Senescence-associated protein p400 is a prognostic marker in renal cell carcinoma

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Abstract. Mutations of the von Hippel-Lindau (VHL) tumor suppressor gene cause hereditary and sporadic renal cell carcinomas (RCCs). The best characterized function of VHL protein is suppression of the α subunit of hypoxia inducible factor (HIF). Additional VHL functions have been reported, including induction of senescence upon loss of VHL mediated by downregulation of the chromatin remodeling factor p400. Induction of senescence either by oncogene activation or inactivation of tumor suppressors is considered a critical feature of mammalian cells by which to suppress tumorigenesis. In the present study, we investigated the relationship between the expression of p400 and patient survival following RCC diagnosis taking advantage of a large and well-documented series of RCC patients with long-term follow-up information. The expression of p400 was measured by immunohistochemistry using a tissue microarray containing tumor tissue samples from 868 RCC patients. Chi-squared tests, Kaplan-Meier curves, Cox regression models and Spearman's rank correlation estimates were used to investigate the possible relationship between p400 expression and Ki-67 proliferative index, clinical and pathological characteristics and patient survival. Complete loss of p400 expression was detected in 64% of all tumor specimens, and decreased p400 expression was associated with advanced tumor stage, higher grade of malignancy and regional lymph node metastasis. Among well-differentiated RCCs, high proliferation (Ki-67 index >10) was found in 12% of carcinomas with an increased p400 expression, compared to 5% of RCCs with decreased p400 expression. Multiple Cox regression indicated that patients with low proliferative tumors and increased p400 expression had a 60% lower cancer-specific mortality risk compared to those affected by low proliferative RCCs with decreased p400 expression. In summary, patients affected by highly proliferative tumors with decreased p400 expression exhibit a poor prognosis by multiple Cox regression. Our data suggest that the highly proliferative, decreased-p400 subgroup of RCCs represents tumors that are characterized by a loss of the tumor-suppressive mechanism of senescence.

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Introduction

Clear-cell renal cell cancer is the most common subtype of kidney cancer and comprises ~70% of renal cell carcinomas (RCCs) (1). Clear-cell renal cell carcinomas (ccRCCs) are associated with Von Hippel-Lindau (VHL) disease, an autosomal dominant disorder caused by germline mutations of the VHL tumor-suppressor gene. By the age of 70, ~70% of patients with VHL disease develop ccRCC, and the mean age of manifestation is 37 vs. 61 years for sporadic ccRCC (2). Furthermore, *vhl* inactivation by methylation or mutation has been reported in up to 91% of sporadic ccRCCs.

The VHL protein (pVHL) targets the α subunit of hypoxia inducible factor (HIF) for ubiquitin-mediated degradation. Thus, inactivation of *vhl* results in accumulation of HIF-α subunits (3). Mammals possess three isoforms of HIF-α (HIF1-α, HIF2-α and HIF3-α) of which HIF1-α and HIF2-α are best characterized (4). Recent findings indicate that HIF2-α is the isoform critical for tumorigenesis of ccRCC (5), whereas HIF1-α may act as a tumor suppressor (6). Apart from the regulation of HIF, pVHL influences p53-mediated cell cycle arrest and apoptosis (7), primary cilium maintenance (8), microtubule-dependent functions (9) and response to DNA damage (10).

Recently, loss of pVHL was reported to induce senescence mediated by retinoblastoma protein and p400 (EP400, E1A binding protein p400) (11). This implies that senescence is a downstream line of defense in the tumorigenesis of VHL-associated malignancies. p400 belongs to the SWI2/SNF2 family of chromatin remodeling proteins, acts as a DNA damage response protein (12) and regulates p21-induced senescence (13) and E1A-induced apoptosis (14,15).

Ki-67 is a proliferation-associated antigen, and Ki-67 labeling is used to estimate proliferation. The MIB1 antibody, which is reactive against Ki-67, can be used on paraffin-embedded tissue (16) and correlates with the mitotic index (17). Prognostic value is confirmed in many tumor entities including RCC (18-21). Although absence of proliferation does not necessarily mean senescence, senescent cells do not proliferate. Therefore, in combination with other markers, Ki-67 is often used to characterize senescent cells (22).

To the best of our knowledge, there is no data concerning the expression of p400 in RCCs, although ccRCCs are the most common tumors associated with loss of pVHL function. We, therefore, investigated the expression of p400 in a large and well documented series of RCCs with long-term follow-up information. Furthermore, we assessed proliferation by Ki-67 labeling and correlated the findings with the results obtained by p400 immunohistochemistry.

