Prostate cancer: Prognostic significance of the association of heterogeneous nuclear ribonucleoprotein K and androgen receptor expression

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Abstract. The management of prostate cancer (PCa) remains challenging because to date, there has been no way to distinguish between indolent and aggressive tumors. Heterogeneous nuclear ribonucleoprotein K (hnRNP K) is implicated in the network of mechanisms that control androgen receptor (AR) expression. We studied the expression of the two proteins in PCa to evaluate their prognostic potential and elucidate the hnRNP K function in PCa progression. HnRNP K and AR expression were analyzed immunohistochemically in 105 patients who had undergone radical prostatectomy. The association between the expression of hnRNP K and/or AR and PSA progression or death was evaluated by univariate and multivariate analyses. The expression of hnRNP K was also investigated *in vitro* using the BPH-1 cell line and two different LNCaP populations that recapitulate the progression of PCa towards a more aggressive disease. AR and hnRNP K were differentially expressed between cancer and normal prostate tissues. A strong association with a good prognosis was evident in PCa exhibiting high percentage of AR-positive cells (>75%) ($p \leq 0.005$) and more interestingly, the combination of high AR and low cytoplasmic hnRNP K expression emerged as the most significant independent prognostic marker for PSA failure-free survival, in a multivariate analysis ($p \leq 0.001$). *In vitro*, a higher expression of hnRNP K and pERK was associated with higher PSA levels, suggesting a relationship between hnRNP K phosphorylation and AR-regulated genes. These results indicate that the interaction between the AR and hnRNP K has an important role in the progression of PCa. Changes of the expression of the two proteins are strongly associated with the clinical outcome and may be a potential prognostic marker.

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Introduction

Prostate cancer (PCa) represents a major health concern in Western countries. In the USA alone, this tumor accounts for 29% of all newly diagnosed cancers (1). This high incidence is due to both the progressive aging of the male population and opportunistic screening for prostate-specific antigen (PSA). The PSA test has two major limitations: i) the lack of specificity because PSA is frequently elevated in benign prostate hyperplasia and prostatitis, and ii) the inability to discriminate between a less aggressive disease, characterized by an indolent behavior, and a more aggressive one with a very poor outcome (2). Several parameters, such as the tumor volume, pathological grade and the Gleason score, have been associated with the malignant potential of PCa (3). However, these parameters have proven to be inadequate, both in the selection of patients who require immediate local treatment and in the discrimination of high-risk patients who might require systemic therapy in the context of a multimodal approach (4-6). Therefore, new diagnostic and prognostic biomarkers are greatly needed for clinicians to improve the risk stratification of patients with PCa.

Recently, studies carried out in our and other laboratories, have shown that heterogeneous nuclear ribonucleoprotein K (hnRNP K) may play a key role in the carcinogenesis process of PCa (7,8). HnRNP K is a protein with pleiotropic functions present primarily in the nucleus (9) and active at the chromatin level, where it is localized at a higher density near transcribed genes compared with silent ones (10). Many human tumors manifest an overexpression of hnRNP K and, in several cases, an aberrant cytoplasmic localization as well. Furthermore, a correlation between the protein expression and the patient's

prognosis has been frequently observed (11-18). In 2004, Nagano *et al* (19) demonstrated that hnRNP K was strongly overexpressed in a primary culture of human PCa with respect to normal cell lines derived from the same patient. In addition, Wang *et al* (20) have shown that a novel transcriptional repressor complex containing Purα and hnRNP K binds to the androgen receptor (AR) gene both in cell lines and in primary human prostate tissues. More recently, evidence has been provided for a role played by hnRNP K in the regulation of AR expression via a post-transcriptional mechanism (7). Using a proteomic approach, we have previously reported that one phosphorylated isoform of hnRNP K present in the nuclear matrix, if co-expressed with another nuclear matrix protein (NM-8), is strongly associated with the clinical outcome of patients following radical prostatectomy (21,22). Moreover, in prostate cancer cell lines, we have demonstrated by immunoprecipitation and confocal microscopy techniques that hnRNP K and AR colocalize in the nucleoplasm in a complex and both proteins are synchronously modulated by treatment with bicalutamide, the anti-androgen widely used in PCa therapy (23).

These results support the hypothesis that hnRNP K is implicated in the network of mechanisms that control AR expression and that this protein could be a good biomarker for disease diagnosis, prognosis and progression in PCa. Based on these observations, in the present study we analyzed, by immunohistochemical analysis, hnRNP K and AR expression both in PCa and benign peritumoral tissues obtained from radical prostatectomy specimens, we have evaluated the diagnostic and prognostic potential of these proteins and whether a relationship between their expression levels also exists. The *in vivo* results were compared with a PCa *in vitro* model.

Materials and methods

Patient characteristics and assessment of clinical outcome. From 1995 to 2007, 105 patients who had undergone radical prostatectomy for biopsy-proven PCa were selected for the present study that was approved by the Ethics Committee of the National Cancer Research Institute of Genoa (OMB06.004). This cohort included all the patients who consented that their tissue specimens could be utilized for this research project and who were subsequently referred to our Unit for treatment or follow up. The patient characteristics are summarized in Table I. All specimens were subjected to a uniform histopathology protocol and clinical stage was reviewed and assigned using the 2011 TNM staging system. Due to the relatively small size of patient population, patients presenting Gleason score 7, 8 and 9 were arbitrarily grouped.

PSA failure-free and overall survivals were the main endpoints of the present analysis. Patients were followed at regular intervals and PSA was determined at each clinical examination. Any PSA level of at least 0.4 ng/ml following prostatectomy, which was confirmed by another assay four weeks later, was considered a biochemical failure. Therefore, PSA failure-free survival was defined by the time elapsed from the date the patient was submitted to prostatectomy to the date PSA progression was documented, and overall survival was the time elapsed from the date of prostatectomy to the date of death, independent of the cause. After a median follow-up time of 10.7 years [95% Confidence interval (CI) 9.7-11.6], 54 (51%) patients were found to have experienced biochemical progression and 21 (20%) had died.

