

Expression of Cadherin-6 as a Novel Diagnostic Tool To Predict Prognosis of Patients with E-Cadherin-absent Renal Cell Carcinoma

Toru Shimazui,¹ Egbert Oosterwijk, Hideyukiz Akaza, Pierre Paul Bringuier, Emiel Ruijter, Hans van Berkel, Jeannette Oosterwijk Wakka, Adrie van Bokhoven, Frans M. J. Debruyne, and Jack A. Schalken

Department of Urology, Institute of Clinical Medicine, University of Tsukuba, Tsukuba 305-8575, Japan [T. S., H. A.], and Urological Research Laboratory [T. S., E. O., P. P. B., H. v. B., J. O. W., A. v. B., F. M. J. D., J. A. S.] and Department of Pathology [E. R.], University Hospital Nijmegen, Nijmegen 6500 HB, the Netherlands

ABSTRACT

In many carcinomas, E-cadherin is considered to be a prognostic marker for patient survivals, and its decreased expression is associated with metastatic disease. Among renal cell carcinomas (RCCs), however, only 20% of tumors express E-cadherin, whereas a much higher percentage express other cadherins, *e.g.*, N-cadherin and cadherin-6 (T. Shimazui *et al.*, *Cancer Res.*, 56: 3234–3237, 1996). Among these cadherins expressed in RCCs, cadherin-6 has been identified as a major cadherin in the renal proximal tubules and in the tumors themselves. Hence, we have investigated the relationship between prognosis and cadherin-6 expression in tumor cells in 43 patients with RCC. Expression of cadherin-6, E-cadherin, and α -catenin was detected immunohistochemically and evaluated microscopically as normal, heterogeneous, or absent. Normal, heterogeneous, and absent expression of cadherin-6 were observed in 19, 16, and 8 of 43 cases, respectively. Coexpression of E-cadherin and cadherin-6 was detected in only 10 cases. Among 30 tumors in which E-cadherin expression was absent, 24 expressed cadherin-6. In addition, the expression pattern of α -catenin correlated more highly with that of cadherin-6 than it did with E-cadherin ($P = 0.0003$ versus 0.025). In survival analyses, aberrant expression of cadherin-6 correlated with poor survivals both among all patients ($P = 0.0009$) and in those with E-cadherin-absent RCC ($P = 0.0008$). These results suggest that cadherin-6 is a major cadherin playing an

essential role in cell-cell adhesion in E-cadherin-absent RCC.

INTRODUCTION

In RCCs,² the existence of a distant metastasis is the most powerful prognostic factor for predicting patient survival (1–4). Although the metastatic cascade from the primary tumor to a distant organ still remains controversial, loss of cell-cell adhesion is considered to play an important role as a first step of this cascade (5). In many carcinomas, E-cadherin functions to preserve epithelial integrity, and decreased expression of this molecule is associated with the presence of metastatic disease and with poor prognosis of patients (6–8).

Although Katagiri *et al.* (9) have reported that normal expression of E-cadherin has a prognostic value in RCC, E-cadherin is expressed in no more than approximately 20% of RCCs (9, 10). And in fact, the majority of RCCs are thought to originate from the epithelia in the renal proximal tubules in which E-cadherin is not expressed. We have reported previously (10) that RCC cell lines expressed mRNA of several cadherins, such as E-cadherin, N-cadherin, and cadherin-6, with the latter two expressed more frequently than E-cadherin. Nouwen *et al.* (11) observed N-cadherin expression in the proximal tubular epithelia of normal kidneys. In our study on RCC cell lines, however, we detected mRNA expressions of N-cadherin in all of the cell lines observed, and these expressions did not seem to correlate with cellular morphology (10). Cadherin-6 was originally isolated from a hepatocellular carcinoma cell line lacking E- and P-cadherin, and it was shown that normal kidney and RCC cell lines expressed cadherin-6 mRNA (12), which demonstrated 97% homology with the cDNA of rat K-cadherin (13). Hence, we hypothesized that cadherin-6, rather than N-cadherin, might be an integral molecule in the cell-cell adhesion in RCC. In this study, we used a newly developed anti-cadherin-6 antibody to investigate the expression pattern and the localization of cadherin-6 in RCC. In addition, we analyzed the prognostic value of cadherin-6 in the particular case of patients with E-cadherin-absent RCC.