Patients and methods

Patients. Tissue samples from 868 patients with primary renal cell carcinoma, treated at the Department of Urology (University of Heidelberg, Germany) between 1987 and 2005, were collected in the Tumor Tissue Bank of the National Center for Tumor Diseases (Heidelberg) and studied after approval by the Ethics Committee of the University of Heidelberg. Samples were graded according to the 4-tiered nuclear grading system and staged based on the TNM classification (2002) by experienced pathologists. No adjuvant treatment was administered for localized disease. Patients with metastasized disease, with a Karnofsky performance index of ≥80 and without medical contraindications received interferon-α- and interleukin-2-based immunotherapy. Clinical follow-up was available for all cases. Patients were prospectively evaluated every 3 months for the first 2 years after treatment; every 6 months for the next 3 years, and yearly thereafter (chest X-ray or thoracic CT scan; abdominal sonography or CT scan or MRI; serum chemistry).

Tissue microarray. A series of tissue microarrays containing 932 primary tumor and corresponding normal tissue samples from 932 patients was assembled as previously described (23).In total, a set of 19 array blocks was generated, each containing 200 tissue core specimens, representing 50 patients per array.

Immunohistochemistry. The tissue microarray slides were dewaxed and rehydrated using xylene and a series of graded alcohols, followed by heat-induced antigen retrieval using a target retrieval solution (S2031; DakoCytomation, Glostrup, Denmark) in a pressure cooker for 15 min. Staining was performed using an automated staining system (Techmate 500; DakoCytomation) with polyclonal anti-P400 rabbit antiserum (1:100; Sigma-Aldrich, St. Louis, MO, USA) for 45 min and avidin-biotin-complex peroxidase technique using aminoethylcarbazole for visualization and hematoxylin for counterstaining. In accordance with the manufacturer's instructions, the following solutions were used: ChemMate Detection kit (K5003; DakoCytomation, containing Dako REAL™ Link, ready-to-use biotinylated goat anti-mouse and anti-rabbit immunoglobulins, and Dako REAL™ AEC/H_2O_2 substrate solution), ChemMate Buffer kit (K5006; DakoCytomation), and for reduction of non-specific avidin/biotin-related staining the Avidin/Biotin Blocking kit (SP-2001; Vector Laboratories, Burlingame, CA, USA). As a negative control for the immunohistochemical staining procedure, the primary antibody was omitted with all other experimental conditions kept constant. Staining intensity was categorized as: 0, negative; 1, low; 2, medium; and 3, high. Staining quantity was categorized as: 0, no expression; 1, positivity in 1-9% of cells; 2, positivity in 10-50% of cells; 3, positivity in 50-100% of cells. For the semi-quantitative immunohistochemical assessment of p400 expression, a score was calculated by multiplying the staining intensity by staining quantity (range, 0-9). Two independent scores were obtained for each patient from two different cylindrical core tissue specimens. If divergent p400 expression scores were observed in the 2 cores, then the higher score was used for further analyses. In addition to the investigation of individual expression categories, a p400 expression score >4 was defined as 'increased p400 expression' and lower scores as 'decreased p400 expression'. Ki-67 labeling was performed as previously published (24).

Statistical methods. The non-normal distribution of expression scores and patient/tumor characteristics motivated the use of non-parametric statistics. Chi-squared tests were used to investigate the possible relationship between the expression of p400 and Ki-67, and clinical and pathological characteristics. Spearman's rank correlation was estimated to quantify the relationship between p400 expression and the Ki-67 proliferative index.

Survival was calculated from the date of nephrectomy to three different events: overall survival (OS, event, death by any cause), cancer-specific survival (CS, event, tumor-related death), and progression-free survival (PFS, event, recurrence, metastasis, or death by any cause). Survival time was censored for patients who did not experience the investigated event; for example, patients alive at last contact (OS, CS and PFS) or patients with a non-tumor-related death (CS and PFS). The association between survival times and p400/Ki-67 expression was first assessed by log-rank tests and represented using Kaplan-Meier plots. In order to account for the influence of established prognostic factors, hazard ratios (HRs) and 95% confidence intervals (CIs) were adjusted for patient gender and age, histology, tumor extent, lymph node metastasis, distant metastasis, grade of malignancy, resection type and Karnofsky performance in a multiple Cox proportional hazard regression. The present study had 80% power to identify an adjusted HR >2.81 or <0.48 (5% type I error probability, 10% of patients with increased p400 expression). Statistical analyses were implemented using R (http://www.rproject.org). Probability values <0.05 were considered to indicate a statistically significant result.