Table I. Patient demographics and tumor characteristics.

Characteristics	N=105	(%)
Median preoperative age, years (range)	64 (48-77)	
Median PSA level at surgery, ng/ml (range)	11 (1.70-167.4)	
PSA ≤10 ng/ml	45	42.9
Tumor stage		
pT2a	2	1.9
pT2b	5	4.8
pT2c	46	43.8
pT3a	24	22.9
pT3b	27	25.7
pT3c	1	0.9
Pelvic nodes involved	23	21.9
Surgical margins involved	41	39.0
Seminal vesicles involved	30	28.6
Gleason score		
<7	34	32.4
=7	37	35.2
>7	34	32.4

Cell culture. The immortalized but non-transformed human prostate epithelial cell line BPH-1 was kindly provided by Dr Pfeffer of our Institute; the human prostate cancer cell line LNCaP was obtained from ATCC (CRL-1740; Rockville, MD, USA). The cells were maintained in phenol red-positive RPMI-1640 containing heat-inactivated 10% fetal bovine serum, 1% penicillin, 1% streptomycin, 1% glutamine, 10 mM HEPES, 1 mM sodium pyruvate and 4.5 mg/ml glucose (Celbio). Two different LNCaP cell populations were utilized: LNCaP cells with a low passage number (less than 28 and designated as LNCaPlp) and with a high passage number (higher than 54 and designed as LNCaPhp). These two populations recapitulate the progression of human PCa towards a more aggressive disease (24).

Immunohistochemical analysis. Immunohistochemistry was carried out on formalin-fixed, paraffin-embedded whole sections using mouse monoclonal antibodies anti-hnRNP K (sc-28380, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, diluted 1:800) and anti-AR (AR441, 200M-14, Cell Marque, CA, USA, diluted 1:50). Anti-hnRNP K was raised against amino acids 1-300 of the protein of human origin whereas anti-AR antibody recognizes both full-length and truncated AR proteins (25). For each antibody, two different sections (3 μm) of the same sample were treated independently. The more representative tumor sections were deparaffinised and in the case of anti-AR antibody, heat-induced antigen retrieval was carried out using

Cell Conditioning 1 solution (CC1, Ventana Medical Systems, S.A. Strasbourg, France) for 30 min.

Immunostaining was carried out using a BenchMark XT automated stainer (Ventana Medical Systems). Sections were incubated for 16 min at 37˚C with the anti-hnRNP K antibody or for 2 h at room temperature with the anti-AR antibody, and then the antigen-antibody complex was detected using the Ventana Medical System/ultraView diaminobenzidine (DAB) detection kit. The sections were counterstained with modified Gill's haematoxylin and mounted in Eukitt (Bio-Optica, Milan, Italy). An appropriate positive tissue control was used for each staining run; the negative control consisted of performing the entire immunohistochemistry procedure on adjacent sections in the absence of primary antibody. For each patient, both PCa and NT adjacent tissues were analyzed. The sections were observed with an Olympus BX41 light microscope (Olympus, Tokyo, Japan). Two observers independently examined the immunostaining of both proteins and the extent of immunochemical reactivity was evaluated exclusively in benign or malignant epithelial cells; stromal cells were not considered.

The expression of proteins was graded according to the number of immunoreactive cells and the staining intensity using the scoring system described by Carpenter *et al* (12), which we have already applied successfully to prostate tissues analyses (8). In this system, the extent of positively labeled nuclei or cytoplasm was ranked as follows: 0, 0%; 1, 1-25%; 2, 26-50%; 3, 51-75%; and 4, 76-100%. Staining intensity was graded into four steps with 0, negative; 1, low; 2, intermediate; and 3, high staining. The results are presented as the sum of the two assessments, thus ranging from 0 to 7 for each cellular compartment. The percentage of AR-positive nuclei was estimated by averaging the values, obtained by Qwin Standard image analysis software (Leica, Cambridge, UK), of 10 fields chosen at random. To study the correlation between AR expression and patient follow-up, the intensity and the percentage of the positive areas were analyzed separately.

Western blot analysis. Cultured cells were mechanically harvested with a sterile plastic disposable cell scraper and recovered by centrifugation at 3000 x g for 15 min. The pellet was washed twice in PBS and resuspend in RIPA lysis buffer containing protease inhibitor and 1 mM dithiothreitol. Cell lysates were prepared as already reported (26). Protein concentration was determined using Bio-Rad (Munich, Germany) protein microassay with bovine serum albumin as a standard. Equal amounts of samples were resolved by SDS-PAGE, transferred to PVDF and probed at 4˚C overnight with the following antibodies: mouse anti-hnRNP K (sc-28380, Santa Cruz Biotechnology Inc., diluted 1:8000); rabbit polyclonal anti-phospho-AKT-1 (pAKT, Ser473, Cell Signaling Technology, Beverly, MA, USA diluted 1:2000) and anti-phospho-ERK 1/2 (pERK 1/2, Thr202/Tyr204, Cell Signaling Technology, diluted 1:2000). After washing, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Milan, Italy) and immunoreactive bands were revealed by enhanced chemiluminescence (Immobilon, Millipore, MA, USA). HPR-conjugated rabbit anti-β-Actin antibody (Cell Signaling Technology, diluted 1:10000) was used as a loading control.

Immunocytochemistry. The cells were grown on chamber slides coated with poly-L-lysine. Cells were washed three times in PBS containing 2% sucrose, fixed for 15 min in 3.7% formaldehyde and treated for 15 min with PBS containing 2% sucrose and 0.2% Triton X-100. Cells were then incubated 5 min with peroxidase-blocking solution (Dako, Milan, Italy) and washed three times with PBS. After 30 min at room temperature in PBS containing 2% BSA, cells were incubated with mouse anti-hnRNP K antiserum (sc-28380, Santa Cruz Biotechnology Inc., diluted 1:1000) for 60 min. Cells were then washed three times with PBS before a 30-min incubation with peroxidase-conjugated anti-mouse IgG (Santa Cruz Biotechnology Inc.). The reaction was visualized using DAB Substrate Chromogen (Dako). The cells were observed by a light microscope (Leica, DM-LB2), photographed in a standardized manner under x40 magnification and stored as TIFF without compression, 24-bit (RGB) with 2040x1536 pixels. The original RGB color images were converted to a grayscale and adjusted homogeneously for contrast. Quantitative evaluation of hnRNP K compartmentalization was manually carried out using ImageJ 1.45 software (http://imagej.nih.gov/ij). The mean gray value, measured in approximately 300 cells, was converted in optical density according to Kim *et al* (27).

