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KPNA2 expression is an independent adverse predictor of biochemical recurrence after radical prostatectomy

Mortezavi, A ; Hermanns, T ; Seifert, H H ; Baumgartner, M ; Provenzano, M ; Sulser, T ; Burger, M ; Montani, M ; Ikenberg, K ; Hofstaedter, F ; Hartmann, A ; Jaggi, R ; Moch, H ; Kristiansen, G ; Wild, P J

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 containing 798 formalin-fixed, paraffin-embedded prostate tissue cores from two different institutes of pathology. TMAs were stained immunhistochemically 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. Efficency of knock down 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 poly(ADP-ribose) polymerase (PARP) cleavage as marker for apoptosis. No significantly increased subG1 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.

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Clinical Cancer Research - Imaging & Diagnosis

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

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Running title: KPNA2 expression in prostate cancer

Key words: KPNA2, NBS1, prostate cancer, biomarker

Conflict of interest: The authors state that no conflict of interest exists.

Abbreviations used: KPNA2, karyopherin alpha 2; RFS, recurrence-free survival; TMA, tissue microarray; PCa, prostate cancer.

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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 demonstrated 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, if KPNA2 immunohistochemistry can also aid in the discrimination of insignificant and significant prostate cancer at the time of the positive biopsy.

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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 containing 798 formalin-fixed, paraffin-embedded prostate tissue cores from two different institutes of pathology. TMAs were stained immunhistochemically 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. Efficency of knock down 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 poly(ADP-ribose) polymerase (PARP) cleavage as marker for apoptosis. No significantly increased subG1 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.

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INTRODUCTION

Accurate prediction of individual prostate cancer (PCa) behaviour 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 ten 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 in order 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 classical 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 immunhistochemistry showed that nuclear KPNA2 overexpression was significantly associated with shorter overall survival and recurrence-free survival. Nuclear KPNA2 expression was a highly significant, independent and adverse risk factor for overall survival in several independent study cohorts (11-13) .

Since 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

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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 & METHODS

Patients and specimen characteristics. Tissue microarrays (TMAs) 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 (non-selected) 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, H&E-stained slides of all specimens were re-evaluated 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 (UICC) and WHO/ISUP criteria (15). The Regensburg TMA contained a series of 330 consecutive (non-selected) 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

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months (0-132) for the cohort from Regensburg (Supplemental Table 1). The study for both cohorts was approved by the local scientific ethics committees (approval no.: StV-Nr. 25/2007).

Immunohistochemical assay. Consecutive 3µm 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., Santa Cruz, CA, USA; 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, Camarillo, CA; no. MHCXCR404, dilution 1:200). The ChemMate detection kit (DAKO, Glostrup, Denmark) was used. After antigen retrieval (microwave oven for 10 min at 250 W) immunohistochemistry was carried out in a NEXES immunostainer (Ventana, Tucson, AZ, USA) according to 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 immunhistochemically for KPNA2 protein. Additionally, cell lines that were used for our in vitro experiments were characterised by analyzing the phosphatidylinositol-3-kinase (PI3)/AKT cascade (Supplemental Figures 1A-1F).

Two surgical pathologist (P.J.W., G.K.) performed a blinded evaluation of the slides for KPNA2 and NBS1 without knowledge of clinical data. Causes of non-interpretable 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 continous readout. The median nuclear KPNA2 immunoreactivity in prostatectomy cases (median 0%) was chosen as cutoff.

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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 four-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 Medium (Invitrogen Corp., Carlsbad, CA, USA) with L-glutamine (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/mlL streptomycin. All cells were grown in a humidified atmosphere incubator with 5% CO_2 at 37 °C. Cells were cultured for two weeks prior to their use in subsequent experiments.

Reverse transfection of siRNAs. Knock down experiments were performed 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 µM; accession no. SI02781051, SI02780631, SI00035539, SI00035525, SI02757524, SI02663570, SI00038479, SI00038493 Qiagen, Hilden, Germany). 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 prior to transfection and combined with diluted Lipofectamine 2000 to form complexes in each well. Cells were washed twice with 5 mL phosphate buffered saline, trypsinized and added directly to the Lipofectamine 2000-RNA

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complexes. Cells were seeded in Nunclon MicroWell plates (Nunc) at a density of 6,000 cells per well (in DMEM with 10% fetal calf serum and antibiotic-free). Transfection occurred while cells were attaching to the well bottom. All Stars negative control siRNA (Qiagen, Hilden, Germany; accession no. SI03650318), H_2O, and All Stars Hs Cell Death siRNA (Qiagen, Hilden, Germany; accession no. SI04381048) were used as negative and positive controls, respectively. As an additional positive control, cells were treated with 5 µM staurosporine (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland; no. S6942) to induce apoptosis twenty-four hours after reverse transfection.

