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Uncoupling of PUMA Expression and Apoptosis Contributes to Functional Heterogeneity in Renal Cell Carcinoma — Prognostic and Translational Implications[1]

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Abstract

Renal cell carcinoma (RCC) is characterized by a profound disruption of proapoptotic signaling networks leading to chemo- and radioresistance. A key mediator of DNA damage-induced apoptosis is the BH3-only protein PUMA. Given its central role in proapoptotic signaling, we analyzed a series of more than 600 precision-annotated primary RCC specimens for PUMA protein expression. We found a reduced expression of PUMA in 22.6% of RCCs analyzed. Unexpectedly, however, PUMA deficiency was not associated with more aggressive tumor characteristic as expected. Instead, a reduced PUMA expression was associated with a lower TNM stage, lower histopathologic grade, and more favorable cancer-specific patient survival. A direct correlation in a separate patient cohort revealed a profound disconnection between PUMA expression and apoptosis as exemplified by the fact that the tumor with the highest level of apoptotic cells was PUMA deficient. In a series of *in vitro* studies, we corroborated these results and discovered the highest propensity to undergo apoptosis in an RCC cell line with virtually undetectable PUMA expression. At the same time, PUMA expression was not necessarily associated with stronger apoptosis induction, which underscores the striking functional heterogeneity of PUMA expression and apoptosis in RCC. Collectively, our findings suggest that PUMA-independent mechanisms of cell death exist and may play an important role in suppressing malignant progression. They underscore the functional heterogeneity of

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RCCs and suggest that PUMA expression alone may not be a suitable predictive biomarker. A better understanding of alternative proapoptotic pathways, however, may help to design novel therapeutic strategies for patients with advanced RCC.

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Introduction

Renal cell carcinoma (RCC) is characterized by a high degree of chemo- and radioresistance. The underlying molecular mechanisms are incompletely understood but very likely entail a profound disruption of proapoptotic signaling networks.

Chemo- or radiotherapy is believed to function mainly through the induction of DNA damage. In particular DNA double strand breaks are highly toxic and trigger an acute cellular response. After induction of a DNA double strand break, a cascade of events is initiated to halt cell cycle progression and activate DNA repair mechanisms. If the damage is too severe to be repaired, cells either enter premature senescence or undergo apoptosis [1]. Activation of p53 has long been shown to be a key event in such cell fate decisions, and a number of critical downstream mechanisms involved in different branches of the p53-mediated response to DNA damage have been identified. Specifically, the induction of p53-dependent apoptosis involves the transcriptional upregulation of PUMA, NOXA, and BAX, which participate in the mitochondrial pathway of cell death [2].

The BH3-only protein PUMA has been identified as a key regulator of p53-dependent as well as p53-independent proapoptotic signaling following genotoxic insults [3–5]. PUMA binds and inhibits antiapoptotic BCL-2 proteins, thereby relieving the inhibition of the proapoptotic proteins BAK/BAX to trigger mitochondrial outer membrane permeabilization and activation of caspases [6]. Members of the BCL-2 network are frequently disrupted in primary RCCs, and furthermore, functional studies have shown that the profound DNA damage resistance of this tumor type involves defects in p53-mediated proapoptotic signaling [7–10]. We have recently shown that RCC cells are characterized by a disconnection between p53 activation and PUMA upregulation that can be reversed by HDAC inhibition [11].

In the present study, we made the surprising discovery that PUMA deficiency correlates with more favorable clinicopathological characteristics and patient survival in a series of more than 600 RCCs and not with more aggressive tumor growth as one would predict from the loss of a proapoptotic protein. To reconcile these findings, we provide evidence for PUMA-independent apoptosis *in vivo* as well as *in vitro*. The striking functional heterogeneity of PUMA expression and apoptosis discourages the use of PUMA as predictive biomarker in RCC but encourages the interrogation of PUMA-independent cell death mechanisms for the development of innovative strategies to resensitize RCCs to DNA radio- or chemotherapy.

