

KPNA2 Expression Is an Independent Adverse Predictor of Biochemical Recurrence after Radical Prostatectomy

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Abstract

Purpose: To analyze rates of expression of karyopherin alpha 2 (KPNA2) in different prostate tissues and to evaluate the prognostic properties for patients with primary prostate cancer.

Experimental Design: Tissue microarrays (TMA) contained 798 formalin-fixed, paraffin-embedded prostate tissue cores from two different institutes of pathology. TMAs were stained immunohistochemically for KPNA2 and NBS1. SiRNA technologies were used to inhibit KPNA2 expression *in vitro*, and the effect of this inhibition on cellular viability was determined. Efficiency of knockdown experiments was determined by Western blot analysis.

Results: KPNA2 expression was significantly upregulated in carcinomas of the prostate, especially in metastatic and castration-resistant prostate cancer samples. Positive nuclear KPNA2 immunoreactivity was identified as a novel predictor of biochemical recurrence after radical prostatectomy ($n = 348$), and was independent of the well-established predictive factors preoperative PSA value, Gleason score, tumor stage, and surgical margin status. These results were validated by analyzing a second and independent prostate cancer cohort ($n = 330$). Further, *in vitro* experiments showed that the cell proliferation and viability of PC3 cells was significantly reduced when KPNA2 expression was inhibited. KPNA2 knockdown did not induce PARP cleavage as marker for apoptosis. No significantly increased sub-G₁ fraction could be found by FACS analysis.

Conclusions: KPNA2 is a novel independent prognostic marker for disease progression after radical prostatectomy. This allows to identify patients who need more aggressive treatment. It can moreover be speculated that patients not suited for surveillance regimens might be identified at initial biopsy by a positive KPNA2 immunohistochemistry. *Clin Cancer Res*; 17(5); 1111–21. ©2011 AACR.

Introduction

Accurate prediction of individual prostate cancer (PCa) behavior is still difficult. The management of patients with localized disease largely consists of three modalities: radical prostatectomy, radiation therapy, and active surveillance. Although cure is achieved for the majority of patients receiving surgery or radiation therapy, still up to 30% of

patients experience disease progression within 10 years. In representative active surveillance studies up to 33% of cases experienced cancer progression (1). An improved identification of high-risk patients is highly desirable to individualize therapy.

Conventional parameters for risk estimation are mainly based on preoperative PSA, the number of positive punch-biopsies, the Gleason score and possibly radiologic features. However, the prognostic value of these established classic markers is limited. Numerous novel molecular markers including MUC1, AZGP1, EZH2, E2F3, Ki67, CD10, RECK, ADAM9, HDAC2, ALCAM, and others have been proposed as prognostic parameters (2–10). A novel candidate biomarker for tumor aggressiveness is the expression of karyopherin alpha 2 (KPNA2). We previously identified KPNA2 overexpression as a possible prognostic marker for breast cancer on mRNA and protein level (11–13). Twenty-four matched pairs of invasive ductal breast cancer and corresponding benign breast tissue were investigated by a combination of laser microdissection and gene expression profiling. Further, analysis by immunohistochemistry showed that nuclear KPNA2 overexpression was significantly associated with shorter overall survival

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Translational Relevance

Biomarkers play an increasingly important role in clinical oncology. However, none of the published tissue biomarkers for prostate cancer has found its way into clinical practice. In this study, we found karyopherin alpha 2 (KPNA2), a marker that we have shown as an independent prognostic marker in breast cancer in recent studies, overexpressed during malignant tumor progression of prostate cancer. Moreover, KPNA2 expression was associated with shortened recurrence-free survival time in univariate and multivariate analyses in two independent patient cohorts treated by radical prostatectomy. The description of KPNA2 as an independent marker of PSA relapse in two independent cohorts of prostate cancer clearly constitutes KPNA2 expression as strong biomarker. This raises the possibility for a better individualized risk stratification to optimize therapy. Further studies have to clarify whether KPNA2 immunohistochemistry can also aid in the discrimination of insignificant and significant prostate cancer at the time of the positive biopsy.

and recurrence-free survival (RFS). Nuclear KPNA2 expression was a highly significant, independent, and adverse risk factor for overall survival in several independent study cohorts (11–13).

Because the relevance of KPNA2 expression as a marker for tumor aggressiveness in other tumor entities is largely unknown, we analyzed the extent of KPNA2 expression in different prostate tissues, and the prognostic relevance of KPNA2 expression in patients with localized PCa receiving radical prostatectomy. Additionally, we analyzed the relationship of KPNA2 expression with its known interaction partner NBS1 (Nijmegen breakage syndrome 1). Finally, the role of KPNA2 on PCa proliferation and apoptosis has been analyzed, *in vitro*.

Materials and Methods

Patients and specimen characteristics

Tissue microarrays (TMA) contained 798 formalin-fixed, paraffin-embedded prostate tissues and were constructed as previously described (14). Specimens were collected between 1993 and 2007 from the Institute of Surgical Pathology, University of Zurich, Switzerland, and the Institute of Pathology, University of Regensburg, Germany. The Zurich TMA (training set) included a series of 348 consecutive (nonselected) radical prostatectomy specimens, 29 hormone-refractory PCa samples, 18 lymph node metastases, 28 distant metastases (bone, lung, urinary bladder), and 45 benign prostatic hyperplasia samples. For the Zurich TMA, hematoxylin and eosin-stained slides of all specimens were reevaluated by two experienced pathologists (P.J.W., H.M.) to identify representative areas. Tumor stage and Gleason score of the Zurich cohort were assigned

according to the International Union Against Cancer and World Health Organization/International Society of Urological Pathology criteria (15). The Regensburg TMA contained a series of 330 consecutive (nonselected) radical prostatectomy specimens and was considered as testing cohort. Tumor stage and Gleason score of the Regensburg cohort were retrieved from the electronic database of the Institute of Pathology, University of Regensburg, Germany. In total, clinical follow-up data were available for 475 of 678 prostatectomy patients (70.1%). Median follow-up of the cohort from Zurich was 71 months (0–163), and 52 months (0–132) for the cohort from Regensburg (Supplementary Table 1). The study for both cohorts was approved by the local scientific ethics committees (approval no.: StV-Nr. 25/2007).