MTT assay. Viability of PC3 and LnCap cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-terazolium bromide (MTT) assay (16). After cells were incubated for 48 h, 100 µL MTT solution (0.1 mg/mL in DMEM with 10% fetal calf serum, antibiotic-free; Sigma-Aldrich, St. Louis, MO, USA) was added to each well and the plates were further incubated for 4 h. The crystal was dissolved in 100 µL lysis buffer (16 µL glycine buffer (0.1M gylcine, 0.1M NaCl, pH 10.5) and 84 µL DMSO) for 10 min. The absorbance was measured at a wavelength of 570 nm using a microplate reader (SpectraMax 190, Molecular Devices, MDS, Sunnyvale, CA, USA). For nomalization, absolute absorbance was divided by the absorbance of wells with All Stars negative control siRNA (Qiagen, Hilden, Germany).

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 (25mM Tris–HCl pH 7.2, 500mM sodium chloride, 10% glycerol, 1% NP-40, 10mM sodium fluoride, 1mM Na3VO4, 1mM PMSF,

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1mM dithiothreitol, 10 mg/ml aprotinin). Protein extracts (50 mg) were run on 8–15% polyacrylamide gels, transferred to nitrocellulose membranes and visualised by immunoblotting with the following antibodies: anti-KPNA2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, no. 6917), anti-tubulin-alpha (anti-rat antibody; kindly provided by Wilhelm Krek, Institute of Cell Biology, ETH Zurich, Switzerland), and anti-PARP (BD Biosciences, Allschwil, Switzerland, 556362). As positive control for the induction of apoptosis, Staurosporine solution (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland, no. S6942, final concentration 5 µm) was added to the cells 24 hours prior to lysation.

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 subG1 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 FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Statistical analyses. To study statistical associations between clinicopathological and immunohistochemical data, contingency table analysis and two-sided Fisher's exact tests were used. For the comparison of two independent samples the non-parametric Mann-Whitney test was calculated. Time to PSA recurrence (cutoff $\geq$ 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

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ng/ml) postoperatively were used for survival analysis. Univariate Cox regression analysis was used to evaluate statistical association between clinicopathological/immunohistochemical data and recurrence-free survival (RFS). RFS curves were calculated using the Kaplan-Meier method with significance evaluated by two-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, Chicago, IL, USA) was used for statistical analyses. P-values <0.05 were 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 hazard ratio of 2.0. Supplemental Figure 2 depicts the estimation of statistical power *versus* total sample size N for different hazard ratios. Accordingly, the available sample size of 341 analyzable patients would be sufficient to detect a difference concerning PSA recurrence with a significance of $p<0.05$ and a power of almost 100%. Calculations were performed using the respective models of the PASS 2008 software (NCSS, Kaysville, UT).

RESULTS

Zurich TMA (training cohort).

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Descriptive and univariate analysis. KPNA2 and NBS1 protein expression in non-malignant 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 Figures 1A-1D. 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 (Figure 2A and Supplemental Figure 3). Nuclear KPNA2 immunoreactivity continuously increased from prostatic hyperplasia to organ-confined PCa to metastatic and castration resistant disease (Figure 2A; $p<0.001$). Regarding NBS1 expression, malignant prostate tissues showed a significantly ($p<0.001$) higher expression compared to prostatic hyperplasia (Supplemental Figure 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 positiv nuclear NBS1 immunostaining ($p<0.001$; Figure 2B). In the subgroup of prostatectomy specimens the same significant association was found ($p=0.004$, Figure 2C). Clinicopathological and immunohistochemical data are summarized in Supplemental Table 1.

Clinicopathological characteristics of the prostatectomy patients were correlated with KPNA2 and NBS1 expression (Table 1A). 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 2A) and log-rank statistics revealed, that KPNA2 expression was highly associated with shorter RFS ($p=0.001$, Figure 2D). The estimated five year

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recurrence-free survival rate of KPNA2 positive cases was 0.607 (standard error 0.055), whereas patients with KPNA2 negative tumors showed a recurrence-free survival rate of 0.794 (standard error 0.034). In a subgroup analysis of patients with moderately differentiated tumors (e.g. Gleason 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 (≥10ng/ml) were significantly associated with shorter RFS time ($p<0.01$, Table 2A).