Material and Methods

Cell Culture and Treatment

RCC cell lines A-498, 786-0, Caki-1, Caki-2, RCC-KP, ACHN, and human embryonic kidney (HEK) 293 cells were all obtained commercially and maintained as recommended by the distributor (CLS). The media were supplemented with 10% fetal bovine serum, 50 U/ml of penicillin, and 50 μg/ml of streptomycin (Life Technologies). Cells were treated with staurosporine (Sigma) or daunorubicin (Toronto Research Chemicals) at the concentrations indicated for 24 hours. DMSO was used as solvent control.

Immunoblotting

Immunoblot analysis was performed as previously described [11]. Antibodies used for immunoblotting were directed against PUMA (Cell Signaling), p53 (DO-1; Santa Cruz), phospho-p53 Ser15 (Cell Signaling), and GAPDH (FL-335; Santa Cruz).

Caspase 3/7 Assay

A luminometric assay (Caspase-Glo 3/7; Promega) was used to measure apoptotic cell death according to manufacturer's instructions.

Immunohistochemistry

Tissue microarrays (TMAs) were obtained through the biobank of the National Center for Tumor Diseases Heidelberg. The tissue specimens provided by the National Center for Tumor Diseases tissue bank were used in accordance to the regulations of the tissue bank and under University of Heidelberg Ethics Committee approvals 206/2005 and 207/2005. Patient samples were collected between 1990 and 2005 with a mean follow-up time of 80.7 months (range 0.3 to 254.7 months). A cohort of 26 patients with varying TNM stages was retrieved from the archives of the Department of Pathology of the University of Heidelberg School of Medicine and reviewed by an expert pathologist (W.R.). The slides were processed as previously described [12] and incubated with a PUMA antibody (Millipore ABC158, 1:100 dilution). Additional antibodies used for immunohistochemistry were directed against cleaved caspase-3 (Cell Signaling) and CD163 (Novocastra). Immunodetection was performed using the Histostain Plus IHC Detection Kit (Life Technologies). Slides were counterstained with hematoxylin.

Statistical Analysis

As an explorative study, all statistical analyses are of descriptive nature. Statistical tests and resulting P values are not adjusted for multiplicity and are therefore interpreted descriptively. A baseline comparison between the two groups with PUMA positivity (group 1) and the group with a reduced PUMA expression (group 2) was done using the standard chi-square test. Prognostic factors for cancer-specific survival were assessed by a univariable and different multivariable Cox regression models. Because of the limited number of patients within the considered subgroups, we only included three covariates in the multivariable models to avoid overfitting. For graphical display, Kaplan-Meier survival curves were generated, and differences between groups were assessed using the log-rank test. Student's t test for independent samples was used to compare experimental groups. P values ≤ .05 were considered significant, and

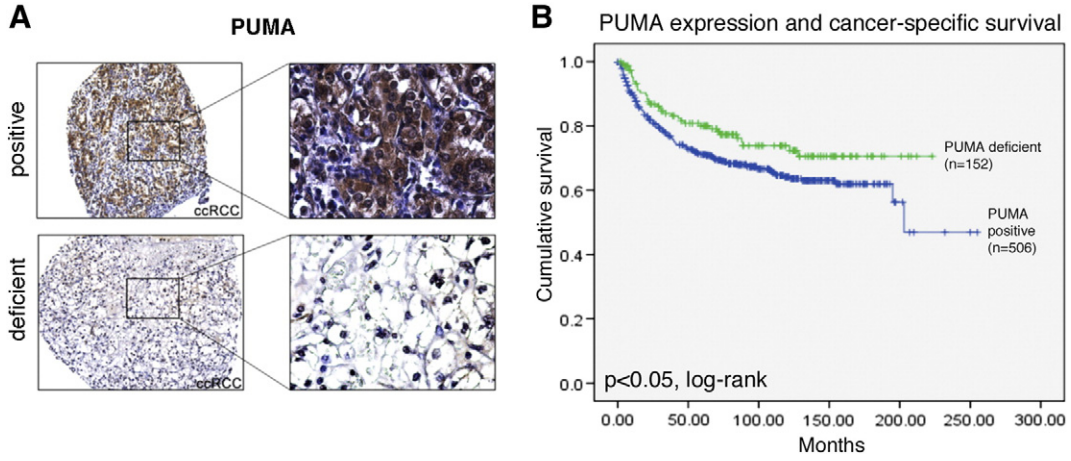

Figure 1. PUMA expression and cancer-specific survival in a series of 673 RCCs.(A) Examples of PUMA-positive and PUMA-deficient RCC tissue specimens from a TMA comprising more than 600 RCCs.(B) Kaplan-Meier curves for patient cohorts with either PUMA-deficient tumors (green) or PUMA-positive tumors (blue). The postoperative cancer-specific survival of 673 patients is shown.