MATERIALS AND METHODS

Forty-three snap-frozen specimens obtained from patients with RCC who underwent nephrectomies at the University Hospital Nijmegen were studied. Details of the patient profiles and follow-up investigations are shown in Table 1. Age distribution ranged from 17 to 80 years, and the average age at the time of surgery was 56.0 years. Histological evaluations were

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¹ To whom requests for reprints should be addressed, at Department of Urology, Institute of Clinical Medicine, University of Tsukuba, 1-1-1, Tennodai, Tsukuba-city, Ibaraki, 305-8575, Japan. Phone and Fax: 81-298-533223.

² The abbreviation used is: RCC, renal cell carcinoma.

Table 1 Patient characteristics and treatments

Case	Age (yr)	Sex	Grade	Pathological stage ^a				Metastases	Treatment ^b	Immunostaining ^c			Prognosis ^d	
				pT	pN	M	pV			Cd6	Ec	α	Month	A/D
1	44	F	3	3B	1	1	1	Lung, bone	Nx	H	(-)	(+)	8	D
2	17	M	3	3	3	1	0	Lung, mediastinum	Nx, LND, IL2	H	(-)	H	68	D
3	57	M	2	2	0	0	0		Nx	H	H	H	72	D
4	42	M	3	4	0	1	1	Lung	Nx, IFN, Cx	H	(-)	H	18	D
5	77	F	2	3B	0	0	1		Nx, LND	(+)	(+)	(+)	15	A
6	63	M	2	2	0	0	0		Nx	(+)	(-)	(+)	84	A
7	60	F	2	3B	0	1	1	Lung	Nx, IFN, RT	H	(-)	(+)	25	D
8	61	M	1	2	0	0	0		Nx	(+)	(-)	(+)	74	A
9	67	M	2	3B	1	1	0	Adrenal gland	Nx	(+)	(-)	(+)	69	A
10	66	F	2	4	0	1	1	Ileocecal	Nx, colectomy	H	(-)	(+)	7	D
11	57	F	1	2	0	0	0		Nx	(-)	(-)	(+)	82	A
12	27	F	1	3A	1	1	1	Ovary, liver, bone	Nx, LND, IFN, RT	(-)	H	H	5	D
13	65	M	2	3B	0	0	1		Nx, Cx	H	(-)	(+)	68	A
14	47	M	1	2	0	0	0		Nx	(+)	(-)	(+)	77	A
15	61	M	2	3B	X	0	0		Nx, LND, IFN	H	(-)	H	9	D
16	46	M	1	3A	X	1	0	Bone	Nx, IFN	(+)	(-)	H	66	A
17	35	F	3	3B	0	0	1		Nx, LND	(-)	(+)	(+)	60	A
18	74	M	1	2	0	0	0		Nx	(+)	(+)	(+)	66	A
19	67	M	2	3B	X	0	2		Nx, IVC	(-)	(-)	H	65	D
20	54	M	2	2	0	0	0		Nx	H	(+)	(+)	60	A
21	56	M	2	3B	1	1	1	Lung, bone	Nx, pneumox, RT	H	(-)	H	8	D
22	69	F	1	2	0	0	0		Nx	(+)	(-)	(+)	67	A
23	52	M	1	3A	0	0	0		Nx	(+)	(-)	(+)	60	A
24	68	M	3	3B	0	1	2	Lung, bone	Nx, IVC, pneumox	H	(-)	H	4	D
25	48	M	1	2	0	0	0		Nx, LND	(+)	(+)	(+)	58	A
26	65	M	2	2	0	0	0		Nx (partial)	(-)	(-)	H	24	D
27	80	F	2	2	X	0	0		Nx	(+)	(-)	(+)	64	A
28	47	M	1	3A	0	0	0		Nx	H	(+)	(+)	56	A
29	46	M	2	3A	0	1	0	Pancreas, kidney	Nx, LND, IL2, IFN	H	(-)	H	30	D
30	80	M	2	3A	0	0	0		Nx	H	(-)	H	28	D
31	69	M	2	3B	3	0	1		Nx, LND	(-)	(-)	H	10	A
32	55	M	2	3A	0	0	0		Nx, LND	(+)	(+)	(+)	61	A
33	59	M	2	2	1	1	0	Bone	Nx, RT	(+)	(+)	(+)	53	D
34	25	F	2	2	0	0	0		Nx	(+)	(-)	(+)	42	A
35	66	M	2	2	0	0	0		Nx, LND	H	(+)	(+)	34	D
36	62	F	2	3B	0	0	1		Nx, LND	(+)	(-)	(+)	42	A
37	28	F	2	2	0	0	0		Nx, LND	(-)	(+)	(+)	21	A
38	49	M	2	3A	1	1	1	Lung	Nx, LND, RT, IL2, IFN	H	(-)	H	13	D
39	55	M	1	2	0	0	0		Nx, LND	(+)	(-)	(+)	36	A
40	62	F	2	3B	0	1	1	Lung	Nx	(-)	(-)	H	18	A
41	51	M	2	3A	0	0	0		Nx, LND	(+)	(-)	(+)	29	A
42	72	M	1	3B	0	0	2		Nx, IVC	(+)	(-)	(+)	22	A
43	73	F	2	3C	0	0	2		Nx, IVC	(+)	(+)	(+)	12	A