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 3A. 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 hazard ratio for KPNA2 expression was 2.129 (95% confidence interval 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 (Supplemental Table 1). In primary PCas, nuclear KPNA2 expression was not associated with any of the parameters (Table 1B). In Kaplan-Meier analysis, KPNA2 expression was significantly associated with shorter RFS

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(Figure 2E, Table 2B). Five year recurrence-free survival rate were 0.769 (standard error 0.066) and 0.589 (standard error 0.050) for 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 3B). Only nuclear KPNA2 expression (p=0.005) remained significant. The hazard ratio for KPNA2 expression was 2.653 (95% confidence interval 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 where KPNA2 expression was inhibited (Figure 3A). Results of the MTT assay could be confirmed in a second experiment, using pooled and non-pooled siRNAs (Figure 3F). In contrast to the androgen-refractory cell line PC3, siRNA mediated KPNA2 knockdown did not have any significant effect on the viability the hormone-dependent cell line LNCaP (Figure 3E). These findings suggest that KPNA2 may be important for the proliferation of androgen-independent PCa cells. In order to show the efficency of our knock down experiments, PC3 and LNCaP cells were transfected with siRNAs against KPNA2 (pooled and non-pooled) and controls, and whole cell lysats were prepared and processed for immunoblotting with KPNA2 antibody (Figure 3B). Specificity of the KPNA2 antibody was determined by immunostaining of paraffin embedded cell pellets (Figure 3C and 3D) with KPNA2 antibody. KPNA2 knockdown did not induce apoptosis. In

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detail, knockdown of KPNA2 did not induce poly(ADP-ribose) polymerase (PARP) cleavage (Figure 4A) as marker for apoptosis. As positive control, PC3 cells, treated with staurosporin, showed cleaved PARP (Figure 4B). No significantly increased subG1 fraction could be found by FACS analysis (Figure 4C). However, knockdown of KPNA2 decreased the number of actively dividing cells as compared to control cells (Figure 4D).

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

DISCUSSION

In the present 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.

Karyopherin alpha 2 (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

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macromolecules by binding cargoproteins, which contain a classical nuclear localization signal (18). Linking a cargoprotein 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 (Nijmegen breakage syndrome 1), a key regulator of the MRE11/RAD50/NBS1 (MRN) 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 androgen receptor (AR). During the development of PCa, cell survival depends primarily on the 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 (Figure 2A).

Furthermore, expression of the cytoplasmic chemokine (C-X-C motif) receptor 4 (CXCR4) in KPNA2 negative vs. positive PCa samples was investigated (Figures 4E-4F). Carver et al. 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

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expression was significantly increased in KPNA2 positive PCa, supporting our hypothesis that KPNA2 expression may be involved in androgen receptor-mediated proliferation in PCa.

Previous investigations could prove KPNA2 overexpression in other solid tumors like 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 the present study shows a reduced cell viability of PC3 PCa cells upon KPNA2 knock down, 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 et al. (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 to normal tissue, which is concordant to our PCa data. Also, inhibition of XPO1 and KPNB1 expression induced cancer cell death, whereas siRNA-mediated KPNA2 knock down 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

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remains challenging. 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, if 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 recurrence-free survival 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.

FIGURE LEGENDS

Figures 1A – 1D. Immunohistochemical expression of KPNA2 (**A & C**) and NBS1 (**B & D**) in hyperplastic and malignant prostate tissue. ***A, B:*** Hyperplastic prostate tissue without nuclear KPNA2 or NBS1 immunoreactivity. ***C, D:*** High grade prostate cancer tissue with strong nuclear KPNA2 and NBS1 expression. Original magnification: 400×; Magnification bars: 20 µM.

Figures 2A-E. ***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-C:***

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Cumulative bar chart representing the correlation between nuclear immunorectivity of NBS1 and KPNA2 in all tissue samples (B) and in the subgroup of prostatectomy specimens (C). ***D-E:*** Kaplan–Meyer curves regarding recurrence-free survival of prostate cancer patients with no KPNA2 expression *versus* patients with positive KPNA2 expression in the training (D) and the testing cohort (E).

Figures 3A-3F. ***A:*** Boxplots showing results of the MTT assay for PC3 cells. Four specific KPNA2 and NBS1 siRNAs were pooled (final concentration 0.05 μM) and all values were normalized to AllStars 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 alpha-tubulin. ***C & D:*** Immunohistochemical KPNA2 expression in Paraffin embedded cell pellets of PC3 (C) and LNCaP cells (D). Original magnification: 400×; Magnification bars: 20 μM. ***E & 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 μM. All values were normalized to AllStars negative control siRNA (Qiagen).