all tests were performed two-sided. Data analysis was performed using the SPSS software package (SPSS) or GraphPad Prism (GraphPad).

Results

PUMA Deficiency and Clinicopathological Characteristics in RCC

Given the importance of PUMA for DNA damage-induced apoptosis, we sought to determine whether and to what extent primary RCCs have an altered baseline PUMA protein expression as a potential cause for primary chemo- and radioresistance.

Using a TMA, a total of 673 RCCs were evaluated (Figure 1 and Table 1). PUMA expression in RCCs was scored as either positive (moderate to strong staining) or deficient (reduced or below detection level; Figure 1*A*).

Table 1. Patient Characteristics (n = 673)

Parameter	n	%
Sex		
Male	420	62.4%
Female	253	37.6%
Age at diagnosis (years)		
<65	366	54.4%
≥65	307	45.6%
Tumor stage		
pT1/2	454	67.5%
pT3/4	219	32.5%
Lymph node metastasis		
N0/pN0	628	93.3%
N+	45	6.7%
Distant metastasis		
M0	573	85.1%
M+	100	14.9%
Fuhrman grade		
G1/2	565	84%
G3/4	108	16%
Histology		
Clear cell	579	86%
Papillary	59	8.8%
Chromophobe	27	4%
Others	8	1.2%

A positive PUMA expression was found in 506 RCCs (75.2%). A reduced expression was found in 152 tumors (22.6%). In addition, an overexpression of PUMA was detected in 15 tumors (2.2%). The latter group showed an overrepresentation of non–clear cell RCCs including 60% papillary and 13.3% chromophobe RCCs and was excluded from further analyses.

A statistical analysis of PUMA-positive RCCs in comparison to PUMA-deficient tumors showed a correlation of the latter with less aggressive tumor characteristics including lower TNM stage as well as Fuhrman grade (Table 2). In line with this, Kaplan-Meier cancer-specific survival curves showed that PUMA deficiency was associated with more favorable cancer-specific patient survival (Figure 1*B*). Cox regression analysis confirmed a borderline negative prognostic impact of PUMA positivity versus PUMA deficiency in a univariable analysis, but different multivariable Cox regression models showed that PUMA expression was not an independent prognostic factor (Table 3).

Taken together, our results demonstrate that PUMA deficiency is associated with more favorable clinicopathological parameters and cancer-specific survival in RCC patients.

Table 2. Correlation of PUMA Expression with Clinicopathological Parameters

PUMA Expression	Group 1: PUMA Positive (n = 506)	Group 2: PUMA Deficient (n = 152)	Significance *
Sex (n; %)	M (327; 64.6%) F (179; 35.4%)	M (85; 55.9%) F (67; 44.1%)	P = .052
Age (n; %)	<65 (284; 56.1%) ≥65 (222; 43.9%)	<65 (77; 50.7%) ≥65 (75; 49.3%)	P = .24
Tumor stage (n; %)	pT1/2 (320; 63.3%) pT3/4 (186; 36.8%)	pT1/2 (122; 80.3%) pT3/4 (30; 19.7%)	P < .001
Lymph node metastasis (n; %)	N0/pN0 (465; 91.9%) pN+ (41; 8.1%)	N0/pN0 (148; 97.4%) pN+ (4; 2.6%)	P = .019
Distant metastasis (n; %)	M0 (421; 83.2%) M+ (85; 16.8%)	M0 (138; 90.8%) M+ (14; 9.2%)	P = .022
Fuhrman grade (n; %)	G1/2 (412; 81.4%) G3/4 (94; 18.6%)	G1/2 (138; 90.8%) G3/4 (14; 9.2%)	P = .006
Histology (n; %)	Clear cell (428; 84.6%) Papillary (47; 9.3%) Chromophobe (23; 4.5%) Others (8; 1.6%)	Clear cell (147; 96.7%) Papillary (3; 2%) Chromophobe (2; 1.3%) Others (0; 0%)	P < .001 (clear cell vs non–clear cell)

* Chi-square test.