Statistical analysis. The t-test was applied to compare mean values of immunoreactive score in PCa with those in non-tumor (NT) tissues. Associations among the principal variables under study, i.e., hnRNP K, AR, preoperative PSA, Gleason score, extracapsular extension, regional lymph nodes, surgical margins status and seminal vesicle involvement, were investigated with the Pearson's correlation coefficient (r) (28). To this aim, variables were categorized as follows: Gleason score (<7 vs. ≥7), extracapsular extension (no vs. yes), surgical margins status (negative vs. positive), seminal vesicles (not involved vs. involved) and regional lymph nodes (not involved vs. involved). PSA was considered as a continuous variable. PSA failure-free and overall survival curves were constructed by means of the Kaplan-Meier method and compared by the log-rank test (29).

To evaluate the possible interactions among all of the variables under study, multi-parametric models were constructed according to the Cox proportional hazard technique (30) by including within the models, all of the covariates that also predicted for either PSA failure-free or overall survival after univariate analysis. The following covariates were included in all models: pre-surgery PSA levels (≤10 ng/ml, >10 ng/ml); extracapsular extension (No, Yes); surgical margins status (negative, positive); involvement of seminal vesicles (No, Yes); Gleason score (<7, ≥7); involvement of regional lymph nodes (pN0, pN1); cytoplasmic hnRNP K (<6, ≥6); AR expression (>75%, ≤75%); AR and cytoplasmic hnRNP K (>75%+hK<6 vs. ≤75% and >75%+hK≥6). The cut-off values used for hnRNP K and AR were those that better discriminated the patient-cohort according to the clinical endpoints under study. A stepwise procedure was used with a significance level of p=0.05 to retain variables in the model (31). Hazard ratio (HR) estimates and their 95% CIs were also calculated. All p-values were two-tailed. The IBM software Statistical Package for Social Sciences (SPSS) version 19.0 for Windows (SPSS Inc. Chicago, IL, USA) was used for data analysis.