Figures 4A-4F. ***A:*** Western blot analysis showing KPNA2, poly(ADP-ribose) polymerase (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 subG1 fraction in PC3 cells with KPNA2 knock-down compared with control cells. PC3 cells were transfected with control siRNA (blue) or pooled siRNAs against KPNA2 (grey) and analyzed for subG1 DNA content, 72 hours after transfection by fluorescence-activated cell sorting. Data are represented as mean % ± standard deviation, n=2. ***D:*** PC3 cells were either transfected with control siRNA (blue) or pooled siRNAs against KPNA2 (grey), and cell

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numbers were determined over the indicated time period. An average of three independent experiments is shown. Data are represented as normalized mean cell numbers (± standard deviation). ***E:*** Cumulative bar charts representing the correlation between CXCR4 and KPNA2 immunorectivity 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: 200×; magnification bars: 20 µM; inset: negative CXCR4 immunoreactivity.

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

A: Zurich tissue-microarray

Variable	Characteristics	Nuclear KPNA2 immunreactivity			NBS1 nuclear immunoreactivity		
		negative	positive	p*	negative	positive	p*
Age at diagnosis (grouped)	< 64 years	83	64	0.367	14	129	0.428
	≥ 64 years	114	70		13	165	
Gleason score (grouped)	5-6	38	18	0.352	6	45	0.515
	7	114	85		14	183	
	8-10	48	36		7	75	
Tumor stage (grouped)	pT2a-c	134	83	0.332	23	186	0.053
	pT3a-b	59	50		4	104	
	pT4	6	6		0	12	
Nodal status	pN0	154	111	0.799	23	238	0.616
	pN1	10	6		0	14	
Surgical margin status	negative	134	86	0.292	19	191	0.399
	positive	62	52		7	108	
Preoperative PSA levels	< 10 ng/mL	81	52	0.906	10	120	1.000
	≥ 10 ng/mL	99	66		13	146	

B: Regensburg tissue-microarray

Variable	Characteristics	Nuclear KPNA2 immunreactivity		
		negative	positive	p
Age at diagnosis (grouped)	< 64 years	58	70	0.437*
	≥ 64 years	55	54	
Gleason score (grouped)	5-6	38	45	0.675**
	7	23	27	
	8-10	23	36	
Tumor stage (grouped)	pT2a-c	46	44	0.348**
	pT3a-b	64	79	
	pT4	3	1	
Nodal status	pN0	92	97	0.491*
	pN1	17	24	
Surgical margin status	negative	69	70	0.510*
	positive	44	54	
Preoperative PSA levels	< 10 ng/mL	54	46	0.080*
	≥ 10 ng/mL	51	71	

* Fisher's exact test or ** Pearson Chi-Square test, two-sided; bold face representing $p<0.05$.

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Table 2. Univariate Cox regression analysis

A: Zurich tissue-microarray

Variable	Characteristics	Recurrence-free survival		
		HR	95% CI	P *
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

B: Regensburg tissue-microarray

Variable	Characteristics	Recurrence-free survival		
		HR	95% CI	P *
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**

[*] P-values <0.05 are marked in bold.

vs , versus.

CI, confidence interval.

HR, hazard ratio.

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Table 3. Multivariate Cox regression analysis

A: Zurich tissue microarray

Variable	Characteristics	Recurrence-free survival		
		HR	95% CI	P [*]
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**

B: Regensburg tissue microarray

Variable	Characteristics	Recurrence-free survival		
		HR	95% CI	P [*]
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**

[*] P-values <0.05 are marked in bold.
vs, versus.
CI, confidence interval.
HR, hazard ratio.

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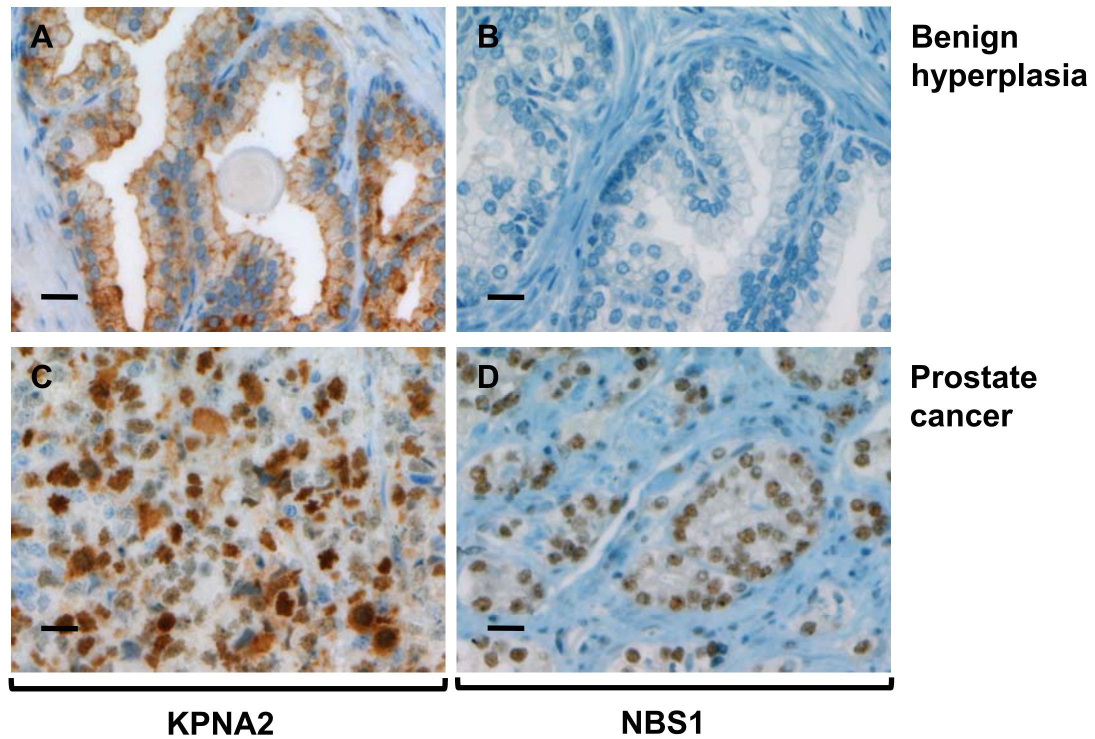