^a pT, primary tumor; pN, regional lymph nodes; M, distant metastasis; pV, venous invasion.^b Nx, nephrectomy; LND, lymph node dissection; IFN, interferon-α; RT, irradiation; pneumox, pneumonectomy.^c Cd6, cadherin-6; Ec, E-cadherin; α, α-catenin; (+), normal; (-), absent; H, heterogeneous.^d A, alive; D, dead.

performed on H&E sections. The tumors were pathologically staged and were graded according to TNM classification (14). Four normal kidney samples were also obtained from noncancerous areas of nephrected kidneys.

Serial frozen sections, 4 μm thick, were immunohistochemically stained using HECD-1 (Takara, Japan), anti-α-catenin mouse serum (8), and 2B6-D8 for E-cadherin, α-catenin, and cadherin-6, respectively. 2B6-D8 was newly developed as a mouse monoclonal antibody against the glutathione *S*-transferase (GST) fusion extracellular domain of human cadherin-6 as follows: (a) a 1233-bp nucleotide fragment (nucleotides 323-1555 corresponding to human cadherin-6 cDNA) was generated using PCR and was cloned into

the Rpr265 vector; (b) the glutathione *S*-transferase fusion protein was purified in an affinity column and was used as an immunogen; (c) female BALB/c mice were immunized against the purified protein, and after the 3rd boost, spleen cells were fused with SP2/0 mouse myeloma cells; and (d) hybridomas were screened, and the 2B6-D8 clone was revealed as a specific clone producing a monoclonal antibody against cadherin-6 that recognized a single 125-kDa band comigrating with cadherin-6 on Western blot (Fig. 1a). Immunostaining was also carried out according to our previous procedure (8). Immunohistochemically, 2B6-D8 demonstrated cell-cell border staining in the SKRC-33 cell line that expressed cadherin-6 alone (Ref. 10; Fig. 1b). In addition, a

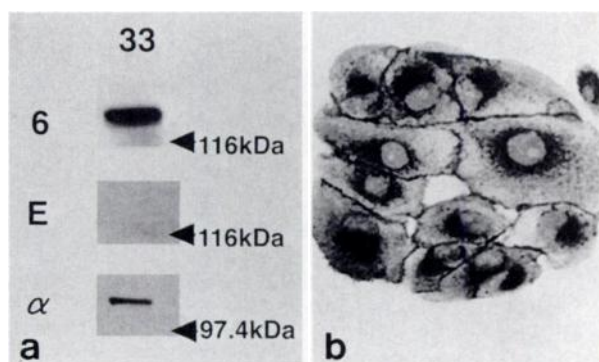


Fig. 1 Cadherin-6 expression in a renal cell carcinoma cell line. In *a*, Western blot analysis shows that the SKRC-33 renal cell carcinoma cell line expressed cadherin-6 (125 kDa) and α -catenin (105 kDa) but not E-cadherin. In *b*, cadherin-6 expression is restricted at the cell-cell contact site by immunofluorescence staining using a monoclonal antibody, 2B6-D8.