Results

Clinical characteristics of the patients. The patient collective included 868 patients with complete clinical and pathological information. The median time of follow-up among the 409 patients who died was 27.2 months. By the end of the follow-up, 251 patients had died from RCC, 158 died due to another

Table I. Clinical and pathological characteristics in the investigated patient collective and their association with survival (results from the multivariate Cox regression).

		Overall survival			Cancer-specific survival			Progression-free survival		
Characteristics	Patients	Global P-value	N[a]	HR with 95% CI	Global P-value	N[a]	HR with 95% CI	Global P-value	N[a]	HR with 95% CI
Gender										
Male	544	**0.0006**	278	**0.69** (0.55-0.85)	**0.01**	174	**0.70** (0.53-0.93)	**0.004**	118	**0.61** (0.44-0.85)
Female	324		131	(Ref.)		77	(Ref.)		53	(Ref.)
Age at diagnosis (years)										
<60	314	**<.0001**	115	**1.64** (1.31-2.05)	0.07	164	1.29 (0.97-1.70)	0.64	56	1.08 (0.78-1.51)
≥60	554		294	(Ref.)		164	(Ref.)		115	(Ref.)
Histology										
Clear-cell	742	0.07	371	(Ref.)	0.10	229	(Ref.)	0.05	157	(Ref.)
Papillary	87		26	0.79 (0.53-1.19)		12	0.84 (0.47-1.55)		10	0.51 (0.26-0.98)
Chromophobe	31		5	0.40 (0.17-0.98)		3	0.36 (0.11-1.14)		2	0.24 (0.06-0.97)
Other	8		1	1.74 (0.80-3.77)		7	1.98 (0.90-4.34)		2	1.01 (0.24-4.26)
Tumor extent (T)										
1	476	**<0.0001**	147	(Ref.)	**<0.0001**	53	(Ref.)	**<0.0001**	47	(Ref.)
2	89		37	1.32 (0.92-1.91)		25	**2.30** (1.42-3.73)		25	**4.03** (2.41-6.73)
3	290		213	**2.07** (1.62-2.64)		162	**3.57** (2.51-5.06)		92	**4.43** (2.92-6.73)
4	13		12	**1.95** (1.04-3.66)		11	**3.16** (1.57-6.38)		7	**26.2** (10.6-64.7)
Lymph node metastasis (N)										
No	804	0.16	352	(Ref.)	0.10	198	(Ref.)	**<0.0001**	145	(Ref.)
Yes	64		57	1.25 (0.91-1.72)		53	1.34 (0.95-1.90)		26	**4.05** (2.49-6.58)
Distant metastasis (M)										
No	728	**<0.0001**	283	(Ref.)	**<0.0001**	131	(Ref.)	**<0.0001**	158	(Ref.)
Yes	140		126	**4.11** (3.18-5.31)		120	**6.20** (4.60-8.36)		13	**0.21** (0.41-21.4)
Grade of malignancy										
1	223	**<0.0001**	86	(Ref.)	**<0.0001**	34	(Ref.)	**0.0003**	35	(Ref.)
2	500		207	1.05 (0.81-1.37)		116	1.16 (0.78-1.73)		91	1.28 (0.85-1.93)
3/4	145		116	**1.91** (1.38-2.64)		101	**2.43** (1.57-3.76)		45	**2.55** (1.55-4.19)
Type of surgery										
Nephrectomy	727	0.78	367	(Ref.)	0.64	235	(Ref.)	**0.02**	147	(Ref.)
Resection	141		42	0.95 (0.68-1.34)		16	0.88 (0.51-1.50)		24	**1.84** (1.12-3.03)
Karnofsky performance										
≥80%	800	**<0.0001**	352	(Ref.)	**0.006**	223	(Ref.)	0.84	155	(Ref.)
<80%	68		57	**2.28** (1.70-3.06)		28	**1.78** (1.18-2.69)		16	1.06 (0.62-1.81)

HR, hazard ratio; CI, confidence interval. [a]N indicates event number. Probability values and hazard ratios considered statistically significant are shown in bold.

cause and 459 were still alive. Table I provides a summary of the clinical and pathological data. As expected, established prognostic factors for RCCs including tumor stage, grade and occurrence of distant metastasis correlated with patient prognosis. For example, 73% (213 out of 290) of patients affected by stage 3 tumors died in comparison to 31% of patients with stage 1 tumors, resulting in an estimated HR of 2.07 (95% CI, 1.62-2.64).

p400 expression, patient prognosis and tumor characteristics. Nuclear p400 was weakly detected in glomerular podocytes, the majority of distal tubules, and, only inconsistently and weakly, in the proximal tubule epithelium (Fig. 1A and B). P400 expression scores were obtained for 787 tumors; 81 samples were excluded due to insufficient tumor tissue and fixation artifacts. The majority of tumors showed complete loss of p400 expression (n=502, 64%), whereas the remaining