Immunohistochemical assay

Consecutive 3- μ m sections were cut from the TMA tissue blocks. The expression of KPNA2 and NBS1 on the Zurich TMA and the expression of KPNA2 on the Regensburg TMA was analyzed immunohistochemically using the following primary antibodies: anti-KPNA2 (goat polyclonal; Santa Cruz Biotechnology, Inc.; no. sc-6917, dilution 1:100), anti-NBS1 (rabbit polyclonal; Cell Signaling Technology; no. 3002, dilution 1:100), and anti-CXCR4 (mouse monoclonal; Invitrogen Corporation; no. MHCXCR404, dilution 1:200). The ChemMate detection kit (DAKO) was used. After antigen retrieval (microwave oven for 10 minutes at 250 W) immunohistochemistry was carried out in a NEXES immunostainer (Ventana) according to the manufacturer's instructions. Normal testicular parenchyma was chosen as internal positive control. For negative controls, the primary antibody was omitted. The specificity of the commercial KPNA2 antibody has been thoroughly validated in former studies (11–13), and was further tested by using the human PCa cell lines PC3 and LNCaP. Cells were trypsinized and embedded in paraffin as a cell pellet. Cell pellets were stained immunohistochemically for KPNA2 protein. Additionally, cell lines that were used for our *in vitro* experiments were characterized by analyzing the phosphatidylinositol-3-kinase (PI3)/AKT cascade (Supplementary Fig. 1A–F).

Two surgical pathologists (P.J.W., G.K.) performed a blinded evaluation of the slides for KPNA2 and NBS1 without knowledge of clinical data. Causes of noninterpretable results included lack of target tissue, presence of necrosis, or crush artifact. Searching for cutoffs in an unbiased way is a major problem in immunohistochemical studies dealing with a continuous readout. The median nuclear KPNA2 immunoreactivity in prostatectomy cases (median 0%) was chosen as cutoff. Accordingly, positive nuclear KPNA2 and NBS1 immunoreactivity was defined as nuclear staining in at least 0.1% of target cells. In the Zurich cohort, cytoplasmic KPNA2 staining of any intensity was found ubiquitous in 440 of 455 (96.7%) informative cases and was not considered in subsequent statistical analyses. No cytoplasmic NBS1 expression could be detected in PCa samples. Cytoplasmic chemokine (C-X-C motif) receptor 4 (CXCR4) expression was

estimated using a semiquantitative 4-step scoring system (0 to 3+): 0, negative; 1+, weak positive; 2+, strong positive; 3+, very strong positive. Evaluation of the slides stained against CXCR4 was done by a surgical pathologist (M.M.).

Cell lines

The PCa cell lines PC3 and LNCaP were obtained from American Type Culture Collection. Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Corp.) with L-glutamine (Invitrogen Corp.) supplemented with 10% fetal calf serum (FCS), 100 units/mL penicillin, and 100 µg/mL streptomycin. All cells were grown in a humidified atmosphere incubator with 5% CO₂ at 37°C. Cells were cultured for 2 weeks before their use in subsequent experiments.

Reverse transfection of siRNAs

Knockdown experiments were done in triplicates in a 96-well plate format with a total volume of 100 µL per well. Four specific KPNA2 and NBS1 siRNAs were pooled or used separately (final concentration 0.05 µmol/L; accession no. SI02781051, SI02780631, SI00035539, SI00035525, SI02757524, SI02663570, SI00038479, SI00038493; Qiagen). SiRNAs were reverse transfected by using Lipofectamine 2000 transfection reagent (Invitrogen) into PC3 and LNCaP cells according to the manufacturer's instructions. Briefly, RNAi molecules were put in each well before transfection and combined with diluted Lipofectamine 2000 to form complexes in each well. Cells were washed twice with 5 mL PBS, trypsinized, and added directly to the Lipofectamine 2000-RNA complexes. Cells were seeded in Nunclon MicroWell plates (Nunc) at a density of 6,000 cells per well (in DMEM with 10% FCS and antibiotic-free). Transfection occurred while cells were attaching to the well bottom. All Stars negative control siRNA (Qiagen; accession no. SI03650318), H₂O, and All Stars Hs Cell Death siRNA (Qiagen; accession no. SI04381048) were used as negative and positive controls, respectively. As an additional positive control, cells were treated with 5 µmol/L staurosporine (Sigma-Aldrich Chemie GmbH; no. S6942) to induce apoptosis 24 hours after reverse transfection.

MTT assay

Viability of PC3 and Lncap cells was determined by MTT assay (16). After cells were incubated for 48 hours, 100 µL MTT solution (0.1 mg/mL in DMEM with 10% FCS, antibiotic-free; Sigma-Aldrich) was added to each well and the plates were further incubated for 4 hours. The crystal was dissolved in 100 µL lysis buffer [16 µL glycine buffer (0.1 mol/L glycine; 0.1 mol/L NaCl, pH 10.5) and 84 µL dimethylsulfoxide] for 10 minutes. The absorbance was measured at a wavelength of 570 nm using a microplate reader (SpectraMax 190; Molecular Devices, MDS). For normalization, absolute absorbance was divided by the absorbance of wells with All Stars negative control siRNA (Qiagen).

Proliferation assays

Cell proliferation capacity of siRNA-transfected cultures was determined by seeding 125,000 PC3 cells in series of 6-well culture dishes and cultivating them for several days. PC3 cells were counted every 24 hours with a coulter counter. For each culture, the cell number at each time point reflects the average of data from three dishes.

Western blot analysis

Cultured cells were lysed in TNN buffer (25 mmol/L Tris-HCl, pH 7.2; 500 mmol/L NaCl; 10% glycerol; 1% NP-40, 10 mmol/L NaF; 1 mmol/L Na₃VO₄; 1 mmol/L phenylmethylsulfonylfluoride, 1 mmol/L dithiothreitol, 10 mg/mL aprotinin). Protein extracts (50 mg) were run on 8% to 15% polyacrylamide gels, transferred to nitrocellulose membranes, and visualized by immunoblotting with the following antibodies: anti-KPNA2 (Santa Cruz Biotechnology, Inc.; no. 6917), anti-α-tubulin (anti-rat antibody; kindly provided by Wilhelm Krek, Institute of Cell Biology, ETH Zurich, Switzerland), and anti-PARP (BD Biosciences; 556362). As positive control for the induction of apoptosis, Staurosporine solution (Sigma-Aldrich Chemie GmbH; no. S6942, final concentration 5 µmol/L) was added to the cells 24 hours before lysis.