mouse monoclonal antibody, RC-3, was used to distinguish renal proximal tubules as described previously (15).

Protein expressions on tumor sections were evaluated by light microscope. If the staining pattern in cancer cells was exclusively at cell-cell borders, the antigen expression was scored as normal. If reactivity was absent (*i. e.*, if there was a complete absence of immunoreactivity) or heterogeneous (*i. e.*, if the tumor was composed of positive and negative areas), antigen expression was scored as abnormal.

Statistical analyses were performed to compare cadherin-6 expression with pathological parameters in restricted patients with E-cadherin-absent RCC. The correlation between cadherin-6 and tumor stage and grade were evaluated by χ^2 test. Survival curves were constructed by the Kaplan-Meier method, and the differences in survival were assessed by the log-rank test. The clinical relevancy of these molecules was analyzed by the Cox proportional hazards model.

RESULTS

Expression of Cell Adhesion Molecules in the Normal Kidney. E-cadherin expression was restricted in distal tubules through collecting tubules, whereas cadherin-6 was expressed in proximal tubules and Henle's thin loop. E-cadherin expression was seen at all of the lateral cell-cell contact sites with the exception of the basal membrane. On the other hand, cadherin-6 expression was located at the basolateral membranes along with α -catenin expression (Fig. 2*a-d*).

Details of the expression of each molecule in RCC specimens are summarized in Table 1. Only 11 tumors showed normal expression of E-cadherin at the cell-cell borders, whereas, in 30 of 32 E-cadherin-aberrant tumors, expression of E-cadherin was completely absent. Normal, heterogeneous, and absent expression of cadherin-6 were observed in 19, 16, and 8 of 43 cases, respectively. Coexpression of E-cadherin and cadherin-6 was detected in 10 cases (Fig. 3, *c* and *d*). Of 30 tumors in which E-cadherin expression was absent, 14 showed normal and 16 showed abnormal expression of cadherin-6 (Table 2, Fig. 3, *a*, *b*, *e*, and *f*). In addition, expression of α -catenin signifi-

cantly correlated with that of cadherin-6 in the E-cadherin-absent group (χ^2 , 11.8; P , 0.0006).

Statistical Analyses in the Subgroup with E-Cadherin-absent RCC. The expression pattern of cadherin-6 correlated with tumor stage and grade (χ^2 , 7.30; P , 0.007 and χ^2 , 10.15; P , 0.006) independent of E-cadherin expression. In the survival analyses, although prognosis of the patients with normal expression of E-cadherin was better than that of those with abnormal expression, the difference was not statistically significant (P = 0.265; Fig. 4*a*). For the restricted patients with absent E-cadherin expression, expression of cadherin-6 strongly correlated with patient survival (P , 0.0008; Fig. 3*b*). Additionally, the Cox proportional hazards regression analysis revealed that the expression pattern of cadherin-6 had a higher risk ratio in prognosis than the pathological parameters for patients with RCC (Table 3).