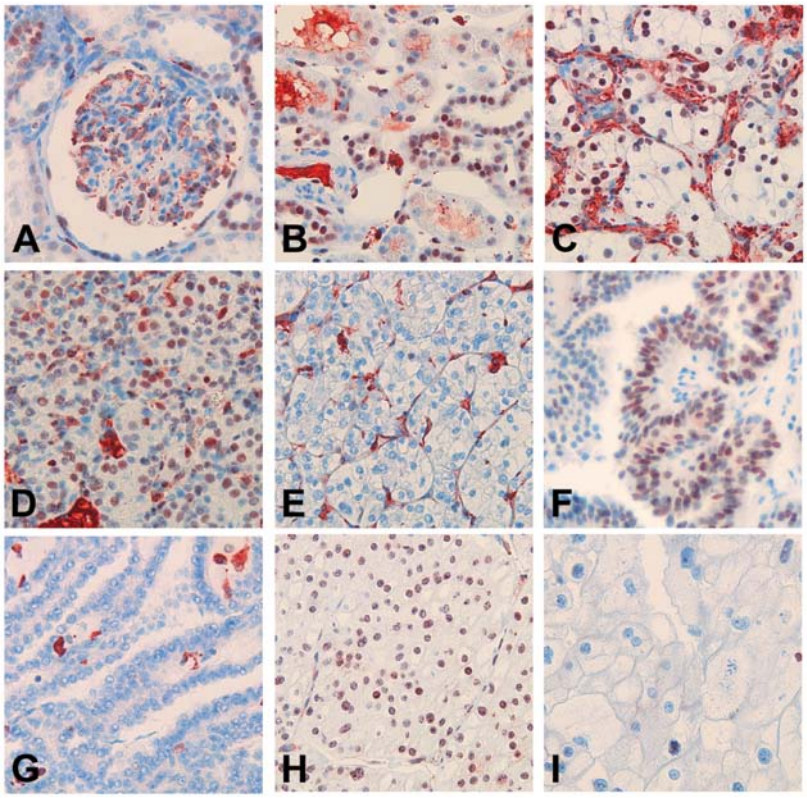

Figure 1. Immunohistochemical detection of p400 protein. Note the variable intensity and quantity of p400 expression in RCCs. Endothelial and immune cells were also stained positive for p400. (A and B) Normal renal tissue; (C-E) clear-cell (conventional) RCCs; (F and G) papillary (chromophil) RCCs; (H and I) chromophobe RCCs. Immunohistochemical scores: C, 9; D, 6; E, 0; F, 6; G, 0; H, 9; I, 0.