Table 3. Cox Regression Analysis.

Cancer-Specific Survival	Univariable		
Variable	HR	95% CI	*P*
PUMA (positive vs deficient)	1.46	1.02-2.09	.04
Age (>65 vs ≤65 years)	1.02	0.77-1.36	.88
Sex (female vs male)	0.62	0.46-0.84	.002
pT (pT3/4 vs pT1/2)	5.04	3.78-6.72	.000
N0/pN0 (+ vs 0)	7.35	5.17-10.45	.000
M (+ vs 0)	10.55	7.85-14.18	.000
Grade (G3/4 vs G1/2)	4.78	3.57-6.41	.000
Histology (clear cell vs non–clear cell)	1.70	1.03-2.79	.037
Cancer-Specific Survival	**Multivariable**		
Variables	HR	95% CI	*P*
PUMA (positive vs deficient)	0.93	0.65-1.35	.71
Sex (female vs male)	0.65	0.48-0.89	.006
pT (pT3/4 vs pT1/2)	4.94	3.69-6.61	.000
PUMA (positive vs deficient)	0.98	0.68-1.43	.93
pT (pT3/4 vs pT1/2)	3.97	2.91-5.43	.000
N0/pN0 (+ vs 0)	3.33	2.28-4.85	.000
PUMA (positive vs deficient)	0.86	0.60-1.23	.41
pT (pT3/4 vs pT1/2)	3.41	2.52-4.62	.000
M (+ vs 0)	7.22	5.29-9.85	.000
PUMA (positive vs deficient)	0.96	0.67-1.39	.84
pT (pT3/4 vs pT1/2)	3.71	2.70-5.09	.000
Grade (G3/4 vs G1/2)	2.59	1.88-3.57	.000
PUMA (positive vs deficient)	0.87	0.60-1.25	.45
pT (pT3/4 vs pT1/2)	4.86	3.62-6.52	.000
Histology (clear cell vs non–clear cell)	1.27	0.77-2.11	.35
PUMA (positive vs deficient)	0.78	0.54-1.12	.18
Grade (G3/4 vs G1/2)	4.52	3.36-6.08	.000
Histology (clear cell vs non–clear cell)	1.51	0.91-2.49	.11

PUMA Expression and the Frequency of Apoptosis in Primary RCCs

To reconcile the perplexing finding that deficiency of the proapoptotic protein PUMA correlates with more favorable tumor characteristics, we directly tested the link between PUMA expression and apoptosis in a cohort of 26 primary clear cell RCCs (ccRCCs). The tumors were chosen to represent a wide range of pTNM stages including 7 pT1N0M0, 10 rarely seen pT1N0M1, and 9 pT3/4 ccRCCS with or without synchronous lymph node or distant metastases.

In addition to PUMA, we stained adjacent sections for cleaved caspase-3 to visualize apoptotic cells and the macrophage marker CD163 (Figure 2). The latter marker has been used to detect tumor-associated macrophages (TAMs) of the M2 phenotype, which have been implicated in tumor growth promotion and metastasis [13,14]. The rationale for this experiment was that PUMA deficiency could lead to a reduced frequency of apoptotic cells and, as a consequence, a diminished infiltration with tumor-promoting M2 TAMs and ultimately an attenuation of malignant progression.