Analysis of apoptosis by propidium iodide staining and flow cytometry

The propidium iodide (PI) flow cytometric assay has been widely used for the evaluation of apoptosis. It is based on the principle that apoptotic cells are characterized by DNA fragmentation and, consequently, loss of nuclear DNA content. Use of a fluorochrome, such as PI, that is capable of binding and labeling DNA makes it possible to obtain a rapid and precise evaluation of cellular DNA content by flow cytometric analysis, and subsequent identification of hypodiploid cells. PC3 cells were transfected with negative control siRNA or pooled siRNA against KPNA2. For sub-G₁ DNA content analysis and calculation of the mitotic index, PC3 cells were processed for flow cytometric analysis (FACS) 72 hours after transfection as described (17). Fluorescence intensities of the samples ($n = 10,000$) were measured, using a FACS Calibur flow cytometer (BD Biosciences).

Statistical analyses

To study statistical associations between clinicopathologic and immunohistochemical data, contingency table analysis and 2-sided Fisher's exact tests were used. For the comparison of two independent samples the nonparametric Mann-Whitney test was calculated. Time to PSA recurrence (cutoff ≥ 0.1 ng/mL) was selected as clinical end point. Only patients with primary PCa undergoing radical prostatectomy and reaching the PSA nadir (<0.1 ng/mL) postoperatively were used for survival analysis. Univariate Cox regression analysis was used to evaluate statistical association between clinicopathologic/immunohistochemical data and RFS. RFS curves were calculated using the Kaplan-Meier method with significance evaluated by 2-

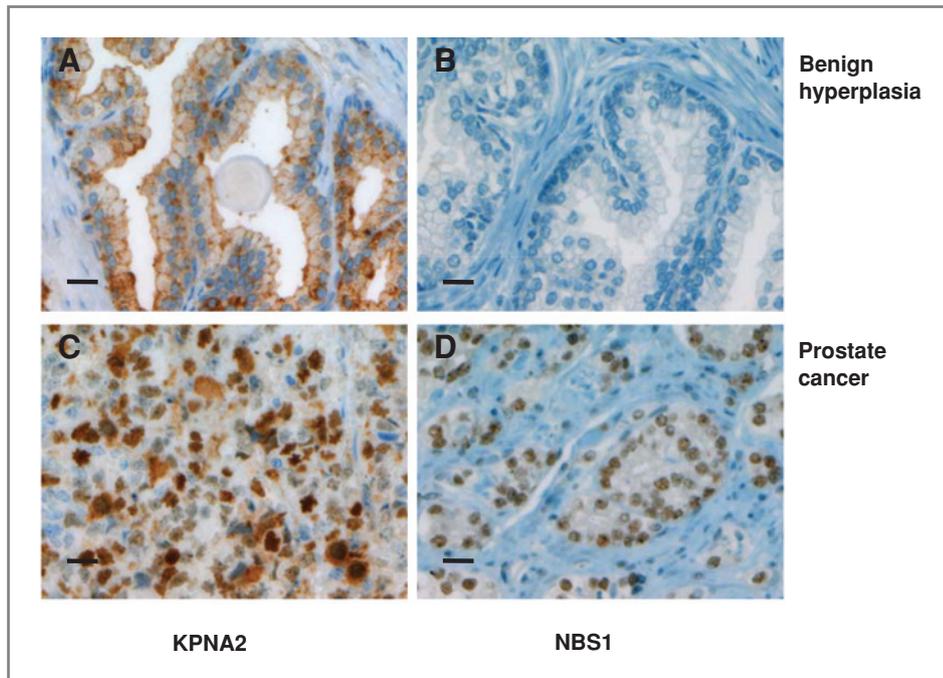


Figure 1. Immunohistochemical expression of KPNA2 (A and C) and NBS1 (B and D) in hyperplastic and malignant prostate tissue. A and B, hyperplastic prostate tissue without nuclear KPNA2 or NBS1 immunoreactivity. C and D, high-grade prostate cancer tissue with strong nuclear KPNA2 and NBS1 expression. Original magnification, $\times 400$; magnification bars, 20 μm .

sided log-rank statistics. Patients were censored at the time of their last tumor-free clinical follow-up visit. A stepwise multivariable Cox regression model was adjusted, testing the independent prognostic relevance of KPNA2 immunoreactivity. The proportionality assumption for all variables was assessed with log-negative log survival distribution functions. SPSS version 17.0 (SPSS) was used for statistical analyses. $P < 0.05$ was considered significant. In case of multiple tests the Bonferroni-Holm procedure was applied.

Statistical considerations regarding sample size

In our training population, KPNA2 expression could be observed in 140 of 341 (41.1%) prostatectomy specimens. We further estimated that the occurrence of KPNA2 expression doubles the risk of PSA recurrence during follow-up, resulting in a HR of 2.0. Supplementary Figure 2 depicts the estimation of statistical power versus total sample size N for different HRs. Accordingly, the available sample size of 341 analyzable patients would be sufficient to detect a difference about PSA recurrence with a significance of $P < 0.05$ and a power of almost 100%. Calculations were done using the respective models of the PASS 2008 software (NCSS).