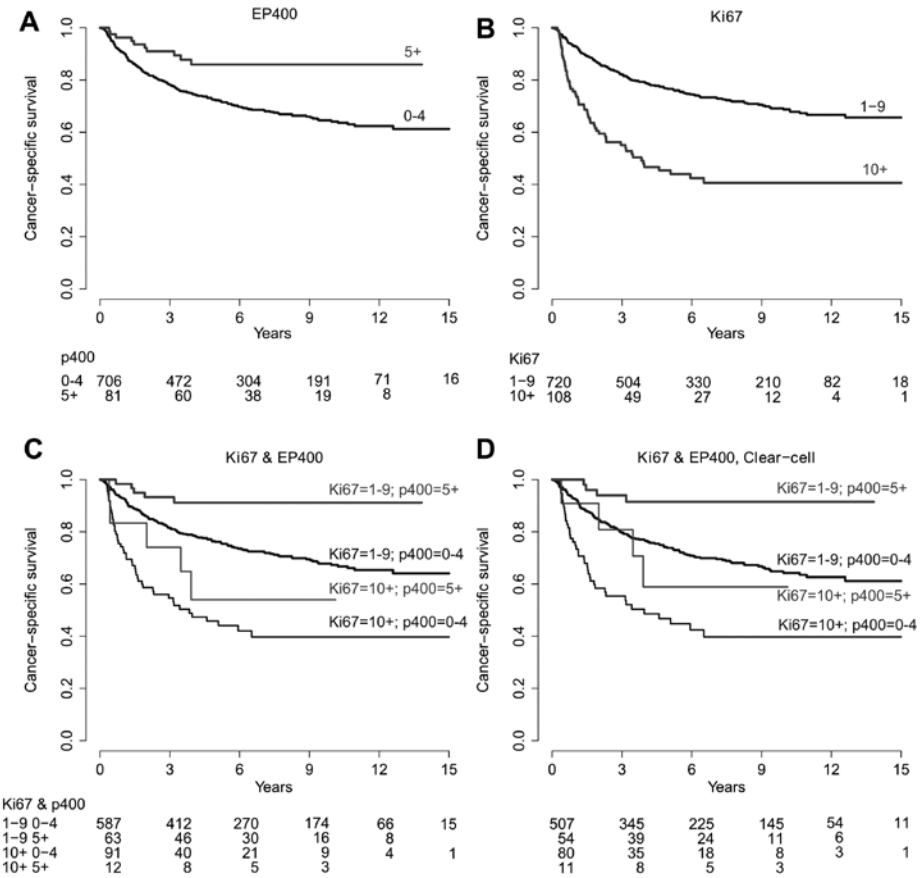

Figure 2. Kaplan-Meier plots for cancer-specific survival according to p400 expression (A), Ki-67 labeling (B) and combination of Ki-67 labeling and p400 expression based on the whole collective (C) and ccRCC only (D). Number of patients at risk is also presented.

Table II. Association between p400/Ki-67 expression and patient survival.

		Overall survival			Cancer-specific survival			Progression-free survival		
Marker	Patients	Global P-value	N[a]	HR with 95% CI	Global P-value	N[a]	HR with 95% CI	Global P-value	N[a]	HR with 95% CI
Raw estimates										
p400										
0	502	**0.02**	247	(Ref.)	**0.02**	156	(Ref.)	**0.02**	114	(Ref.)
1	73		39	1.09 (0.78-1.53)		21	0.95 (0.60-1.50)		13	0.78 (0.44-1.39)
2	61		34	1.15 (0.80-1.64)		20	1.11 (0.69-1.76)		8	0.56 (0.27-1.14)
3,4	70		29	0.83 (0.56-1.22)		15	0.67 (0.39-1.14)		11	0.65 (0.35-1.20)
5-9	81		21	**0.51** (0.32-0.79)		10	**0.37** (0.20-0.71)		6	**0.29** (0.13-0.66)
Ki-67										
1	216	**<0.0001**	99	(Ref.)	**<0.0001**	53	(Ref.)	**0.002**	34	(Ref.)
2	151		73	1.02 (0.75-1.38)		44	1.15 (0.77-1.72)		31	1.28 (0.79-2.09)
3	252		102	0.81 (0.62-1.07)		58	0.86 (0.60-1.25)		52	1.24 (0.81-1.92)
5	101		47	1.03 (0.72-1.45)		32	1.31 (0.85-2.04)		19	1.25 (0.71-2.19)
10+	108		74	**2.18** (1.61-2.95)		56	**2.92** (2.00-4.25)		30	**2.61** (1.60-4.28)
Adjusted estimates										
p400										
0	502	0.11	247	(Ref.)	0.14	156	(Ref.)	0.32	114	(Ref.)
1	73		39	1.19 (0.84-1.68)		21	1.16 (0.73-1.86)		13	0.84 (0.46-1.51)
2	61		34	1.43 (1.00-2.06)		20	1.61 (1.00-2.59)		8	0.73 (0.35-1.50)
3,4	70		29	1.16 (0.78-1.72)		15	1.02 (0.59-1.79)		11	1.11 (0.59-2.08)
5-9	81		21	0.72 (0.45-1.13)		10	0.61 (0.32-1.17)		6	0.44 (0.19-1.02)
Ki-67										
1	216	0.16	99	(Ref.)	0.20	53	(Ref.)	0.40	34	(Ref.)
2	151		73	0.93 (0.69-1.27)		44	0.99 (0.66-1.48)		31	1.32 (0.81-2.16)
3	252		102	0.78 (0.59-1.03)		58	0.76 (0.52-1.12)		52	1.30 (0.84-2.01)
5	101		47	0.82 (0.57-1.17)		32	0.86 (0.54-1.35)		19	1.29 (0.73-2.27)
10+	108		74	1.11 (0.81-1.54)		56	1.21 (0.81-1.80)		30	1.69 (1.01-2.82)

HR, hazard ratio; CI, confidence interval. [a]N indicates event number. Probability values and hazard ratios considered statistically significant are shown in bold.

tumors showed nuclear positivity with variable intensity and percentage of nuclei (Fig. 1C-I). Cytoplasmic staining was considered as unspecific.

When tumors were grouped according to p400 expression, univariate survival analysis revealed an improved OS, CS and PFS in patients affected by tumors with increased p400 expression (5+, Table II). Kaplan-Meier plots are depicted in Fig. 2. Global probability values from multiple regression analysis on OS, CS and PFS did not reach statistical significance (Table II).