We identified 11 PUMA-deficient ccRCCs (<1 positive cell per 40 × high-power field, HPF) of 26 tumors analyzed (42.3%; Figure 3). The 15 tumors that were PUMA positive (57.7%) showed an average of 42 positive cells per 40 × HPF. The average frequency of apoptotic cells was 12.7 per HPF in PUMA-deficient tumors and 5.2 in PUMA-positive tumors, but this trend toward more apoptosis in PUMA-deficient tumors did not reach statistical significance ($P > .05$). The average frequency of CD163-positive cells was 23.6 per HPF in PUMA-deficient tumors and 20.3 in PUMA-positive tumors, which was also not statistically significant ($P > .05$; Figure 3). Remarkably, the highest level of apoptotic cells and TAM infiltration was detected in a PUMA-deficient ccRCC (Figure 3). The average frequency of CD163-positive TAMs in tumors in which no apoptotic cells were detected ($n = 9$) was 15.2 per HPF versus 25.1 per HPF in tumors in which apoptotic cells were present ($n = 17$; $P > .05$).

These findings underscore that PUMA expression and apoptosis are frequently uncoupled in RCC.

Functional Heterogeneity of PUMA Expression and Apoptosis Induction In Vitro

To corroborate these tissue-based results on a functional level, we used a panel of six RCC cell lines and noncancerous HEK293 cells for further analyses (Figure 4). First, we determined the baseline

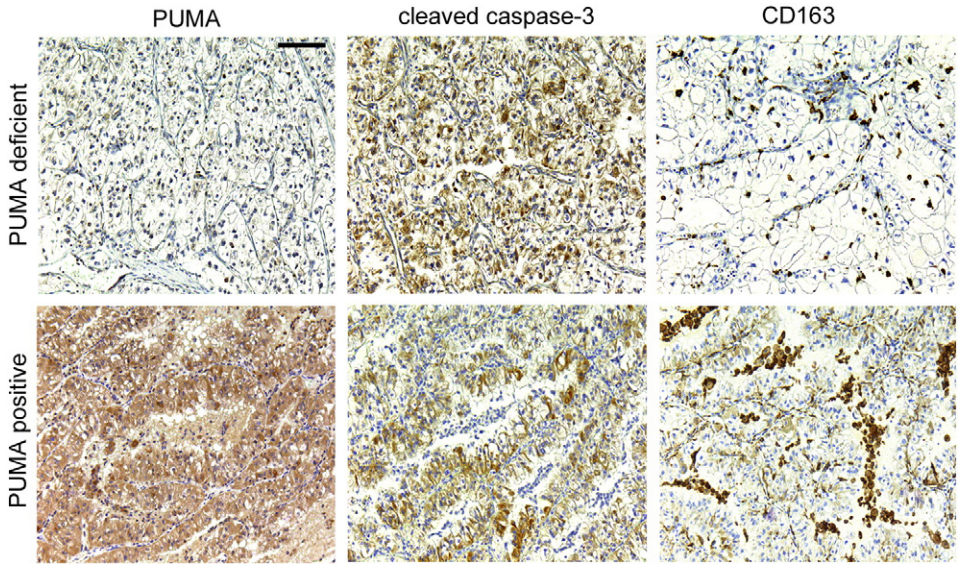

Figure 2. PUMA, cleaved caspase, and CD163 expression in ccRCCs.Immunohistochemical analysis of a PUMA-deficient and PUMA-positive ccRCC for cleaved caspase-3 and CD163. Scale bar = 50 μm.