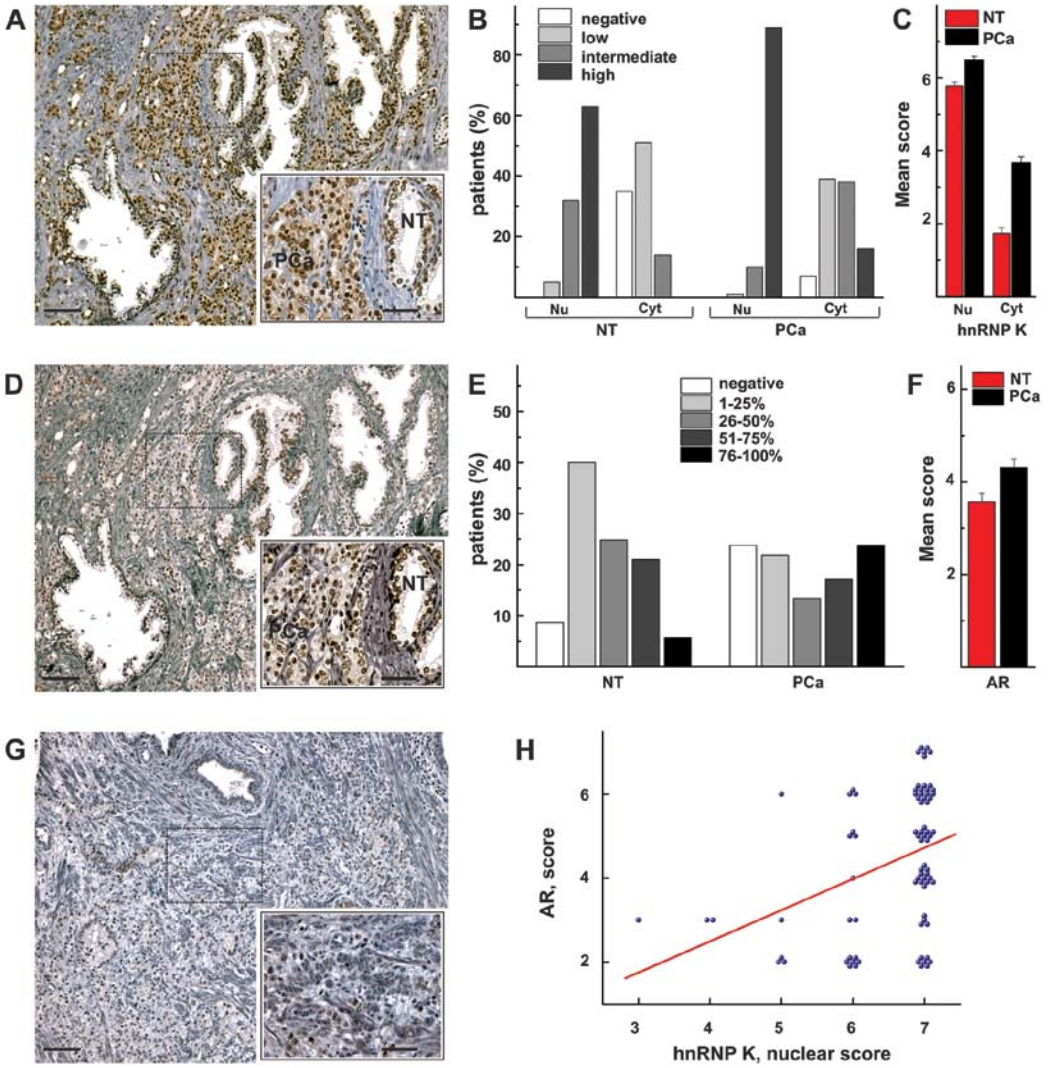


Figure 1. The analysis of hnRNP K and AR expression in prostate tissues. Consecutive PCa sections were stained using anti-hnRNP K (A) or anti-AR antibodies (D and G) and were evaluated by immunohistochemistry. Representative images of PCa specimens are reported; higher magnifications of the areas enclosed in boxes are shown in the inset. (D) An example of positive and (G) of negative staining with the anti-AR antibody. The bars correspond to 100 μm in (A), (D) and (G) and 50 μm in the insets. PCa and NT mark tumor and non-tumor area, respectively. (B) The distribution of cytoplasmic (Cyt) and nuclear (Nu) hnRNP K scores in the different patients analyzed. The scores were banded as follows: 0, negative; 1, 2, 3, low; 4, 5, intermediate; 6, 7, high. (C) Comparison of the scores of hnRNP K between NT and PCa tissues. The ordinate represents the mean score ± SE. HnRNP K expression was significantly higher ($p<0.0001$) in PCa compared with NT in both cellular compartments. (E) Distribution of the percentage of area positive for AR expression in NT and PCa in all patients analyzed. (F) Comparison of AR scores between NT and PCa. AR expression was significantly higher ($p=0.004$) in PCa. Only patients with a score >0 were considered. In the same patients, regression analysis (H) demonstrated a significant direct correlation between nuclear hnRNP K and AR expression ($r=0.363$, $p=0.0009$).

Results

HnRNP K and AR expression in PCa and paired NT tissues. HnRNP K was expressed in all of the specimens examined but with a different compartmentalization. In PCa, the protein displayed a more frequent and strong immunoreactivity, both cytoplasmic and nuclear, compared with NT tissues, where it was primarily localized in the nucleus with very little cytoplasmic staining observed (Fig. 1A). More than 85% of patients had tumor cells with a high nuclear score (≥6), and only 7% had negative cytoplasm. In contrast, in approximately 60% of the NT samples examined, hnRNP K was expressed with an intermediate score (4 or 5) in the nucleus, while in 35% of cases, it was not present in the cytoplasm (Fig. 1B). The difference in expression between PCa and paired NT tissues was highly significant ($p<0.00001$) in both cellular compartments (Fig. 1C), confirming the potential diagnostic value of the hnRNP K protein level.

Specific immunostaining of the AR was exclusively visible in the nuclei (Fig. 1D). The staining intensity was almost homogeneous both in NT and PCa tissues where more than 70% of patients had a low or intermediate intensity. In contrast, the distribution of the percentage of the area that reacted positively to the antibody was more flattened (kurtosis -1.27) in tumor than in NT specimens (kurtosis -0.39), suggesting a great heterogeneity of the malignant tissues among different patients (Fig. 1E). The tissue sections from 25 PCas (24%) showed negative staining (Fig. 1G), whereas in the remaining 80 samples, the percentage of AR-positive cells, as well as the total score, was statistically higher in malignant than non-malignant epithelial cells ($p=0.002$ and $p=0.004$, respectively) (Fig. 1F).

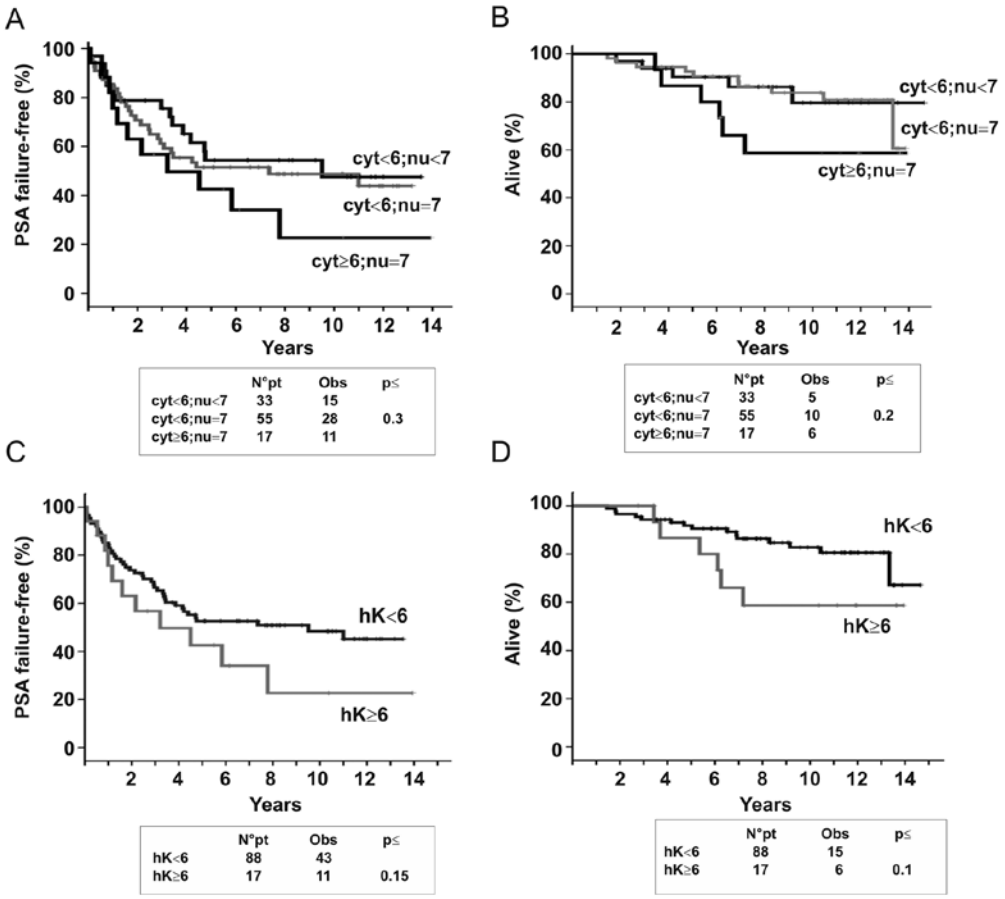


Figure 2. The analysis of the prognostic value of hnRNP K. Kaplan-Meier plots for PSA failure-free (A and C) and overall survival (B and D) in 105 PCa patients are reported as a function of hnRNP K score obtained considering both cytoplasmic (cyt) and nuclear (nu) expression (A and B) or only the cytoplasmic hnRNP K (hK) score (C and D).