Table III shows the distribution of p400 expression according to tumor/patient characteristics. The proportion of tumors with increased p400 expression decreased with tumor extent (P=0.02), lymph node metastasis (P=0.01) and was lower in tumors treated with nephrectomy (P<0.0001).

Comparison of p400 and Ki-67 expression. As loss of p400 may induce senescence in RCCs, we used Ki-67 labeling to estimate the proliferation index. Although the absence of proliferation is not equivalent with senescence, tumors with a high amount of senescent cells are expected to show less proliferation.

Ki-67 labeling was obtained for 828 tumors. The expression of Ki-67 was not available for 34 patients with data on p400 expression.

As expected, univariate survival analysis revealed a less favorable CS in patients affected with tumors characterized by a high proliferation rate (Ki-67 >10%). Kaplan-Meier plots are depicted in Fig. 2. Global probability values from multiple regression analysis on OS, CS and PFS did not reach statistical significance (Table II). Kaplan-Meier plots stratified by p400 and Ki-67 expression are shown in Fig. 2. The distribution of patients by Ki-67 expression and tumor/patient characteristics is shown in Table III.

Notably, patients affected by low proliferative tumors showed a particularly good prognosis in the case of increased p400 expression (cancer-specific HR, 0.28; 95% CI, 0.12-

Table III. Relationship between clinical and pathological characteristics and Ki-67/p400 expression.

	p400			Ki-67			Ki-67 and p400		
Characteristics	0-4 n (%)	5+ n (%)	P-value	1-9 n (%)	10+ n (%)	P-value	Other n (%)	10+ and 0-4 n (%)	P-value
Gender									
Male	442 (91)	44 (9)	0.47	449 (87)	69 (13)	0.76	404 (87)	59 (13)	0.48
Female	269 (89)	32 (11)		271 (87)	39 (13)		258 (89)	32 (11)	
Age at diagnosis (years)									
<60	249 (89)	31 (11)	0.32	259 (89)	31 (11)	0.14	237 (91)	23 (9)	**0.05**
≥60	462 (91)	45 (9)		461 (86)	77 (14)		425 (86)	68 (14)	
Histology									
Clear-cell	606 (90)	65 (10)	0.42	623 (87)	95 (13)	**0.005**	572 (88)	80 (12)	**0.01**
Papillary	74 (94)	5 (6)		74 (89)	9 (11)		68 (89)	8 (11)	
Chromophobe	26 (84)	5 (16)		19 (100)	0 (0)		19 (100)	0 (0)	
Other	5 (83)	1 (17)		4 (50)	4 (50)		3 (50)	3 (50)	
Tumor extent (T)									
1	382 (88)	54 (12)	**0.02**	412 (91)	40 (9)	**<0.0001**	384 (93)	31 (7)	**<0.0001**
2	76 (92)	7 (8)		73 (87)	11 (13)		67 (86)	11 (14)	
3	241 (94)	15 (6)		228 (81)	52 (19)		204 (82)	45 (18)	
4	12 (100)	0 (0)		7 (58)	5 (42)		7 (64)	4 (36)	
Lymph node metastasis (N)									
No	656 (90)	76 (10)	**0.01**	678 (88)	89 (12)	**<0.0001**	626 (89)	74 (11)	**<0.0001**
Yes	55 (100)	0 (0)		42 (69)	19 (31)		36 (68)	17 (32)	
Distant metastasis (M)									
No	595 (90)	67 (10)	0.31	616 (89)	76 (11)	**<0.0001**	566 (90)	66 (10)	**0.001**
Yes	116 (93)	9 (7)		103 (76)	32 (24)		96 (79)	25 (21)	
Grade of malignancy									
1	172 (87)	26 (13)	0.08	201 (95)	11 (5)	**<0.0001**	181 (96)	8 (4)	**<0.0001**
2	421 (91)	43 (9)		427 (89)	51 (11)		403 (91)	42 (9)	
3/4	118 (94)	7 (6)		92 (67)	46 (33)		78 (66)	41 (34)	
Type of surgery									
Nephrectomy	616 (93)	45 (7)	**<0.0001**	601 (86)	95 (14)	0.23	554 (87)	81 (13)	0.19
Resection	95 (75)	31 (25)		119 (90)	13 (10)		108 (92)	10 (8)	
Karnofsky performance									
≥80%	650 (90)	74 (10)	0.07	668 (87)	96 (13)	0.16	614 (88)	80 (12)	0.11
<80%	61 (97)	2 (3)		52 (81)	12 (19)		48 (81)	11 (19)	

Probability values considered statistically significant are shown in bold.