Results

Zurich TMA (training cohort)

Descriptive and univariate analysis. KPNA2 and NBS1 protein expression in nonmalignant and PCa tissue samples was investigated by immunohistochemical analysis of the Zurich TMA containing 468 specimens from patients with benign or malignant prostatic disease. A total of 455 (97.2%) could be evaluated for KPNA2 and 440 (94.0%)

for NBS1 immunostaining. Representative KPNA2 and NBS1 staining patterns are summarized in Figure 1A–D. In general, KPNA2 and NBS1 expression in at least 0.1% of nuclei was detectable in 193 of 455 (42.4%) and in 391 of 440 (88.9%) of analyzable cases, respectively (Fig. 2A; Supplementary Fig. 3). Nuclear KPNA2 immunoreactivity continuously increased from prostatic hyperplasia to organ-confined PCa to metastatic and castration-resistant disease (Fig. 2A; $P < 0.001$). Regarding NBS1 expression, malignant prostate tissues showed a significantly ($P < 0.001$) higher expression compared with prostatic hyperplasia (Supplementary Fig. 3). For the different types of metastases (lymph node, bone, lung, bladder), differences in expression levels of KPNA2 and NBS1 were not detectable (data not shown). Regarding all tissue samples on the Zurich TMA, positive nuclear KPNA2 immunostaining was significantly associated with positive nuclear NBS1 immunostaining ($P < 0.001$; Fig. 2B). In the subgroup of prostatectomy specimens the same significant association was found ($P = 0.004$; Fig. 2C). Clinicopathologic and immunohistochemical data are summarized in Supplementary Table 1.

Clinicopathologic characteristics of the prostatectomy patients were correlated with KPNA2 and NBS1 expression (Table 1, Zurich TMA). In primary PCas, nuclear KPNA2 and NBS1 expression was not associated with any of the parameters (age at diagnosis, Gleason score, tumor stage, nodal status, surgical margin status, preoperative PSA level).

Univariate Cox regression analysis (Table 2, Zurich TMA) and log-rank statistics revealed that KPNA2 expression was highly associated with shorter RFS ($P = 0.001$; Fig. 2D). The estimated 5-year RFS rate of KPNA2-positive cases was

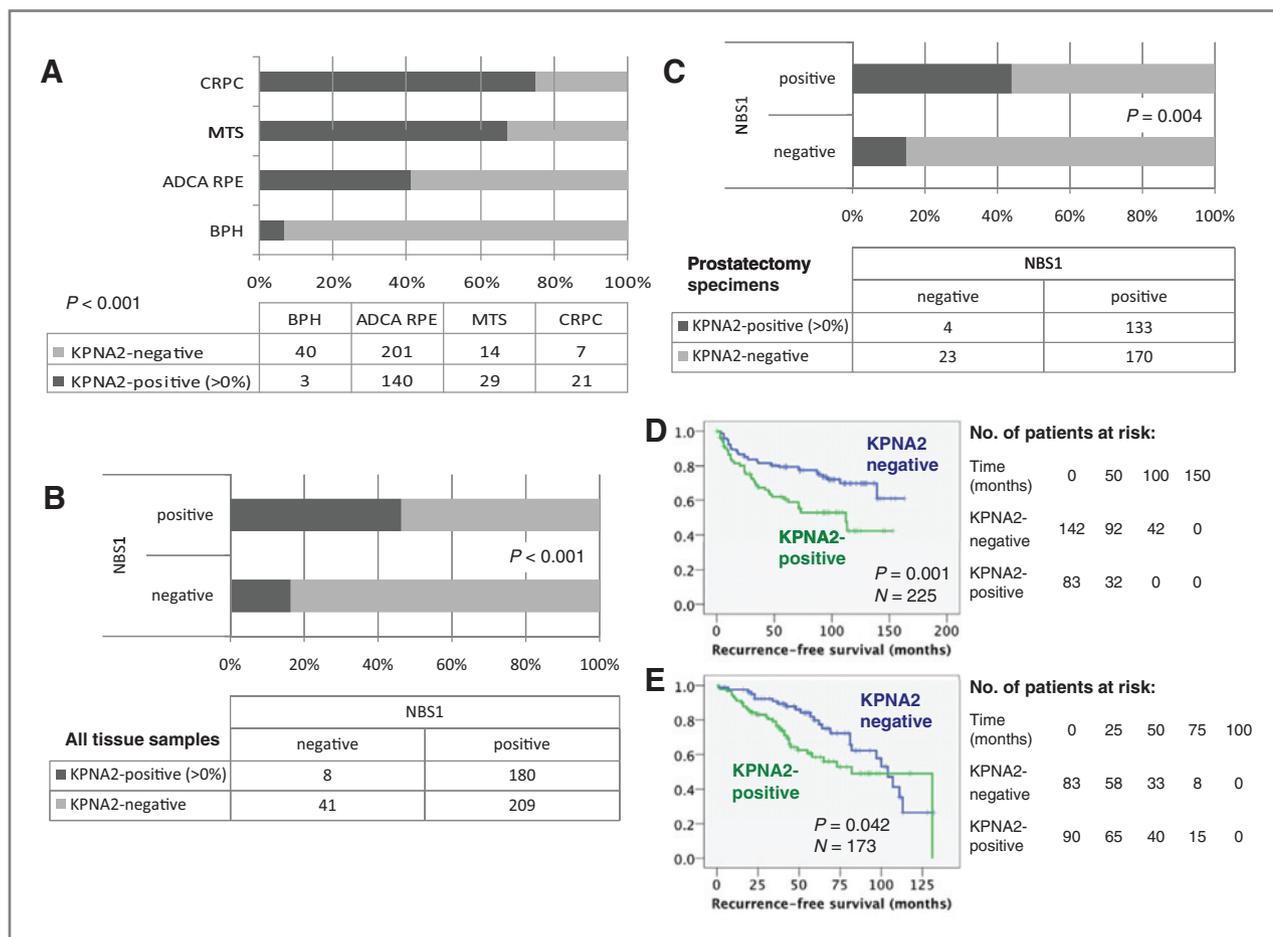


Figure 2. A, cumulative bar chart representing nuclear immunoreactivity for KPNA2 in different tissue types. BPH, benign prostate hyperplasia; ADCA RPE, organ-confined prostate cancer; MTS, prostate cancer metastasis; CRPC, castration-resistant prostate cancer. B and C, cumulative bar chart representing the correlation between nuclear immunoreactivity of NBS1 and KPNA2 in all tissue samples (B) and in the subgroup of prostatectomy specimens (C). D and E, Kaplan–Meier curves regarding RFS of prostate cancer patients with no KPNA2 expression versus patients with positive KPNA2 expression in the training (D) and the testing cohort (E).