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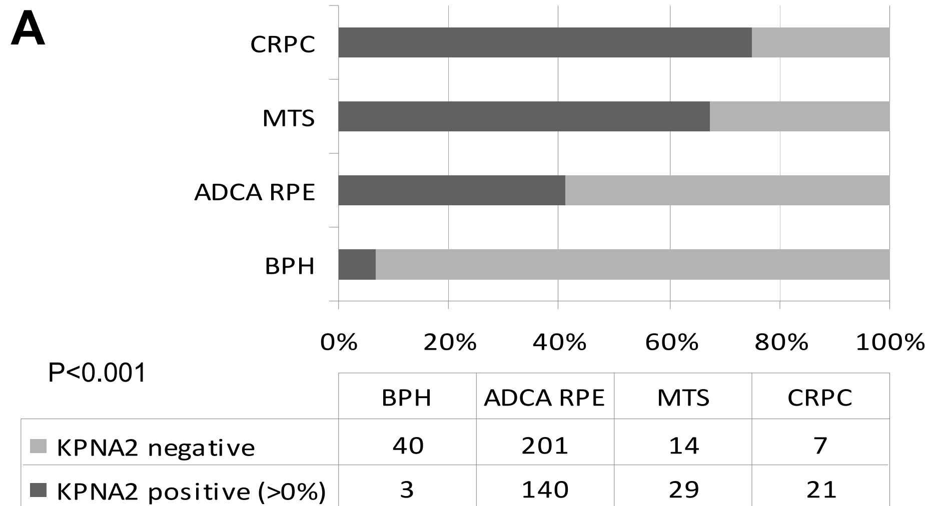
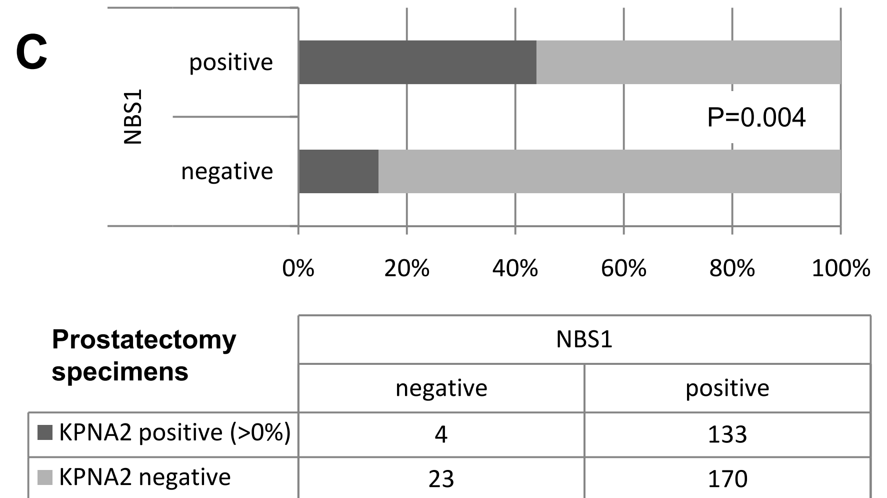
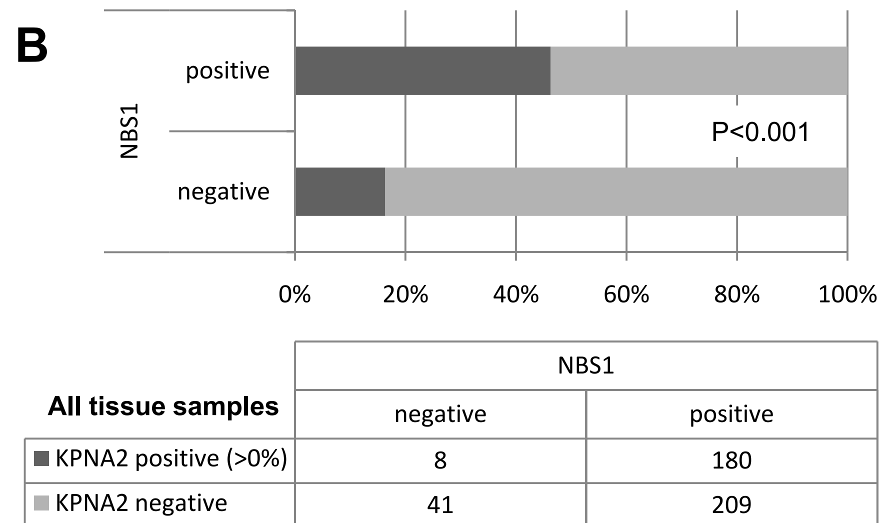