0.69, compared to decreased p400 expression; Table III). Approximately 12% (91/753) of the patients were diagnosed with highly proliferative tumors that showed a decreased p400 expression. These patients had a poor prognosis (cancer-specific HR, 2.79; 95% CI, 2.01-3.87, compared to low proliferative tumors with a decreased p400 expression). Cancer-specific survival differences among the 4 groups combining Ki-67 and p400 expression categories remained statistically significant after adjustment for established prognostic factors in the multiple regression survival analysis (Table IV).

The expression of p400 and Ki-67 showed a weak positive correlation (Spearman's rank correlation rho=0.10; 95% CI, 0.03-0.17; P=0.006). This correlation was higher among grade I tumors (rho=0.25; 95% CI, 0.11-0.37; P=0.004) and nearly absent among grade 3 tumors (rho=-0.01; 95% CI, 0.19-0.16; P=0.88; data not shown).

In the present, study high proliferation was only noted in 6% of low grade carcinomas. In contrast, this fraction increased up to 12% in low grade RCCs with high p400 expression, which outnumbers the fraction of high proliferative intermediate

Table IV. Combined effect of Ki-67 and p400 expression on patient survival.

Characteristics	Patients	Overall survival			Cancer-specific survival			Progression-free survival		
		Global P-value	N[a]	HR with 95% CI	Global P-value	N[a]	HR with 95% CI	Global P-value	N[a]	HR with 95% CI
Whole study population										
Raw estimates										
Ki-67 1-9, p400 0-4	587	**<0.0001**	275	(Ref.)	**<0.0001**	159	(Ref.)	**0.0001**	116	(Ref.)
Ki-67 1-9, p400 5+	63		14	**0.47** (0.27-0.80)		5	**0.28** (0.12-0.69)		4	**0.29** (0.11-0.80)
Ki-67 10+, p400 0-4	91		63	**2.29** (1.74-3.01)		47	**2.79** (2.01-3.87)		26	**2.23** (1.45-3.42)
Ki-67 10+, p400 5+	12		6	1.27 (0.56-2.84)		5	1.73 (0.71-4.21)		2	0.96 (0.24-3.87)
Adjusted estimates										
Ki-67 1-9, p400 0-4	587	0.07	275	(Ref.)	**0.02**	159	(Ref.)	0.13	116	(Ref.)
Ki-67 1-9, p400 5+	63		14	0.58 (0.34-1.01)		5	**0.40** (0.16-0.98)		4	0.35 (0.13-0.97)
Ki-67 10+, p400 0-4	91		63	1.27 (0.95-1.70)		47	**1.43** (1.02-2.02)		26	1.29 (0.80-2.07)
Ki-67 10+, p400 5+	12		6	1.19 (0.53-2.70)		5	1.53 (0.62-3.79)		2	1.23 (0.30-4.99)
Clear-cell only										
Adjusted estimates										
Ki-67 1-9, p400 0-4	507	0.19	252	(Ref.)	0.06	149	(Ref.)	0.13	108	(Ref.)
Ki-67 1-9, p400 5+	54		13	0.64 (0.37-1.13)		4	0.37 (0.14-1.02)		3	0.30 (0.09-0.93)
Ki-67 10+, p400 0-4	80		56	1.25 (0.92-1.70)		41	1.40 (0.97-2.02)		24	1.29 (0.79-2.12)
Ki-67 10+, p400 5+	11		5	1.02 (0.42-2.50)		4	1.26 (0.46-3.47)		2	1.24 (0.31-5.06)

HR, hazard ratio; CI, confidence interval. [a]N indicates event number. Probability values and hazard ratios considered statistically significant are shown in bold.

Table V. Interaction between Ki-67 and p400 regarding tumor grade.

Ki-67-labeling	P400 expression	Grade		
		1, n (%)	2, n (%)	3/4, n (%)
1-9	0-4	155 (82)	361 (81)	71 (60)
1-9	5+	23 (12)	36 (8)	4 (3)
10+	0-4	8 (4)	42 (9)	41 (34)
10+	5+	3 (2)	6 (1)	3 (3)

carcinomas (all G2) (10%) and nearly equals the fraction of high proliferative carcinomas based on the whole collective (13%) (Table V).

Discussion

Inactivation of *vhl* is a pivotal event in the carcinogenesis of the majority of either sporadic or hereditary clear-cell renal cell carcinoma (ccRCC) (25,26). In addition to the well-known function of regulating hypoxia inducible factor (HIF), VHL protein (pVHL) influences several additional intracellular processes including senescence. Recently, Young *et al* (27) reported activation of an HIF-independent senescence program mediated by loss of pVHL, downregulation of p400, stabilization of $p27^{KIP}$ and activation of Rb.