DISCUSSION

It is generally considered that E-cadherin functions as an invasion suppressor gene and correlates with the survival of patients with many different carcinomas, *e.g.*, bladder tumors and prostate, breast, skin, colon, and gastric cancers (5–7, 16, 17). Katagiri *et al.* (9) have reported that normal E-cadherin expression is associated with a better prognosis of patients with RCC. However, it has been shown (9, 10) that most RCCs (70–80%) demonstrated absent E-cadherin expression and only 20% exhibited normal E-cadherin expression as compared with the normal expression rate of E-cadherin in other carcinomas. As mentioned above, this can probably be attributed to the fact that the renal proximal tubules, which are presumably an origin of RCC, do not express E-cadherin. Although N-cadherin has been identified as one of the cell adhesion proteins expressed in the renal proximal tubules (11), Tani *et al.* (18) have demonstrated that E- and N-cadherin expression do not correlate with tumor grade in RCC. Thus, it remains unclear whether or not aberrant expression of the cadherin molecules expressed in RCC is associated with poor prognosis of patients. Recently, Paul *et al.* (19) demonstrated that cadherin-6 is expressed in renal proximal tubular epithelia and that RCC and its aberrant expression seem to correlate with dedifferentiation and progression of RCC. In this study, we, therefore, extended their research to focus on the correlation between the expression pattern of cadherin-6 and the prognosis of patients with RCC.

When looking at the expression of each cadherin, coexpression of E-cadherin and cadherin-6 was observed in only 10 of 43 tumors. This may be explained by up-regulation of E-cadherin during the carcinogenesis of RCC arising from renal proximal tubules. Another explanation is that some RCCs may be derived from the segment of the nephron that coexpresses both E-cadherin and cadherin-6. Even if the up-regulation of E-cadherin expression is an aberrant phenomenon in RCC, its homogeneous cell border expression seems to be associated with less metastatic disease and better prognosis of patients (9).

For this reason, we focused on cadherin-6 expression in the subgroup with E-cadherin-absent tumors. In this group, aberrant cadherin-6 expression correlated with distant metastatic disease (10 of 16) whereas normal cadherin-6 expression did not (2 of 14). This finding suggested that cadherin-6 acted as a metastasis

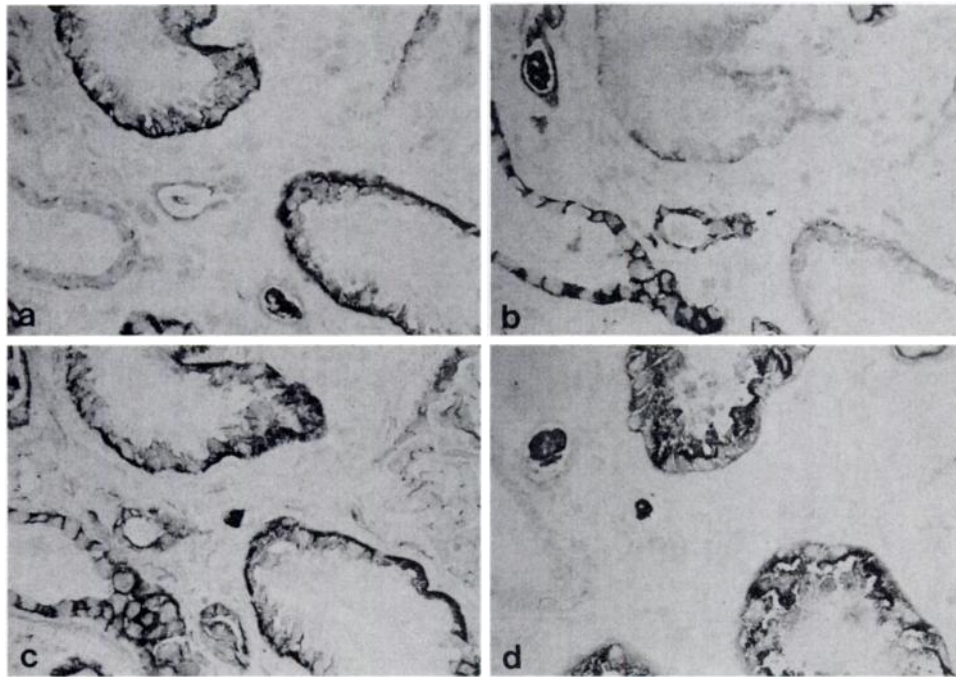


Fig. 2 Expression of Cadherin and catenin in the normal kidney. Renal proximal tubules stained by RC-3 (d) express cadherin-6 (a) and α -catenin (c) at the basolateral site of the epithelium but do not express E-cadherin (b). E-cadherin is expressed at the cell-cell border in the renal distal tubular epithelia (b).