0.607 (SE = 0.055), whereas patients with KPNA2-negative tumors showed a RFS rate of 0.794 (SE = 0.034). In a subgroup analysis of patients with moderately differentiated tumors (e.g., Gleason score 7), nuclear KPNA2 staining was associated with shorter RFS times ($P < 0.001$; data not shown). NBS1 immunostaining was not associated with shorter RFS ($P = 0.175$). Besides, higher Gleason scores (>7), increased tumor stage (pT3a–pT4), positive surgical margin status, and increased preoperative PSA levels (≥ 10 ng/mL) were significantly associated with shorter RFS time ($P < 0.01$; Table 2, Zurich TMA).

Multivariate analysis. In a multivariate analysis, a Cox regression model was developed for assessment of the RFS rate. Characteristics of variables are shown in Table 3 (Zurich TMA). Because of model assumptions (noninformative censoring, proportional hazards), only nuclear KPNA2 expression, Gleason score, tumor stage, surgical margin status, and preoperative PSA levels were considered.

All variables, including nuclear KPNA2 expression ($P = 0.002$), remained significant. The HR for KPNA2 expression was 2.129 (95% CI, 1.332–3.403).

Regensburg TMA (testing cohort)

Descriptive and univariate analysis. KPNA2 protein expression was investigated by immunohistochemical analysis of a Regensburg TMA containing 330 prostatectomy specimens from patients with PCa. A total of 237 cores (71.8%) could be evaluated for KPNA2. Applying the same criteria for positive KPNA2 immunoreactivity as established for the training cohort, KPNA2 expression was detected in 124 of 237 (52.3%) analyzable samples (Supplementary Table 1). In primary PCAs, nuclear KPNA2 expression was not associated with any of the parameters (Table 1, Regensburg TMA). In Kaplan–Meier analysis, KPNA2 expression was significantly associated with shorter RFS (Fig. 2E; Table 2, Regensburg TMA). Five-year RFS rates were 0.769 (SE = 0.066) and 0.589 (SE = 0.050) for

Table 1. Clinicopathologic characteristics in relation to KPNA2 and NBS1 immunoreactivity in radical prostatectomy specimens

Variable	Characteristics	Nuclear KPNA2 immunoreactivity			Nuclear NBS1 immunoreactivity		
		Negative	Positive	<i>P</i>	Negative	Positive	<i>P</i>
Zurich TMA							
Age at diagnosis (grouped)	<64 y	83	64	0.367 ^a	14	129	0.428 ^a
	≥64 y	114	70		13	165	
Gleason score (grouped)	5–6	38	18	0.352 ^a	6	45	0.515 ^a
	7	114	85		14	183	
	8–10	48	36		7	75	
Tumor stage (grouped)	pT2a-c	134	83	0.332 ^a	23	186	0.053 ^a
	pT3a-b	59	50		4	104	
	pT4	6	6		0	12	
Nodal status	pN0	154	111	0.799 ^a	23	238	0.616 ^a
	pN1	10	6		0	14	
Surgical margin status	Negative	134	86	0.292 ^a	19	191	0.399 ^a
	Positive	62	52		7	108	
Preoperative PSA levels	<10 ng/mL	81	52	0.906 ^a	10	120	1.000 ^a
	≥10 ng/mL	99	66		13	146	
Regensburg TMA							
Age at diagnosis (grouped)	<64 years	58	70	0.437 ^a			
	≥64 years	55	54				
Gleason score (grouped)	5–6	38	45	0.675 ^b			
	7	23	27				
	8–10	23	36				
Tumor stage (grouped)	pT2a-c	46	44	0.348 ^b			
	pT3a-b	64	79				
	pT4	3	1				
Nodal status	pN0	92	97	0.491 ^a			
	pN1	17	24				
Surgical margin status	Negative	69	70	0.510 ^a			
	Positive	44	54				
Preoperative PSA levels	<10 ng/mL	54	46	0.080 ^a			
	≥10 ng/mL	51	71				

NOTE: Bold face representing $P < 0.05$.^aFisher's exact test.^bPearson's 2-sided χ^2 test.

KPNA2-negative and -positive cases, respectively. Besides, only increased tumor stage ($P = 0.003$) was significantly associated with shorter RFS time.

Multivariate analysis. To further verify the prognostic value of the KPNA2 assay, another Cox regression model was developed (Table 3, Regensburg TMA). Only nuclear KPNA2 expression ($P = 0.005$) remained significant. The HR for KPNA2 expression was 2.653 (95% CI, 1.340–5.253).

MTT and proliferation assays

siRNA technologies were used to inhibit KPNA2 expression, and the effect of this inhibition on cellular function

was determined. PC3 and LNCaP PCa cells were reverse and transiently transfected with siRNAs against KPNA2. To determine whether inhibition of expression had biological relevance, a cell proliferation assay was performed on the presence of controls and KPNA2 siRNAs. PC3 cell proliferation and viability was significantly reduced in cells in which KPNA2 expression was inhibited (Fig. 3A). Results of the MTT assay could be confirmed in a second experiment, using pooled and nonpooled siRNAs (Fig. 3F). In contrast to the androgen-refractory cell line PC3, siRNA-mediated KPNA2 knockdown did not have any significant effect on the viability of the hormone-dependent cell line LNCaP (Fig. 3E). These findings suggest that KPNA2 may be important for the proliferation of androgen-independent PCa

Table 2. Univariate Cox regression analysis

Variable	Characteristics	Recurrence-free survival		
		HR	95% CI	P ^a
Zurich TMA				
Age at diagnosis		1.006	0.964–1.050	0.772
Gleason score (grouped)	5–7 vs 8–10	2.972	1.823–4.844	<0.001
Tumors stage (grouped)	pT2a-c vs pT3a-4	2.336	1.625–3.356	<0.001
Surgical margin status	Negative vs positive	3.268	2.069–5.161	<0.001
Preoperative PSA level	< 10 ng/mL vs ≥ 10 ng/mL	2.317	1.432–3.748	0.001
Nuclear KPNA2 immunoreactivity	0% vs > 0%	2.075	1.314–3.277	0.002
Nuclear NBS1 immunoreactivity	0% vs >0%	2.013	0.732–5.538	0.175
Regensburg TMA				
Age at diagnosis		0.978	0.942–1.016	0.257
Gleason score (grouped)	5–7 vs 8–10	1.400	0.816–2.403	0.222
Tumors stage (grouped)	pT2a-c vs pT3a-4	2.074	1.279–3.362	0.003
Surgical margin status	Negative vs positive	0.114	0.717–1.807	0.582
Preoperative PSA level	<10 ng/mL vs ≥10 ng/mL	1.320	0.818–2.132	0.255
Nuclear KPNA2 immunoreactivity	0% vs >0%	1.703	1.013–2.864	0.044