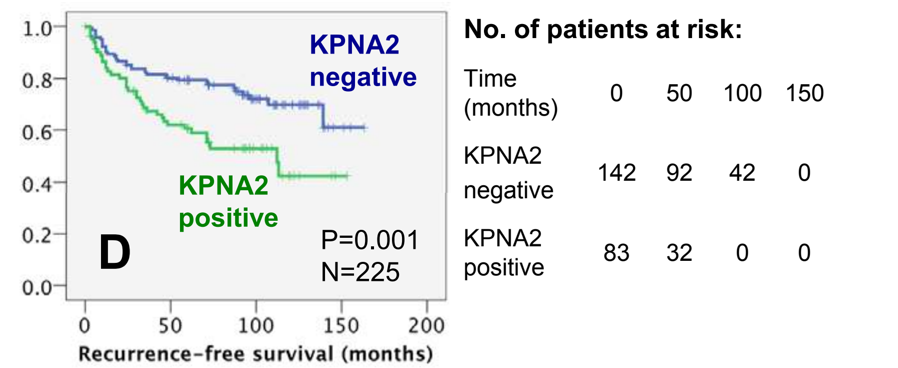
	BPH	ADCA RPE	MTS	CRPC
KPNA2 negative	40	201	14	7
KPNA2 positive (>0%)	3	140	29	21



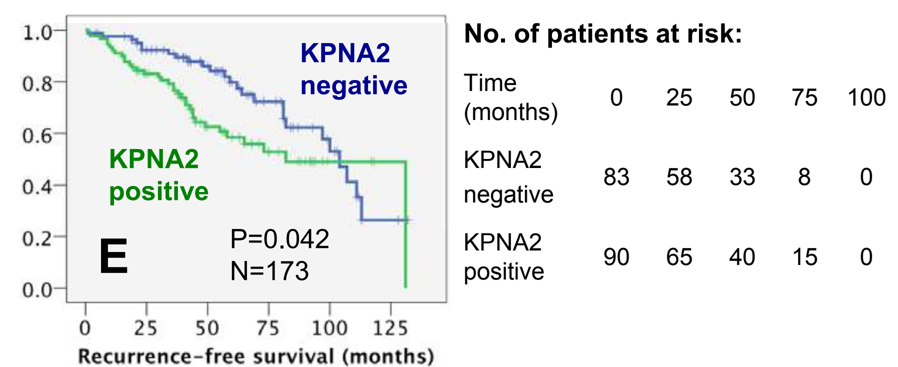
Prostatectomy specimens	NBS1	
	negative	positive
KPNA2 positive (>0%)	4	133
KPNA2 negative	23	170



All tissue samples	NBS1	
	negative	positive
KPNA2 positive (>0%)	8	180
KPNA2 negative	41	209



Time (months)	0	50	100	150
KPNA2 negative	142	92	42	0
KPNA2 positive	83	32	0	0



Time (months)	0	25	50	75	100
KPNA2 negative	83	58	33	8	0
KPNA2 positive	90	65	40	15	0