Since we found that hnRNP K and AR colocalize in the nucleoplasm of prostate cancer cell lines (LNCaP and TRAMP) giving rise to a complex (23), we analyzed whether a correlation between the expression levels of the two proteins also exists in human tissue. As shown in Fig. 1H, in AR-positive PCas, protein expression was directly correlated with the level of nuclear hnRNP K (r=0.363, p=0.0009), and the correlation was also maintained when all PCas were considered (r=0.256, p=0.009). No relationship was demonstrated between cytoplasmic expression of hnRNP K and AR. This result suggests that the interaction between the two proteins found *in vitro* is a more widespread behavior, which is also present *in vivo*.

Patient characteristics and hnRNP K expression. Statistical analyses revealed no specific correlations between cytoplasmic or nuclear expression levels of hnRNP K and clinicopathological characteristics of patients (Table II).

To evaluate whether the expression of hnRNP K was correlated with PSA failure-free and/or overall survival, a Kaplan-Meier analysis was performed. We stratified the patients into three groups based on the hnRNP K expression level within both of the cellular compartments. Group 1, which included 17 patients whose tumors presented high cytoplasmic (≥6) and nuclear expression (=7), showed the worst outcome, whereas in other groups (group 2: cytoplasmic <6 and nuclear <7 and group 3: cytoplasmic <6 and nuclear =7), the clinical outcome was independent of nuclear expression and the curves for both PSA failure-free and overall survival were almost superimposable (Fig. 2A and B). Therefore, for the following analysis, we considered only the cytoplasmic expression of hnRNP K and divided patients into two groups, those with low (<6, 88 patients) and those with high (≥6, 17 patients) expression. As illustrated by Kaplan-Meier curves shown in Fig. 2C and D, patients with high cytoplasmic hnRNP K levels showed a trend for a highest risk of biochemical failure (HR=1.63, 0.84-3.16 CI 95%, p=0.15) and death (HR=2.23, 0.86-5.81 CI 95%, p=0.1) confirming that the aberrant cytoplasmic accumulation of the protein has an important role in the aggressiveness of PCa, as already reported for several human tumors (12-18).

Patient characteristics and AR expression. A significant inverse correlation of AR expression with the Gleason score ≥7 (r=-0.238; p≤0.010) and with PSA (r=-0.249; p≤0.010) was found (Table II). The first observation is in agreement with previous results showing a direct relationship between a higher degree of AR-positive cells and lower Gleason score (32). Whereas, the inverse correlation between the AR score and the level of PSA could depend on a limitation of immunohistochemical methods that only requires the presence of an immunoreactive epitope and does not distinguish between a functional vs. non-functional AR.

Table II. Relationship between the expression levels of hnRNP K and AR and clinicopathological characteristics of patients.

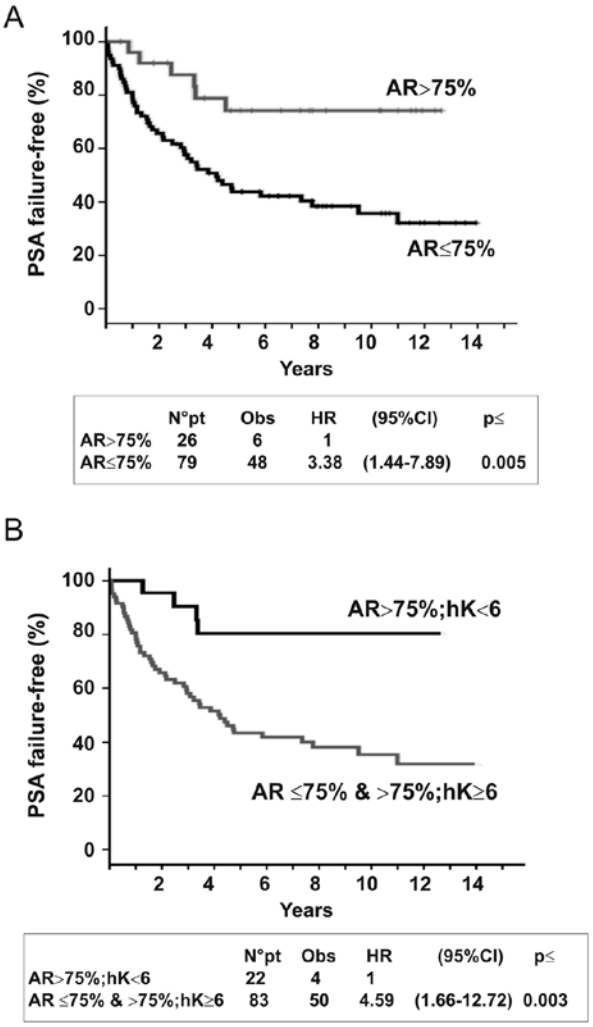
	Cytoplasmic hnRNP K		Nuclear hnRNP K		AR	
Characteristics	r	p-value	r	p-value	r	p-value
PSA	0.145	≤0.1	0.126	≤0.2	-0.249	0.01
Extra-prostatic extension	0.029	≤0.7	0.191	≤0.051	0.024	0.8
Pelvic nodes involved	0.022	≤0.8	0.142	≤0.1	-0.049	0.6
Surgical margins involved	-0.038	≤0.7	0.007	≤0.9	-0.021	0.8
Seminal vesicles involved	0.073	≤0.4	0.165	≤0.1	0.044	0.6
Gleason score <7	-0.063	≤0.5	0.027	≤0.8	-0.110	0.3
Gleason score ≥7	-0.055	≤0.6	0.043	≤0.7	-0.238	0.01

Table III. PSA failure-free according to principal clinicophatological variables and AR or AR in combination with cytoplasmic hnRNP K expression.