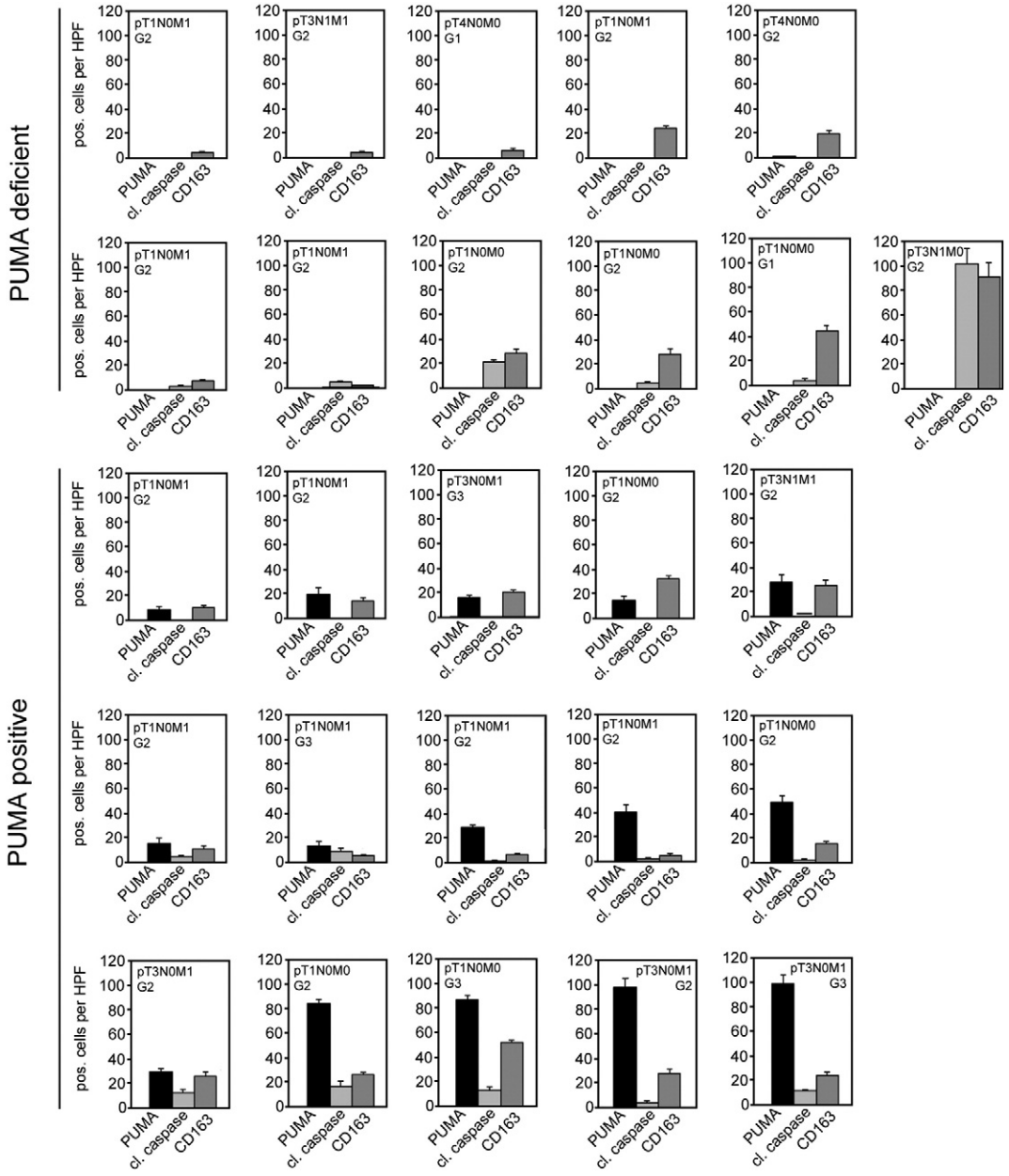

Figure 3. PUMA expression and apoptosis induction are disconnected in ccRCCs.Quantification of staining results from a total of 26 ccRCCs. pTNM and Fuhrman grade are indicated for each tumor. Each bar indicates mean and standard error of the number of positively stained cells from 10 HPFs (40 ×).

expression of PUMA by immunoblotting and found significant differences between cell lines (Figure 4*A*). To exploit this fact, we determined apoptotic cell death, as measured by caspase 3/7 activties, following a 24-hour exposure of cells to proapoptotic stimuli using either the protein kinase C inhibitor staurosporine or the DNA damaging agent daunorubicin at two concentrations (Figure 4*B*). The highest induction of apoptosis (9.6-fold) was detected in A-498 cells treated with 1 μM daunorubicin, a cell line with virtually undetectable PUMA protein expression. The second highest induction of apoptosis (6.2-fold; 1 μM daunorubicin) was found in Caki-1 cells with very low PUMA protein expression. Under these short-term treatment conditions, HEK293, which had the highest level of PUMA protein expression, did not undergo enhanced apoptosis, most likely because these cells require a longer time interval as suggested by previous experiments[11]. PUMA-expressing ACHN cells showed a 4.1-fold increase of apoptosis in response to 1 μM daunorubicin, which was clearly below A-498 and Caki-1 cells, both of which had significantly lower PUMA expression. There was a trend toward more pronounced apoptotic responses to staurosporine than daunorubicin in 786-0, Caki-2, and RCC-KP cells. This underscores that the mode of apoptosis induction is relevant for the cellular response. The fact treatment with 10 μM daunorubicin led to less apoptosis in some RCC cells reflects the well-known fact that higher drug concentrations shift peak caspase activation and/or lead to nonapoptotic cell death. To rule out nonspecific effects that may have influenced these results, e.g., differences in cell cycle distribution, we

