

ERG, erythroblast transformation-specific-related gene; ISUP, International Society of Urological Pathology; HR, hazard ratio; CI, confidence interval. *Statistically significant (P<0.05).

monoclonal antibody), sunitinib, and sorafenib (multi-tyrosine kinase inhibitors), and temsirolimus (mTOR inhibitor) [26]. Furthermore, angiopoietin, a glycoprotein that is also associated with cancer neovascularization, has been proposed as a candidate therapeutic target for RCC [27].

RCC is a highly vascularized tumor and useful for studying angiogenesis. In earlier studies, MVD was used to assess angiogenesis in RCCs [28-31]. However, the significance of MVD in RCC is unclear due to conflicting results in previous studies [28-31]. Various other findings, such as endothelial cell proliferation fraction [12] and MVA [13, 14], have been evaluated. However, determining the endothelial cell proliferation fraction is complex and results for MVA have been equivocal. Yao [12] divided blood vessels within ccRCCs into undifferentiated (CD31+/CD34-) and differentiated (CD34+) microvessels and reported that a higher undifferentiated MVD in ccRCC was significantly associated with higher tumor grades and shorter survival. Sato [13] reported the MVA of immature vessels (CD34+/α-SMA-; mature vessels, CD34+/SMA+) was positively associated with tumor grade (P<0.0001), Tstage (P<0.0001), and American Joint Committee on Cancer stage (P<0.0001). In addition, MVA was significantly higher in tumors with metastasis than in those without metastasis (P<0.0001). However, distinguishing blood vessel types based on CD31, CD34, or SMA staining results is difficult [32].

Endothelial cells play a pivotal role in angiogenesis in RCC. In the present study, various vascular markers, including CD31, CD34, nestin, and ERG, were examined in RCC specimens. CD31 and CD34 are commonly used as VE markers in MVD studies. CD31 is a glycoprotein involved in endothelial cell intercellular junctions [33] and migration. CD34 is a glycoprotein that functions as a cell-cell adhesion factor and facilitates opening of the vascular lumen [34]. CD31 and CD34 are expressed in tumor vessels irrespective of their size or maturity [9]. Nestin is a class VI intermediate filament present in VE cells. Unlike CD34 and CD31, which are expressed in various VE cells, nestin is only expressed in proliferating VE cells [35]. Thus, nestin indicates neovascularization. However, the use of nestin as a biomarker has several limitations. Researchers have attempted to demonstrate a relationship between nestin-positive vessels and the clinical outcome of patients. In colorectal carcinomas [35] and prostate cancers [36], nestin expression was associated with a worse prognosis. However, gastric cancer [37] and pancreatic cancer [38] studies failed to prove this relationship. In addition, the utility of nestin as a prognostic marker has not been assessed in a large-scale study. In the present study, loss of nestin expression was associated with a higher ccRCC stage. However, a significant association between nestin expression and prognosis was not found.

As mentioned above, ERG is essential for postnatal vascular development and is involved in tumor angiogenesis and growth [17]. Haber [21] compared immunostaining for CD31, CD34, α-SMA, and ERG in CNS tumors and found that ERG was exclusively and strongly expressed in VE cells but not in stromal or tumor cells. Conversely, α-SMA was expressed in abluminal cells of hyperplastic vessels and CD31 and CD34 showed weak or moderate

Table 7. Immunohistochemical staining for erythroblast transformation-specific-related gene

	Negative	Equivocal	Positive
ccRCC (n = 184)	58 (31.5%)	28 (15.2%)	98 (53.2%)
chRCC (n = 14)	11 (78.6%)	2 (14.3%)	1 (7.1%)
Oncocytoma (n = 4)	0 (0.0%)	0 (0.0%)	5 (100.0%)

Data shown are n (%). ccRCC, clear-cell renal cell carcinoma; chRCC, chromophobe renal cell carcinoma.

reactivity [21]. The use of CD31 and CD34 as endothelial cell markers is limited because they are not only expressed in endothelial cells. CD31 shows reactivity in platelets and blood leukocytes adherent to vascular walls. Likewise, CD34 can show positivity in some CNS tumor cells. In contrast, ERG is expressed specifically in VE cells. The loss of ERG expression was observed in some intratumoral blood vessels in ccRCCs and chRCCs. We found that a loss of ERG expression was associated with poor prognosis in ccRCCs. ERG is a specific indicator of VE cells and associated with blood vessel stability because it controls vascular junctional integrity by regulating Wnt/beta-catenin signaling and the expression of VE-cadherin, an adhesion molecule involved in endothelial cell-cell junctions. *In vivo*, vessels formed by the activity of VEGF are highly permeable and unstable. ERG can promote the stabilization of VEGF-induced vessels and contribute to angiogenesis *in vivo*. Conversely, the deletion of ERG results in vascular defects [17]. Thus, non-ERG-expressing vessels in RCCs could be regarded as unstable and highly permeable cells that are immature.

In conclusion, loss of ERG and nestin expression is associated with tumor progression, and loss of ERG is a powerful prognostic marker for ccRCC. Our study results indicate the degree of ERG expression determined based on IHC allows determination of the quality of angiogenesis in RCCs and is superior to MVD, MVA, and other markers such as CD31, CD34, and SMA.

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Disclosure of conflict of interest

None.

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