	Univariate analysis			Multivariate analysis			Multivariate analysis		
Variables	HR	(95% CI)	p-value	HR	(95% CI)	p-value	HR	(95% CI)	p-value
PSA									
≤10 ng/ml	1.0			1.0			1.0		
>10 ng/ml	1.88	(1.06-3.35)	≤0.03	1.21	(0.64-2.28)	≤0.5	1.18	(0.63-2.23)	≤0.6
Extra-prostatic penetration									
No	1.0			1.0			1.0		
Yes	2.98	(1.67-5.32)	≤0.000	3.2	(1.41-7.26)	≤0.005	3.18	(1.39-7.24)	≤0.006
Pelvic nodes involved									
pN0	1.0			1.0			1.0		
pN1	2.92	(1.66-5.11)	≤0.000	1.29	(0.63-2.61)	≤0.5	1.29	(0.63-2.65)	≤0.5
Surgical margins status									
Negative	1.0			1.0			1.0		
Positive	2.45	(1.43-4.19)	≤0.001	1.69	(0.88-3.24)	≤0.1	1.6	(0.84-3.07)	≤0.2
Seminal vesicles involved									
No	1.0			1.0			1.0		
Yes	1.71	(0.98-2.99)	≤0.06	0.47	(0.23-0.95)	≤0.04	0.47	(0.23-0.96)	≤0.04
Gleason score									
<7	1.0			1.0			1.0		
≥7	2.71	(1.36-5.39)	≤0.004	1.41	(0.63-3.09)	≤0.4	1.63	(0.75-3.55)	≤0.2
AR									
>75%	1.0			1.0					
≤75%	3.38	(1.44-7.89)	≤0.005	4.25	(1.76-10.26)	≤0.001			
AR and cytoplasmic hK									
>75% + hK <6	1.0						1.0		
≤75% and >75% + hK ≥6	4.59	(1.66-12.72)	≤0.003				5.71	(2.01-16.23)	≤0.001

hK, hnRNP K.

Since there has been no consensus for measuring the AR expression by immunohistochemistry in clinically localized PCa (33), we performed a Kaplan-Meier analysis considering the intensity and the percentage of AR-positive cells separately. No correlation was observed between the staining intensity in tumor cells and PSA failure-free survival (data not shown), whereas when we considered the percentage of AR-positive cells a strong association with a worse prognosis was evident



	N°pt	Obs	HR	(95%CI)	p≤
AR>75%	26	6	1		
AR≤75%	79	48	3.38	(1.44-7.89)	0.005

	N°pt	Obs	HR	(95%CI)	p≤
AR>75%;hK<6	22	4	1		
AR ≤75% & >75%;hK≥6	83	50	4.59	(1.66-12.72)	0.003

Figure 3. The analysis of the prognostic value of co-expression of AR and hnRNP K. Kaplan-Meier plots for PSA failure-free in 105 PCa patients are reported as a function of AR expression (A) or the co-expression of AR and cytoplasmic hnRNP K (B).

in PCa exhibiting low percentage of AR-positive cells (<75%) (HR=3.38, 1.44-7.89 CI 95%, p=0.005) (Fig. 3A). This variable was the most significant (p=0.001) in a multi-parametric model build up by including all variables significantly correlated with the risk of PSA progression at univariate analysis (Table III). No statistical correlation was observed between the percentage of area positive for AR staining and overall survival (data not shown).

Effect of the association of hnRNP K and AR expression on clinical outcome. We hypothesized that patient prognosis is most likely dependent on an interplay between AR and cytoplasmic hnRNP K. Therefore, we performed an analysis combining these two variables and stratifying patients into four distinct groups, depending on the level of expression of each protein: AR>75% and hnRNP K<6; AR≤75% and hnRNP K<6; AR>75% and hnRNP K≥6; AR≤75% and hnRNP K≥6. The combination of higher AR (>75%) and lower hnRNP K (<6) expression was strongly associated with a good prognosis (Fig. 3B) and demonstrated even better than considering AR alone (Fig. 3A). Moreover, this association emerged as the most significant independent prognostic marker for PSA failure-free survival in a multivariate analysis (Table III).

HnRNP K expression in an in vitro model of human PCa. It is possible to mimic the natural history of PCa utilizing cell lines reflecting the various steps of the tumor development (24). Therefore, to understand the role of hnRNP K in PCa progression we studied hnRNP K expression in BHP-1, LNCaPlp and LNCaPhp human prostate cells. Light microscopy and western blot analysis (Fig. 4) showed that hnRNP K was weakly expressed in BPH-1 cells, where it was localized mainly in the nucleus whereas the cytoplasm was faintly stained. In LNCaP cancer cells a significant increase of protein expression was detected and it was localized both in the nucleus and in the cytoplasm. More interestingly, hnRNP K expression was higher in LNCaPhp cells with respect to less aggressive LNCaPlp (Fig. 4A and B). These data are in agreement with *in vivo* observations reported above. Since it is known that ERK phosphorylation drives cytoplasmic hnRNP K accumulation (34) and in PCa progression upregulated ERK activity is often correlated with AKT activation (35), we studied the expression of p-AKT and p-ERK in this *in vitro* model. pAKT expression was absent in BPH-1 cells and present in both LNCaP cell populations where no difference in the protein level was observed. pERK 1/2 expression was weakly detectable in BPH-1 and was elevated in cancer cells. In LNCaPhp the protein expression was approximately 2.5 times higher than in LNCaPlp (Fig. 4B). This result is consistent with the more elevated cytoplasmic localization of hnRNP K in this cell line. Of note, higher hnRNP K and pERK expressions were associated with higher PSA level (Fig. 4B), suggesting a relationship between hnRNP K phosphorylation and AR-regulated genes.