In the present study, we investigated the expression status of p400 in a large series of RCCs and compared the findings with the proliferation rate and clinical and pathological parameters. We found that loss of p400 expression was observed in the majority of RCCs. Notably, the proportion of carcinomas with decreased p400 expression increased with advancing tumor stage (T1, 88%; T2, 92%; T, 94%; T4, 100%) and loss of differentiation (G1, 87%; G2, 91%; G3, 94%) and was more often encountered in carcinomas with established regional lymph node metastasis. Furthermore, the proportion of carcinomas with compete loss of p400 increased with advancing tumor stage (T1, 59%; T2, 63%; T3/4, 72%; P=0.002) and loss of differentiation (G1, 58%; G2, 63%; and G3, 78%; P<0.0001) and was more often encountered in carcinomas with established regional lymph node metastasis (P=0.02) (data not shown).

Induction of senescence by inactivation of tumor-suppressor genes such as *vhl* (28,29), *nf1* (30) or *pten* (31) but also by activation of oncogenes such as *ras* (32) or *braf* (22) has been reported and is considered an important mechanism of mammalian cells by which to limit tumor development (33). Cellular senescence denotes a stable loss of proliferative capacity, and Ki-67 labeling in combination with other markers is recommended to detect senescent cells (33). Comparison of p400 expression and proliferation, measured by Ki-67 immunohistochemistry, showed a statistically significant positive

correlation in well-differentiated carcinomas and was almost null among G3 carcinomas. In addition, high proliferation (Ki-67 index 10+) was found in 12% of carcinomas with a positive p400 expression, in contrast to only 5% in p400-negative low grade RCCs. This may indicate that retained p400 expression is required for proliferation in low grade carcinomas whereas in dedifferentiated tumors other mechanisms might contribute to the escape of senescence.

In human cells, loss of p400 also triggers the p53/p21-dependent senescence pathway (13) and, although RCCs rarely harbor p53 mutations, repression of the p53 pathway in RCC-derived cell lines has been reported (34). Burrows *et al* (35) reported that Polybromo-1 (BAF180, PBRM1) is required for p53-induced senescence; hence mutations of *pbrm1* may provide a mechanism to antagonize p53 function in cancer cells. *Polybromo-1* has recently gained attention as truncated mutations have been identified in up to 41% of ccRCCs (36), indicating that *pbrm1* may be a second major cancer-related gene in the carcinogenesis of ccRCCs. Therefore, mutations in *pbrm1* may be an additional mechanism in RCCs to escape senescence induced by loss of *vhl*. Given that kidney cancer is not a single tumor entity but comprises a number of different types of cancer (25) and ccRCCs by themselves show substantial genetic heterogeneity (37), it is quite likely that a variety of other pathomechanisms contribute to the escape from senescence induced by loss of VHL.

Importantly, the present study showed that patients affected by highly proliferative RCCs with decreased p400 expression have a very unfavorable clinical course with a 5-year cancer-specific survival rate of only 44% (standard error, SE 0.06) in contrast to 76% (SE, 0.02) among low proliferative RCCs with decreased p400 expression, and 92% (SE, 0.04) in low proliferative RCCs with an increased p400 expression. The proliferation rate obtained by Ki-67 labeling is an established well-known prognostic factor in RCCs (18). In our collective, high proliferation (10+) was also an unfavorable prognostic marker. However, after implementing p400 in the multivariate model, only highly proliferative carcinomas with decreased p400 protein levels exhibited worse clinical outcome (HR, 2.79; 95% CI, 2.01-3.87) in contrast to the subgroup of tumors characterized by high proliferation rate and increased p400 expression (HR, 1.73; 95% CI, 0.71-4.21), both hazard ratios taking low proliferative tumors with decreased p400 expression as reference.

Young *et al* (27) showed that induction of senescence triggered by loss of VHL was mediated by downregulation of p400 and a downstream pathway including Skp2, $p27^{KIP}$ and Rb. Our data suggest that at least in low grade RCCs a retained p400 is demanded for high proliferation. Therefore, patients with highly proliferative and p400-positive RCCs may in particular benefit from new pro-senescence therapy strategies. For example, the SKP1-CUL1-F-box protein (SCF)-SKP2 complex inhibitor (MLN4924) is currently in phase I trials and has the ability to induce senescence by stabilization of $p27^{KIP}$ and inhibits tumor growth *in vitro* and *in vivo* (38,39).

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