Fig. 3 Several combinations of expression pattern between cadherin-6 and E-cadherin are observed in RCC specimens. Case 6 RCC shows normal cell-cell border staining of cadherin-6 (a) with absent E-cadherin (b). In case 32, both cadherin-6 (c) and E-cadherin (d) are homogeneously expressed at the cell-cell border. Heterogeneous cadherin-6 (e) staining is observed in case 38, whereas E-cadherin (f) is absent.

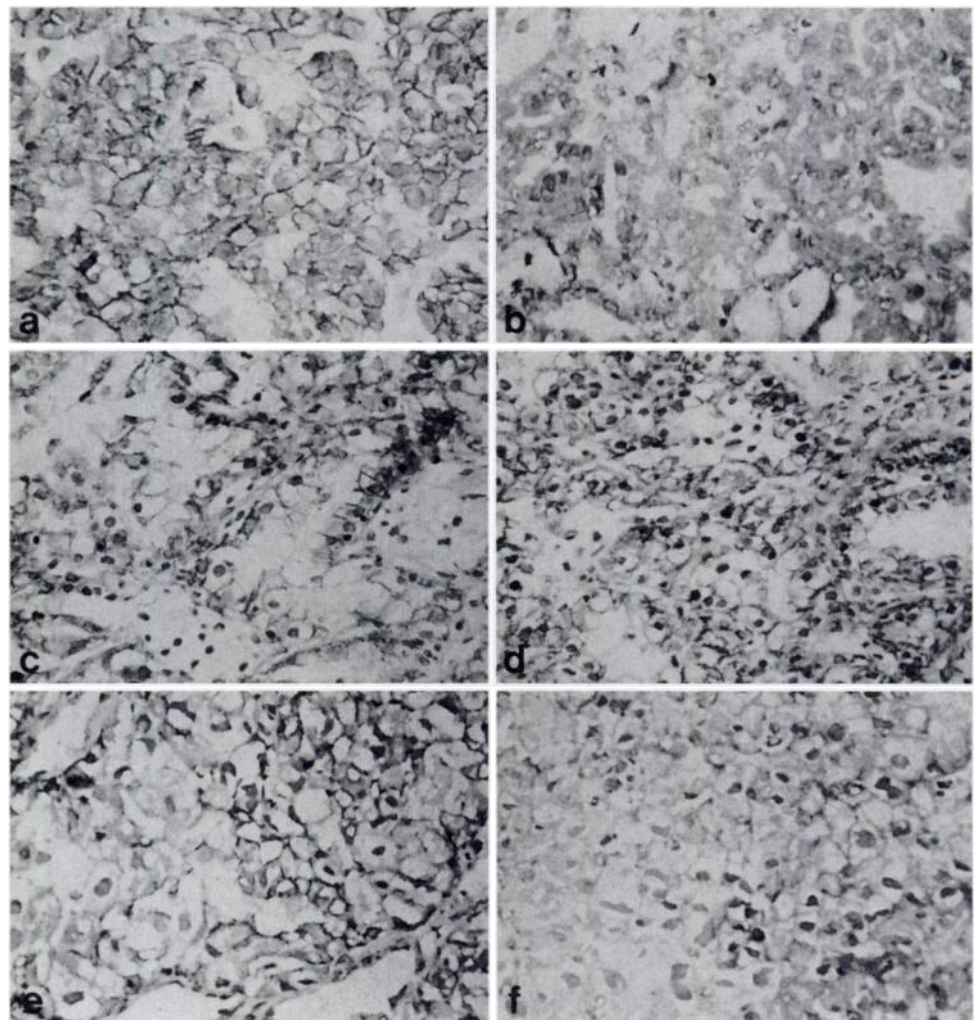


Table 2 Correlation between E-cadherin and cadherin-6 in RCC

Cadherin-6	E-Cadherin			Total
	Normal	Heterogeneous	Absent	
Normal	6	0	13	19
Heterogeneous	3	1	12	16
Absent	2	1	5	8
Total	11	2	30	43