Discussion

Despite extensive research efforts, Gleason grade, tumor stage and PSA are still the only parameters utilized for the stratification of patients in prognostic groups (36). The pathogenesis of PCa is complex and heterogeneous, and an understanding of the mechanisms that regulate this process and its principal actors is fundamental to the identification of new prognostic markers and new therapeutic strategies.

In this study, we have demonstrated that AR and hnRNP K are more highly expressed in cancer than normal prostate tissues. Moreover, we found that a high percentage of AR-positive cells (>75%) was strongly associated with a favorable prognosis and this prognostic value increased when it was associated with low cytoplasmic expression of hnRNP K.

While there is a widespread consensus that the ligand-stabilized AR is nuclear and that AR expression is more variable in PCa with respect to NT, the prognostic value of AR expression in the epithelia of PCa and its clinical relevance is still debated (33). In this study, heterogeneous expression of AR, due to the presence of both negative and positive cells in PCa (AR cell positive ≤75%), was significantly associated with shorter PSA-free survival in agreement with well-established results demonstrating that a high variability of AR protein content in PCa cells correlates significantly with a worse prognosis (37,38).

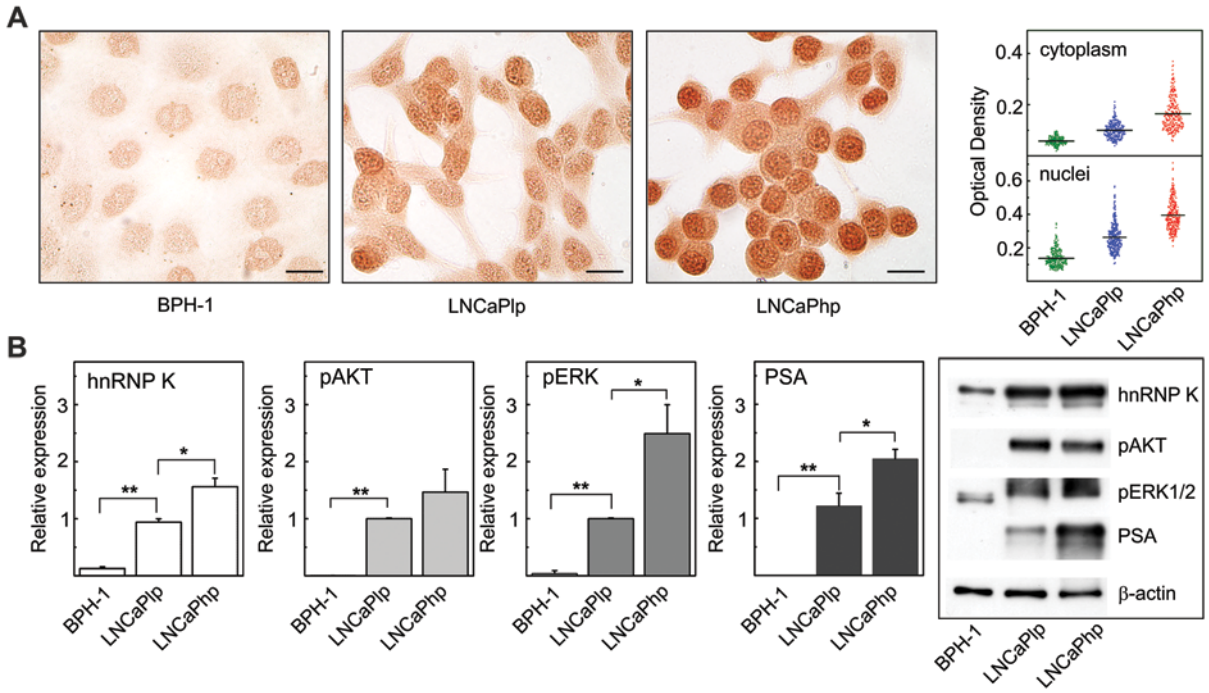


Figure 4. HnRNP K localization and expression in prostate cells. (A) Representative light microscopy images showing immunocytochemical staining for hnRNP K in BPH-1, LNCaPlp and LNCaPhp cells. A significant increase of protein expression both into the nucleus and the cytoplasm is evident as a function of aggressiveness of the cells. Scatter plot (right panel) shows the distribution of the optical densities. Horizontal line indicates the mean values obtained measuring for each cell line approximately 300 cells. The differences were statistically significant, $p<0.001$. (B) Western blot analysis of hnRNP K, pAKT, pERK 1/2 and PSA expression. The ordinates represent the mean ± SE of relative expression with respect to LNCaPlp cells; the data were obtained by three different experiments ($^{*}p<0.05$; $^{**}p<0.01$). Representative western blots are shown on the right. β-actin was used as equal loading marker.