^aP < 0.05 are marked in bold.

cells. To show the efficiency of our knockdown experiments, PC3 and LNCaP cells were transfected with siRNAs against KPNA2 (pooled and nonpooled) and controls, and whole cell lysates were prepared and processed for immunoblotting with KPNA2 antibody (Fig. 3B). Specificity of the KPNA2 antibody was determined by immunostaining of paraffin-embedded cell pellets (Fig. 3C and D) with KPNA2 antibody. KPNA2 knockdown did not induce apoptosis. In detail, knockdown of KPNA2 did not induce PARP cleavage (Fig. 4A) as marker for apoptosis. As positive control, PC3 cells, treated with staurosporin, showed cleaved PARP

(Fig. 4B). No significantly increased sub-G₁ fraction could be found by FACS analysis (Fig. 4C). However, knockdown of KPNA2 decreased the number of actively dividing cells as compared with control cells (Fig. 4D).

Accordingly, KPNA2 expression may be involved in androgen receptor (AR)-mediated proliferation in PCa. To address this question, expression of the cytoplasmic CXCR4 in KPNA2-negative versus -positive PCa samples was investigated (Fig. 4E and F). Cytoplasmic CXCR4 expression was significantly increased in KPNA2-positive PCa samples.

Table 3. Multivariate Cox regression analysis

Variable	Characteristics	Recurrence-free survival		
		HR	95% CI	P ^a
Zurich TMA				
Gleason score (grouped)	5–7 vs 8–10	2.119	1.250–3.590	0.005
Tumors stage (grouped)	pT2a-c vs pT3a-pT4	2.016	12.38–3.284	0.005
Surgical margin status	Negative vs positive	2.519	1.558–4.071	<0.001
Preoperative PSA level	< 10 ng/mL vs ≥ 10 ng/mL	1.954	1.150–3.321	0.013
Nuclear KPNA2 immunoreactivity	0% vs >0%	2.129	1.332–3.403	0.002
Regensburg TMA				
Gleason score (grouped)	5–7 vs 8–10	0.851	0.425–1.706	0.650
Tumors stage (grouped)	pT2a-c vs pT3a-pT4	1.970	0.926–4.192	0.078
Surgical margin status	Negative vs positive	0.826	0.418–1.633	0.583
Preoperative PSA level	<10 ng/mL vs ≥10 ng/mL	1.674	0.849–3.304	0.137
Nuclear KPNA2 immunoreactivity	0% vs >0%	2.653	1.340–5.253	0.005

^aP < 0.05 are marked in bold.