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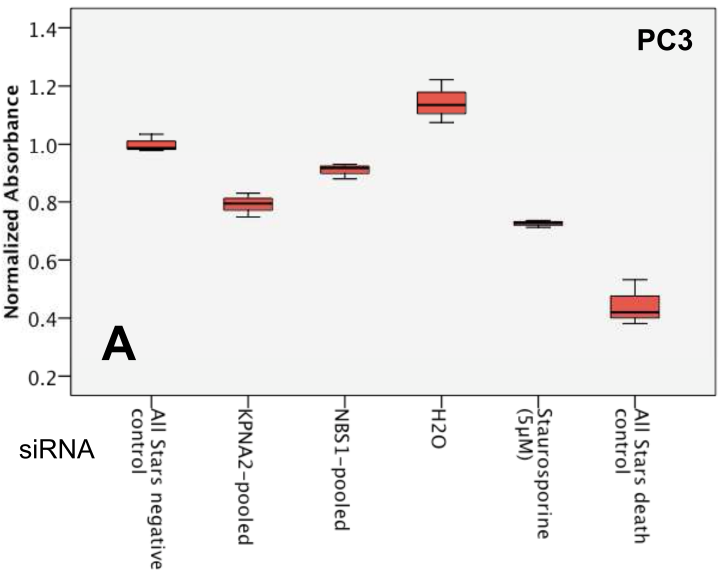