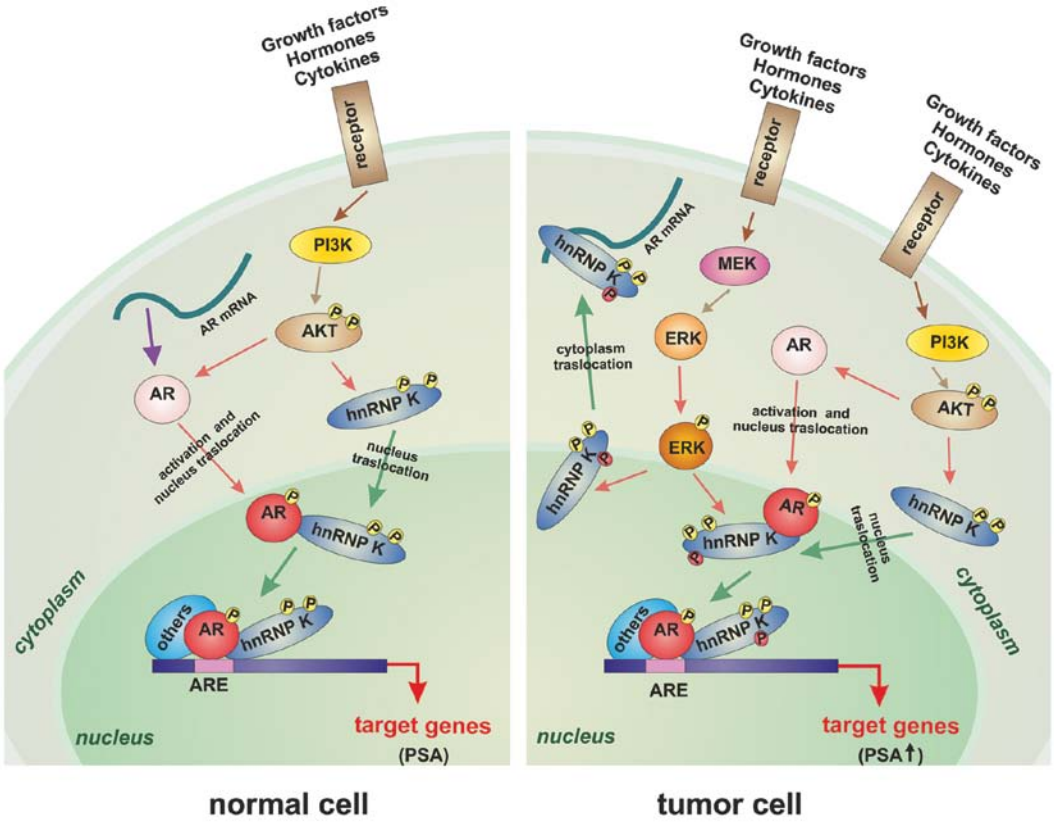


Figure 5. A possible model for the interaction of AR with hnRNP K in normal and tumor prostate cells. In normal cells activation of AKT results in phosphorylation of hnRNP K that migrates in the nucleus where binds AR facilitating its interaction with the transcription apparatus. In PCa cells both the increased hnRNP K expression level and the modification of phosphorylation status of this protein by augmented levels of pERK could modify the nuclear interaction between AR and hnRNP K, cause an increase of the PSA level and promote the migration of the hnRNP K into the cytoplasm, where it accumulates and is incapable of correctly regulating AR mRNA translation.

The interaction between AR and hnRNP K could have a pivotal role in the development and progression of PCa. The modular structure of hnRNP K causes a functional versatility that allows it to interact with both nucleic acids and proteins, thus acting as a 'docking platform' to co-ordinate nucleic acid metabolism and to facilitate cross-talk between signaling pathways (39). It has been demonstrated that hnRNP K is an inhibitor of AR mRNA translation (7) and Shi *et al* (40) have reported that in the aging rat liver and in oxidatively stressed hepatoma cells, the transcriptional downregulation of the AR involves hnRNP K, which participates in the activation of the complex that governs AR stimulation; in this model, silencing hnRNP K decreased AR expression. Moreover, we have shown in two cell lines (human LNCaP and mouse TRAMPC2 cells) that the AR and hnRNP K colocalize in the nucleoplasm forming a complex ligand-dependent (23). Notably, in a loss-of-function screening, it has been found that hnRNP K is a potential target for metastasis and that the cytoplasmic accumulation of this protein was essential for its role in promoting metastasis (41). Additionally, the knockdown of hnRNP K expression gives rise to a loss of the angiogenic and migratory phenotype of prostate carcinoma cells (42). Therefore, hnRNP K could have different roles in PCa as a function of its cellular compartmentalization.

It is known that the cytoplasmic localization of hnRNP K is phosphorylation-dependent and that the translation-regulatory function of the protein depends on its cytoplasmic localization. The increase of the phosphorylation grade of hnRNP K that occurs during PCa progression (8,21,22) could be responsible for its cytoplasmic accumulation. This accumulation is not peculiar to PCa but seems to be a general characteristic, indeed, it has been observed in several human tumors, and it is often associated with a worse prognosis (12-18). Moreover, utilizing an *in vitro* model, in this study we have demonstrated that the aggressiveness of cancer cell lines correlates with an increase in cytoplasmic expression of hnRNP K and increased levels of pERK 1/2.

Somatic mutations, gene amplification, increased protein stability and an altered level or function of coregulators are the principal molecular events that have been proposed to act in determining the level of AR in PCa. HnRNP K is one of the dozens of AR-interacting coregulators (43) and post-translational modifications of AR coregulators play a critical role in the formation of the transcriptional complex and growth factor-induced enhancement of AR transcription (44). We have recently demonstrated *in vitro* that the bond between AR and hnRNP K depends on the phosphorylation of the latter (45). Therefore, we propose a model where alterations in the expression level and phosphorylation status of hnRNP K could modify the nuclear interaction between AR and hnRNP K, cause an increase of the PSA level and promote the migration of the hnRNP K into the cytoplasm, where it accumulates and is incapable of correctly regulating AR mRNA translation, as shown schematically in Fig. 5. This hypothesis is supported by several experimental observations. Habelhah *et al* (34) have demonstrated that ERK efficiently phosphorylates hnRNP K primarily in the nucleus, after which phosphorylated protein translocates to the cytoplasm and inhibits mRNA translation. Interestingly, increased expression of the Ras/Raf/MEK/ERK pathway has been associated with PCa progression (46). Furthermore, altered levels of pAKT in the AKT/hnRNP K/AR/β-catenin pathway are critical for neuroendocrine differentiation (47). Finally, more recently Gao *et al* (48) have demonstrated that hnRNP K overexpressing cells show enhanced malignant and metastatic properties by regulation of extracellular matrix components through the ERK signaling pathway.

Our results indicate that the association of AR and hnRNP K expression has a potential prognostic value in PCa. The possibility of detecting the expression of these two proteins with immunohistochemistry, an easy-to-handle technique, could extend the study of PCa progression to material obtained through core biopsies and could significantly improve upon the sensitivity and specificity with which PCa is diagnosed. Determining the expression levels of AR and hnRNP K could allow for the stratification of patients into different prognostic subgroups and could provide a rationale for developing new chemotherapeutic agents directed against phosphorylated hnRNP K.

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