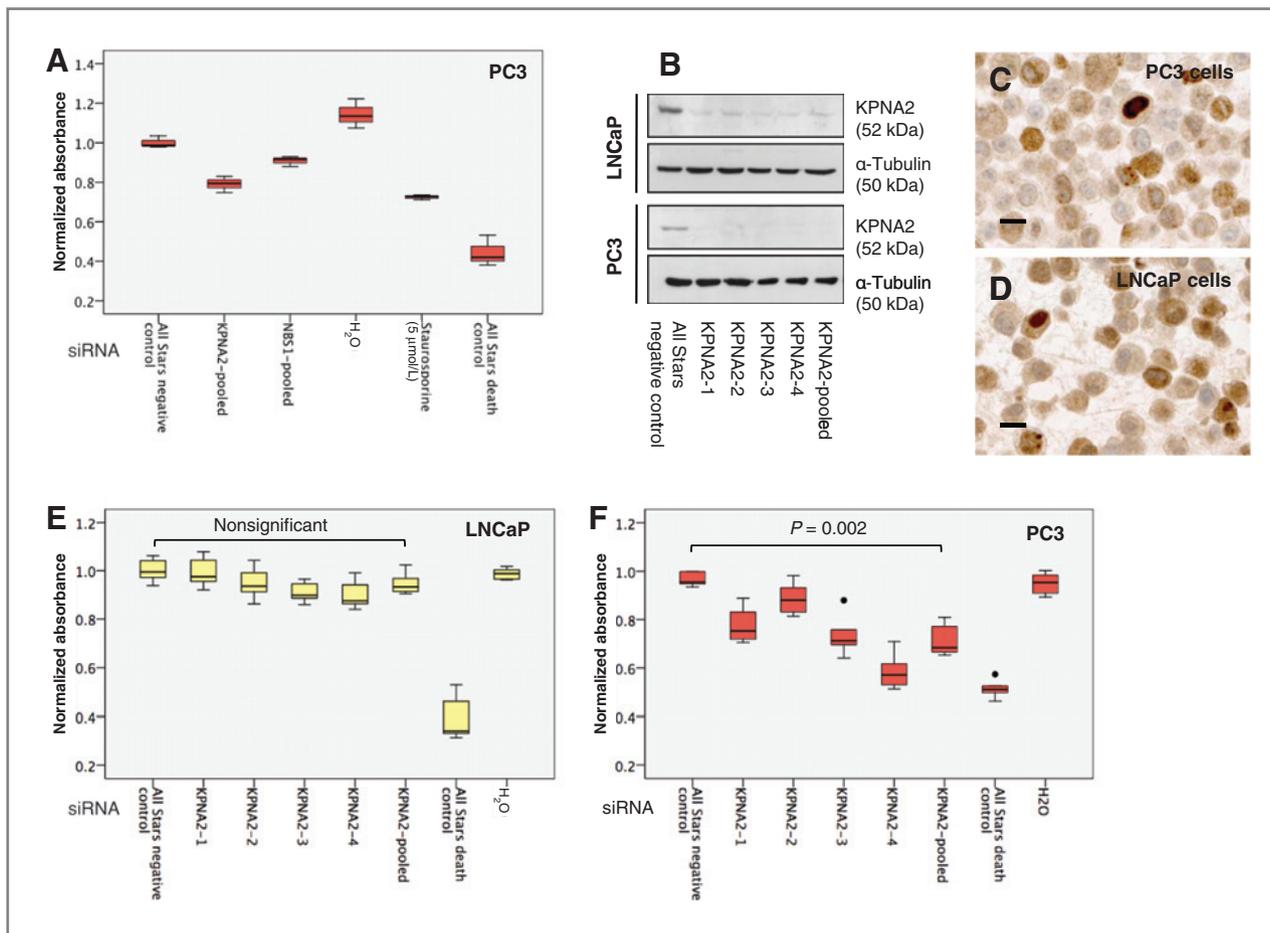


Figure 3. A, boxplots showing results of the MTT assay for PC3 cells. Four specific KPNA2 and NBS1 siRNAs were pooled (final concentration 0.05 μmol/L) and all values were normalized to All Stars negative control siRNA (Qiagen). B, Western blot analysis of PC3 and LNCaP cells after control knockdown (All Stars negative control) or knockdown of KPNA2. Blots were probed with antibodies against KPNA2 and α-tubulin. C and D, immunohistochemical KPNA2 expression in paraffin-embedded cell pellets of PC3 (C) and LNCaP cells (D). Original magnification, ×400; magnification bars, 20 μm. E and F, boxplots showing results of the MTT assay using LNCaP and PC3 cells. Four specific KPNA2 siRNAs were either pooled or used separately at a final concentration of 0.05 μmol/L. All values were normalized to All Stars negative control siRNA (Qiagen).

Discussion

In this study, KPNA2 was significantly upregulated in adenocarcinomas of the prostate, especially in metastatic and castration-resistant PCA. Moreover, positive nuclear KPNA2 immunoreactivity was identified as a novel predictor of biochemical recurrence after radical prostatectomy, which was independent of the well-established predictive factors preoperative PSA value, Gleason score, tumor stage, and surgical margin status. These results were confirmed in a second independent testing cohort. The classical markers high Gleason score, high tumor stage, positive surgical margins, and increased preoperative PSA levels were relevant for the recurrence probability as well.

KPNA2 is a member of the karyopherin (importin) family, which plays a central role in nucleocytoplasmic transport. It acts as an adaptor in the nuclear import of macromolecules by binding cargoproteins, which contain a

classical nuclear localization signal (18). Linking a cargo-protein and Karyopherin beta, KPNA2 initiates the transport through the nuclear core complex (19). In particular, KPNA2 is a major adaptor for the nuclear localization of NBS1, a key regulator of the MRE11/RAD50/NBS1 complex (20). This complex plays an important role in the early processing of double-strand breaks (DSB; 21). NBS1 has two important roles based on its subcellular localization, with KPNA2 being the major effector to determine the subcellular localization of NBS1. In the nucleus, NBS1 acts as tumor suppressor involved in DNA DSB repair, maintaining genome stability. In the cytoplasm, however, NBS1 provides an oncogenic role, which promotes tumorigenesis through binding and activation of the phosphatidylinositol 3-kinase/AKT pathway (22).

Another recently described KPNA2-mediated transport mechanism is the nuclear import of the AR. During the development of PCA, cell survival depends primarily on the