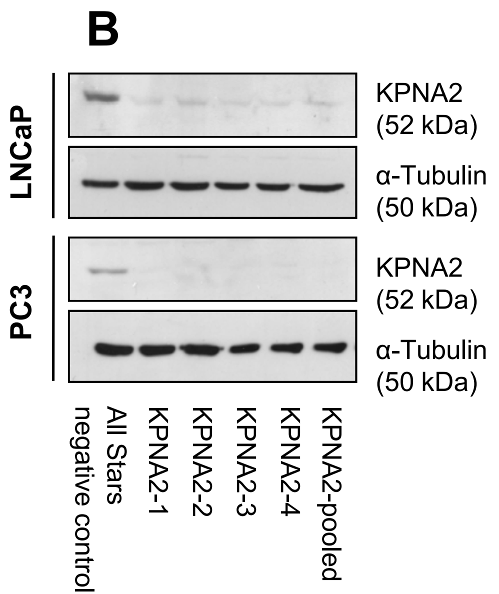

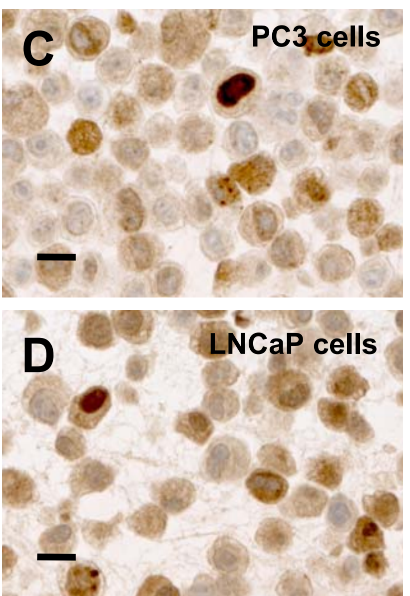

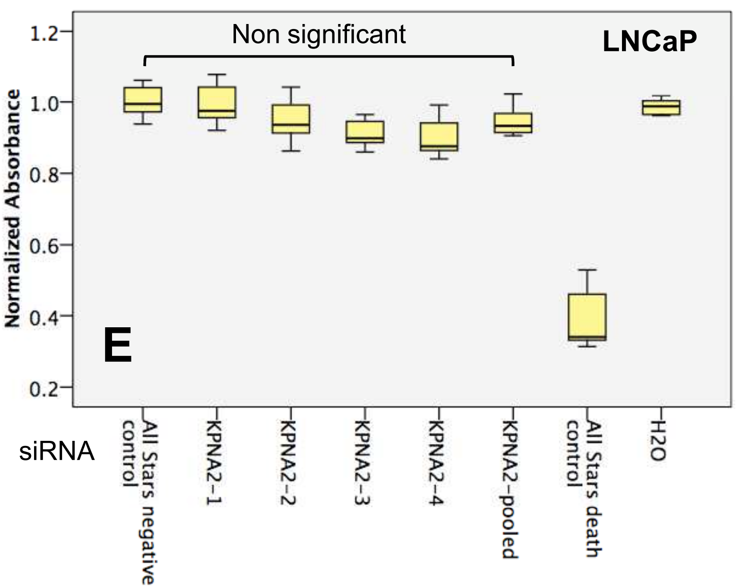

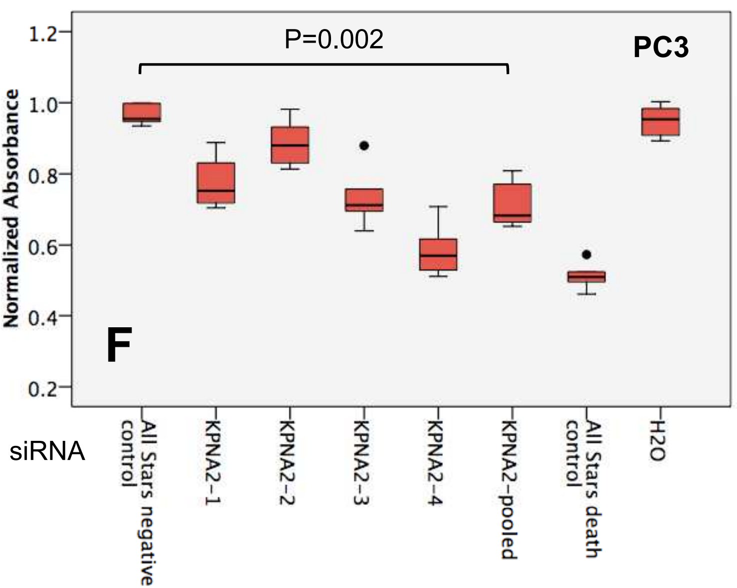

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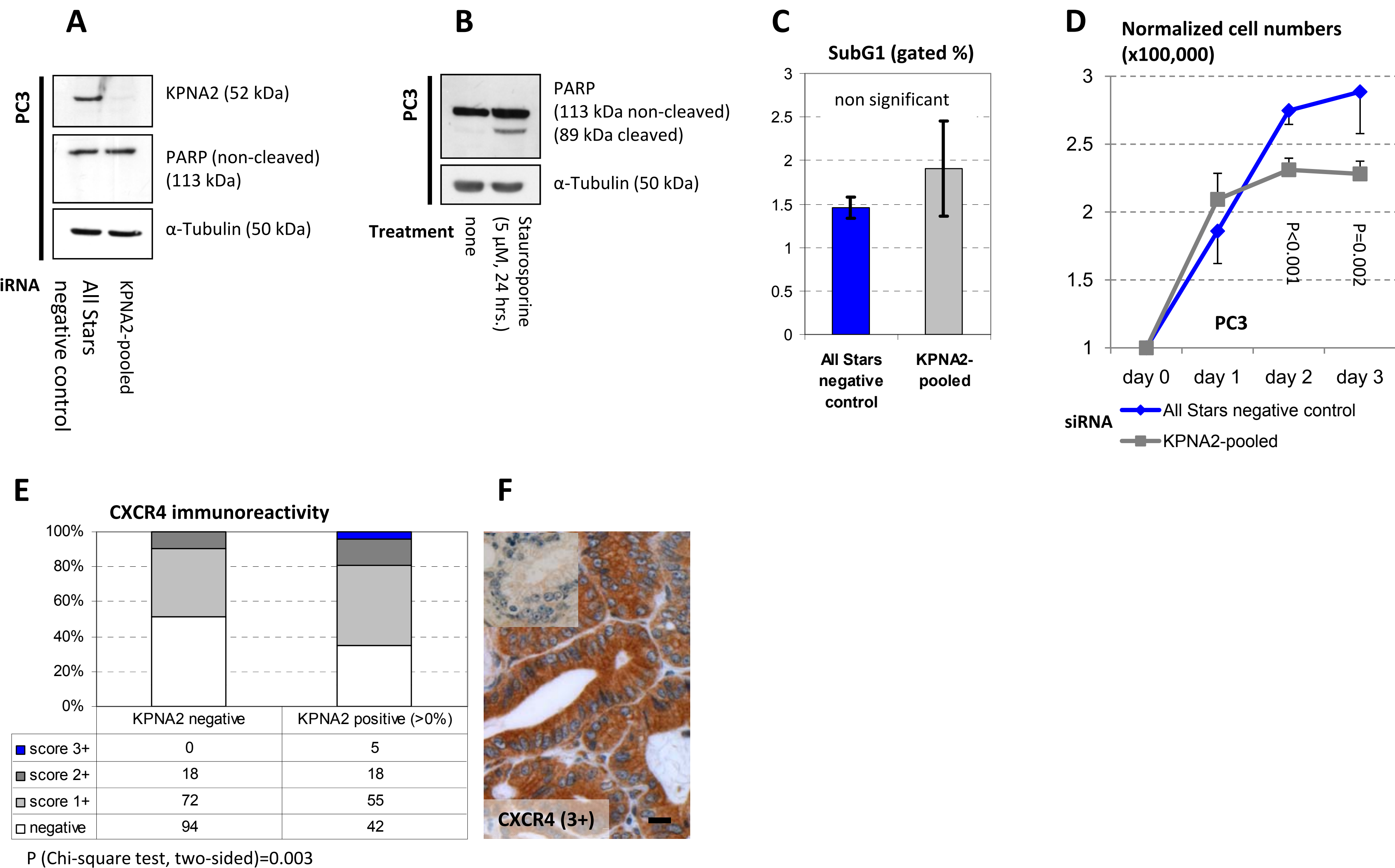

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