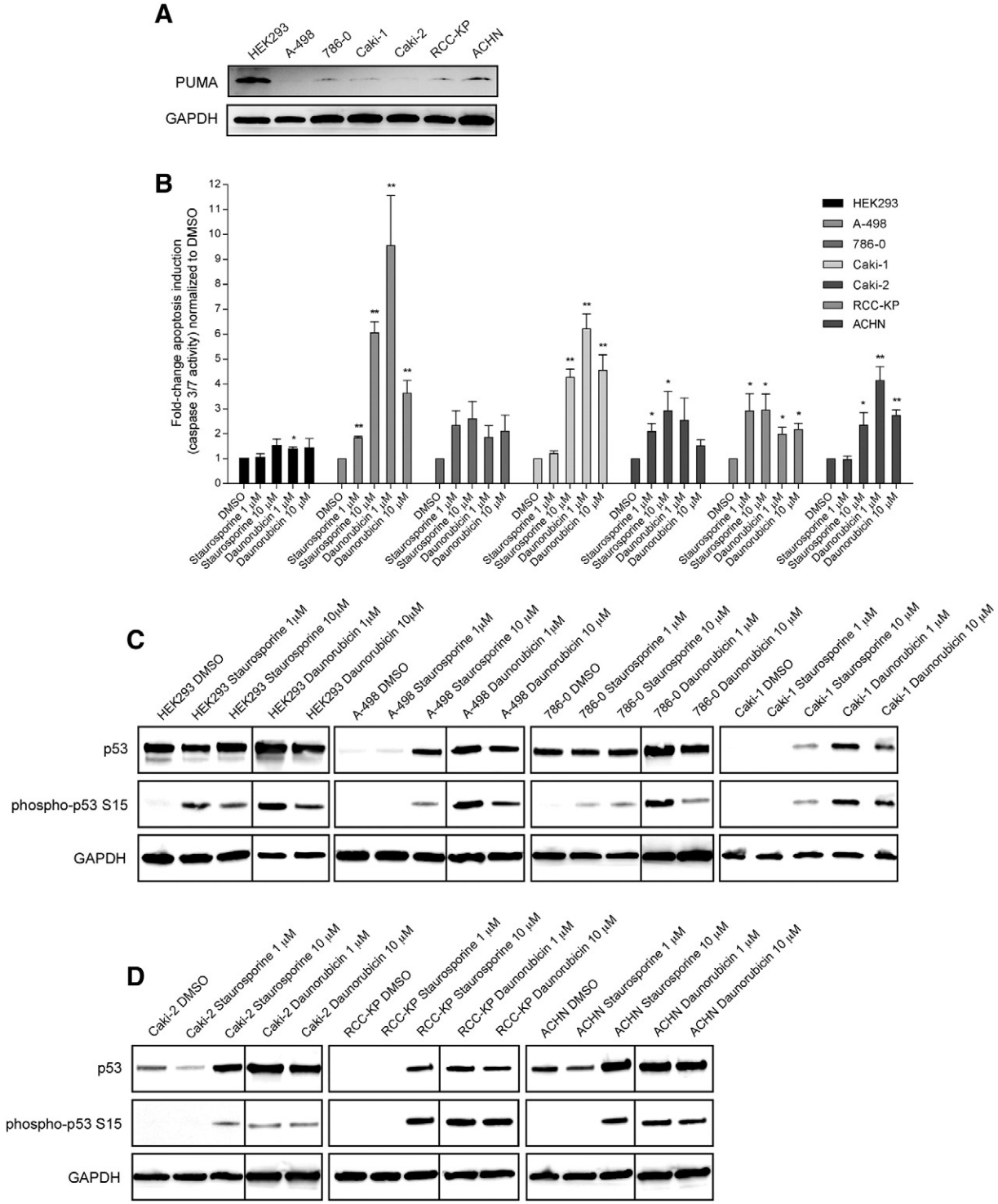

Figure 4. PUMA expression and apoptosis are uncoupled *in vitro*.(A) Immunoblot analysis of PUMA expression in six RCC cell lines and noncancerous HEK293 cells. Immunoblot for GAPDH is shown to demonstrate protein loading.(B) Quantification of apoptosis induction by staurosporine or daunorubicin (24 hours) at the concentrations indicated. Fold changes are shown after normalization to DMSO. Asterisks indicate the level of statistical significance (*$P \leq .05$; **$P \leq .005$).(C, D) Immunoblot analysis of RCC cell lines and HEK293 cells for p53 and phospho-p53 S15 under the same experimental conditions as in (B). GAPDH is shown to demonstrate protein loading.

performed an immunoblot analysis to determine p53 activation (Figure 4, *C* and *D*). Our results show that all cell lines show an activation of p53 as measured by serine 15 phosphorylation.

Taken together, these results highlight the remarkable functional heterogeneity of RCC cells and the profound disconnect between PUMA expression and apoptosis induction.

Discussion

PUMA is a key mediator of p53-induced apoptosis following various insults including genotoxic stress [5]. Here, we analyzed PUMA protein expression in a series of more than 600 RCCs and found an unexpected correlation between PUMA deficiency and more favorable clinicopathological characteristics including cancer-specific

patient survival. To reconcile these findings, we show that loss of PUMA does not necessarily confer apoptosis resistance. In fact, the average frequency of apoptotic cells was higher in PUMA-deficient tumors in comparison to PUMA-positive tumors, although this difference did not yield statistical significance. In addition, a series of *in vitro* experiments underscores the remarkable functional heterogeneity of RCC cells and showed the highest level of apoptosis in PUMA-deficient cells.