Table 3 Cox proportional hazards model in the patients with E-cadherin-absent RCC

Values	χ^2	P	Hazards ratio
Cadherin-6	4.93	0.027	12.66
Stage	0.57	0.45	3.46
Nodal status	0.037	0.85	0.860
Distant metastasis	0.012	0.91	0.831
Grade	0.16	0.69	1.39

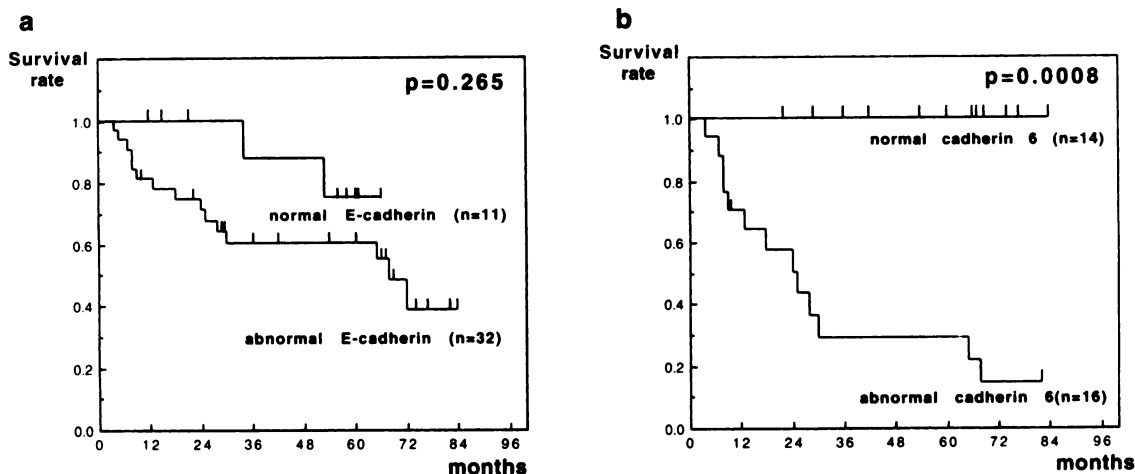


Fig. 4 Survival curves demonstrate no SD in survival rates between patients with tumors showing normal and abnormal expression of E-cadherin (*a*; $P = 0.265$). Among patients with E-cadherin-absent tumors, the survival rate of those with tumors showing abnormal cadherin-6 expression was significantly lower than that of those showing normal cadherin-6 expression (*b*; $P = 0.0008$).

suppressor molecule in RCC. Expression of cadherin-6 significantly decreased with an increase of tumor grade and stage much as in the study by Paul *et al.* (19). These relationships between cadherin-6 and pathological parameters were highly pronounced in the E-cadherin-absent cancer group.

Cadherin-6 expression significantly correlated with α -catenin expression in our series. This suggested that cadherin-6 may have been forming a functional cell adhesion complex with α -catenin in RCC, as it did in our previous *in vitro* investigation (10). Paul *et al.* (19) revealed in an immunoprecipitation study that α - and β -catenin are coprecipitated with cadherin-6 in RCC cell lines, suggesting that the binding to catenins is likely to be important for proper functioning of cadherin-6. On the other hand, some tumors expressed neither E-cadherin nor cadherin-6 but did express α -catenin (*i.e.*, tumors 11, 19, 26, 31, and 40). This fact may indicate that other cadherins, *e.g.*, N-cadherin, expressed and formed a cell adhesion complex with α -catenin (20). Additional immunohistochemical studies will be needed to clarify the diversity of cadherin expression in RCC.

In conclusion, we revealed that cadherin-6 is specifically expressed in the epithelial cells of renal proximal tubules and is retained in most RCCs. Thus, cadherin-6 could be considered one of the major cadherins expressed in RCC, and its expression could be considered to have greater prognostic value than tumor stage or grade in this disease. These immunohistochemical observations constitute the first report on the prognostic value of cadherin-6 for patients with RCC.

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