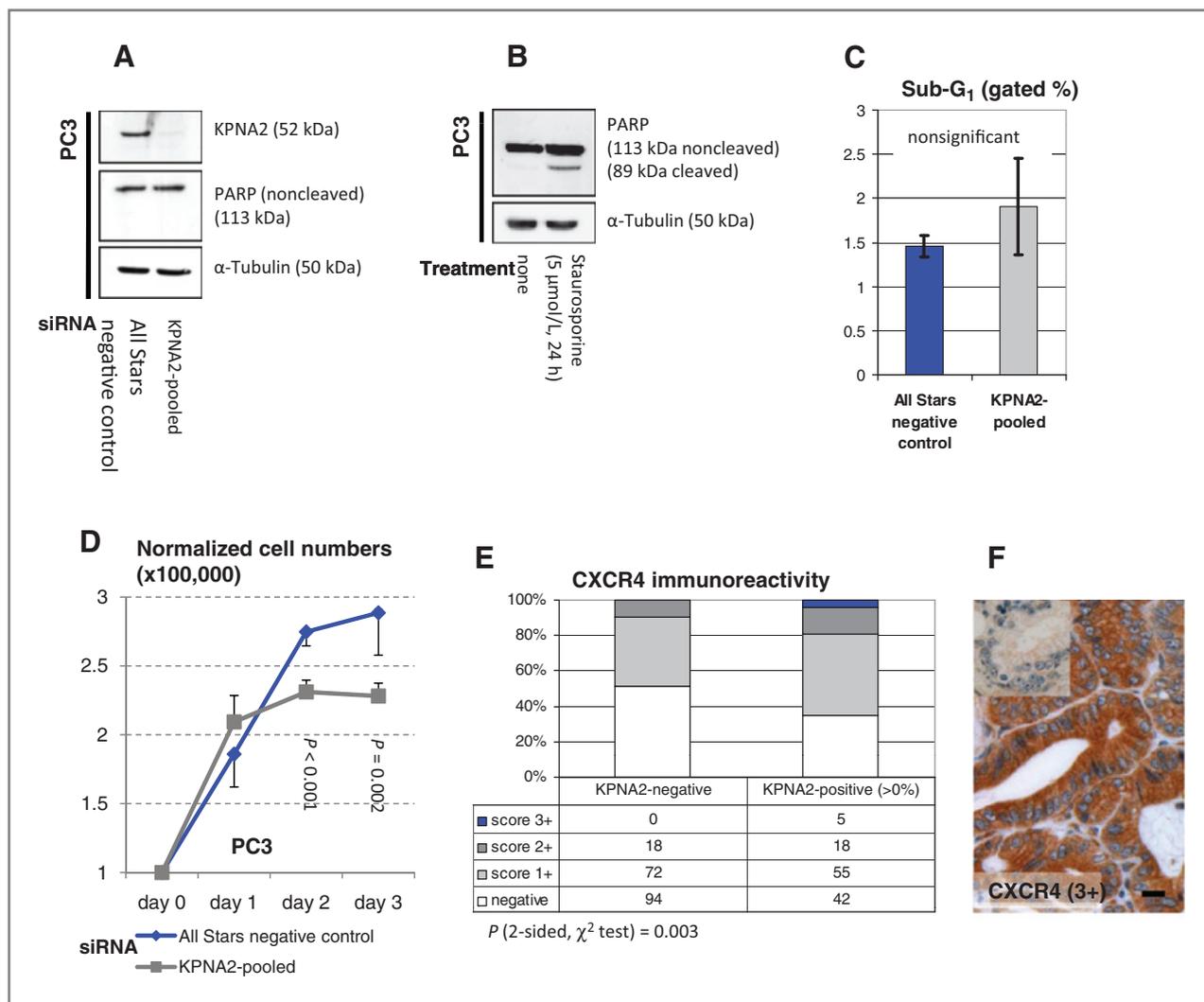


Figure 4. A, Western blot analysis showing KPNA2, PARP, and α -tubulin levels after KPNA2 knockdown. Knockdown of KPNA2 did not result in PARP cleavage. B, control experiment showing cleaved PARP after staurosporine treatment of PC3 cells. C, FACS assay showing the sub-G₁ fraction in PC3 cells with KPNA2 knockdown compared with control cells. PC3 cells were transfected with control siRNA (blue) or pooled siRNAs against KPNA2 (gray) and analyzed for sub-G₁ DNA content, 72 hours after transfection by fluorescence-activated cell sorting. Data are represented as mean \pm SD; $n = 2$. D, PC3 cells were either transfected with control siRNA (blue) or pooled siRNAs against KPNA2 (gray), and cell numbers were determined over the indicated time period. An average of 3 independent experiments is shown. Data are represented as normalized mean cell numbers (\pm SD). E, cumulative bar charts representing the correlation between CXCR4 and KPNA2 immunoreactivity in the subgroup of prostatectomy specimens, respectively. F, prostate cancer tissue of a radical prostatectomy specimen with strong immunohistochemical CXCR4 expression (cytoplasmic intensity score 3+). Original magnification, $\times 200$; magnification bars, 20 μ m; inset, negative CXCR4 immunoreactivity.

AR (23). Binding of the active androgen dihydrotestosterone to the AR leads to its KPNA2 dependent translocation into the nucleus, where it activates the transcription of a wide range of target genes (24). Considering this, KPNA2 overexpression might be an indicator for increased translocation of the AR and be therefore associated with highly proliferative and hormone-refractory PCa. Accordingly, in this study nuclear KPNA2 immunoreactivity continuously increased from prostatic hyperplasia to organ-confined PCa to metastatic and castration-resistant disease (Fig. 2A).

Furthermore, expression of the cytoplasmic CXCR4 in KPNA2-negative versus -positive PCa samples was investi-

gated (Fig. 4E and F). Carver and colleagues have recently shown that CXCR4, a candidate gene strongly associated with cell migration, was upregulated in the presence of ERG overexpression. Thus, androgens may induce CXCR4 through ERG factor expression in TMPRSS2-ERG fusion-positive PCa cells (25). In our study, CXCR4 expression was significantly increased in KPNA2-positive PCa, supporting our hypothesis that KPNA2 expression may be involved in AR-mediated proliferation in PCa.

Previous investigations could prove KPNA2 overexpression in other solid tumors such as melanoma and breast cancer and also demonstrated an association with shorter

survival rates (11, 13, 26). Recently, KPNA2 expression has been confirmed in a randomized patient cohort of high-risk breast cancer patients. KPNA2 expression was an independent negative prognostic factor for event-free and overall survival (12). Considering this last clinical trial, the data confirmed that KPNA2 positivity defines an aggressive breast cancer phenotype.

The preliminary functional data of this study shows a reduced cell viability of PC3 PCa cells on KPNA2 knockdown, suggesting that KPNA2 is essential for PCa cellular function. KPNA2 knockdown did not induce apoptosis but reduced cell proliferation and viability of androgen-independent PC3 cells. This clearly merits further study. Van der Watt and colleagues (27) examined the expression of karyopherins, exportin 1 (XPO1), Karyopherin beta 1 (KPNB1), and KPNA2 in cervical tissue and cell lines. Individual siRNAs were used to investigate the functional significance of these proteins. They also found KPNA2 overexpressed in cervical cancer compared with normal tissue, which is concordant to our PCa data. Also, inhibition of XPO1 and KPNB1 expression induced cancer cell death, whereas siRNA-mediated KPNA2 knockdown had no significant effect (27). The partially different cellular responses to the inhibition of KPNA2 in prostate and cervical cancer cell lines could be due to tissue-specific tumor etiologies and also warrant further study.

Molecular tissue biomarkers play an increasingly important role in clinical oncology. They could help to detect the disease at an early stage, guide treatment decisions, and help to identify subpopulations of patients who are most likely to benefit from a specific therapy (28). However, prognostic evaluation of a specific cancer patient by analysis of cancer tissue for protein expression remains chal-

lenging. Up to now, none of the published tissue biomarkers for PCa has found its way into clinical practice. The description of KPNA2 as an independent marker of PSA relapse in two independent cohorts of PCa clearly constitutes KPNA2 expression as strong biomarker. Further studies have to clarify whether KPNA2 immunohistochemistry can also aid in the discrimination of insignificant and significant PCa at the time of the positive biopsy, which we speculate is likely.

In summary, we found KPNA2 overexpressed during malignant tumor progression of PCa. Moreover, KPNA2 expression was associated with shortened RFS time in univariate and multivariate analyses in two independent patient cohorts treated by radical prostatectomy. This raises the possibility for a better individualized risk stratification to optimize therapy. The clinical application of these findings has to be investigated in further studies using prostate biopsy material.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

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KPNA2 Expression Is an Independent Adverse Predictor of Biochemical Recurrence after Radical Prostatectomy

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