Although our finding that patients with PUMA-deficient tumors had a survival advantage was initially perplexing, the presence of PUMA-independent apoptosis as shown here both *in viv*o and *in vitro* may explain this result. How precisely caspase-dependent apoptosis in response to genotoxic stress is executed when PUMA is absent or reduced requires further experimental analysis. Although the existence of PUMA-independent caspase activation and apoptosis induction has previously been reported in colon cancer cells as well as neuronal cells [15,16], the underlying mechanisms remain unclear. There is the possibility of autoactivation of caspases under certain stress conditions such as proteasome inhibition [16]. Whether this explains the results shown here remains to be tested, but it is noteworthy that RCC cell lines with apoptosis induction following DNA damage also responded to staurosporine, which suggests a more general propensity to undergo apoptosis or not. Another possible scenario is that p53 activation triggers a death receptor pathway-mediated induction of apoptosis as previously reported [17]. However, our finding that p53 activation was basically identical in the presence or absence of apoptosis speaks against this notion.

Almost all members of pro- and antiapoptotic proteins of the BCL-2 family have been shown to be altered in RCC [7–9,18,19]. This report extends this knowledge by adding PUMA to the group of proapoptotic proteins that are frequently disrupted in RCC. Nevertheless, the remarkable functional heterogeneity of RCC cells requires further consideration. PUMA-deficient cells can be either highly susceptible to rapid apoptosis induction as shown here or resistant to cell death unless PUMA is reactivated by HDAC inhibition as previously shown [11]. Conversely, PUMA-proficient cells may or may not show a propensity to undergo apoptosis under conditions used here with ACHN cells as examples of the former and HEK293 cells as examples of the latter. It is hence conceivable that PUMA expression status alone may not be suitable to predict the response to DNA damage-inducing therapies. However, our report also underscores the importance of PUMA-independent cell death pathways in RCC. Understanding and harnessing these pathways should hence be an integral part of innovative strategies to resensitize RCC cells to DNA damaging agents to improve oncological treatment modalities for advanced RCC patients.

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