

Heterogeneous Nuclear Ribonucleoprotein K Is a Novel Regulator of Androgen Receptor Translation

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Abstract

The regulation of androgen receptor (AR) expression in prostate cancer is still poorly understood. The activation of the epidermal growth factor receptor (EGFR) in prostate cancer cells was previously shown to lower AR expression by a rapamycin-sensitive, posttranscriptional mechanism involving the AR mRNA 5'-untranslated region (5'-UTR). In a search for an intermediate within the EGFR/phosphoinositide 3-kinase/Akt/mammalian target of rapamycin pathway that regulates AR at this site, we identified the nucleic acid-binding protein, heterogeneous nuclear ribonucleoprotein K (hnRNP-K), by mass spectrometric analysis of Akt immune complexes from lipid raft-enriched subcellular fractions. We show here that hnRNP-K is a novel inhibitor of AR mRNA translation that regulates androgen-responsive gene expression and prostate cancer cell proliferation. A functional hnRNP-K binding site involved in down-regulating AR protein levels was identified in the AR mRNA 5'-UTR. Further analysis revealed that hnRNP-K is also able to inhibit AR translation in the absence of the 5'-UTR, consistent with the presence of additional predicted hnRNP-K binding sites within the AR open reading frame and in the 3'-UTR. Immunohistochemical analysis of a human prostate cancer tissue microarray revealed an inverse correlation between hnRNP-K expression and AR protein levels in organ-confined prostate tumors and a substantial decline in cytoplasmic hnRNP-K in metastases, despite an overall increase in hnRNP-K levels in metastatic tumors. These data suggest that translational inhibition of AR by hnRNP-K may occur in organ-confined tumors but possibly at a reduced level in metastases. HnRNP-K is the first protein identified that directly interacts with and regulates the AR translational apparatus. [Cancer Res 2009;69(6):2210-8]

Introduction

Prostate cancer is the most commonly diagnosed malignancy among men in the United States (1). Androgens, the male sex steroids, mediate growth and development of reproductive tissues

and are also prominently involved in prostate cancer as well as in certain other pathologic conditions (2-5). Androgens bind and activate the androgen receptor (AR), a 110-kDa nuclear receptor transcription factor (5). Prostate cancer is initially androgen dependent early in the course of the disease. However, progression to a state of castration resistance typically occurs in the case of nonlocalized disease after androgen-deprivation therapy (6-11). AR is expressed in primary prostate cancer and also in castrate-resistant tumors, where AR expression may actually increase with progression to metastases (12). Recent work from a number of laboratories confirms the continuing involvement of the AR throughout disease progression (13-15).

Our group recently showed that activation of the epidermal growth factor receptor (EGFR) by the prostate stromal growth factor HB-EGF regulates AR expression by a rapamycin-sensitive mechanism in LNCaP human prostate cancer cells (16). EGFR activation by HB-EGF suppressed AR protein levels, whereas rapamycin, an inhibitor of the serine-threonine kinase mammalian target of rapamycin (mTOR), antagonized this effect. Rapamycin alone also potently increased AR protein levels. Suppression of AR expression by EGFR activation was also seen in LNCaP xenografts *in vivo* in a separate study (17). The sensitivity to the mTOR inhibitor suggests that a repressive signal to AR passes through the phosphoinositide 3-kinase (PI3K)/Akt/mTOR pathway. In a search for intermediates within this pathway, we immunoprecipitated the serine-threonine kinase Akt from lipid raft-enriched subcellular fractions isolated from LNCaP cells and identified coprecipitating proteins by mass spectrometry. Using this approach, we recently found that the serine/threonine kinase, Mst1/STK-4, a proapoptotic protein, is a novel inhibitor of Akt that functions by a mechanism involving direct interaction between the two kinases (18). Here, we describe the application of a similar strategy that resulted in the finding that the nucleic acid binding protein, heterogeneous ribonucleoprotein K (hnRNP-K), regulates AR expression by a posttranscriptional mechanism.

HnRNP-K was originally identified as a polycytidylic acid binding protein (PCBP) that exhibits a high-affinity, sequence-specific interaction with poly(C) RNA (19). PCBPs comprise two subsets in mammalian cells: (a) hnRNP-K and hnRNP-J and (b) the α -complex proteins (20). HnRNP-K contains three conserved KH (K homology) nucleic acid binding domains and a kinase interacting region that mediates interaction with a variety of protein targets (21). HnRNP-K resides in both the nucleus and the cytoplasm and has been linked to a variety of cellular processes, including mRNA translation, transcription, RNA processing, RNA shuttling and stabilization, chromatin remodeling, and cell survival (22-26).

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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These diverse functions arise from the capability of hnRNP-K to bind both RNA and DNA. Here, we present the first evidence that hnRNP-K is an endogenous inhibitor of AR protein translation, along with additional data consistent with a role for this protein in prostate cancer.

Materials and Methods

Materials. R1881 was purchased from Sigma Chemical Co. AR and hnRNP-K antibodies are from Santa Cruz Biotechnology. EGFR, lamin, α -tubulin, and Akt antibodies are from Cell Signaling Technology. KDR antibody is a generous gift from Dr. Soumitra Pal (Children's Hospital Boston). ECL detection kit was from New England Nuclear, Inc. Smart pool small interfering RNA (siRNA) for hnRNP-K is from Dharmacon, Inc. Casodex is a gift from Zeneca Pharmaceuticals. The TNT Quick Coupled Transcription/Translation Systems is from Promega. LightShift Chemiluminescent EMSA Kit and Biotin Labeling Kit are from Pierce.

Cell culture. LNCaP cells were cultured in RPMI 1640 and COS or HeLa cells in DMEM supplemented with 10% fetal bovine serum, 2% glutamine, and 1% antibiotics. Cell proliferation was determined by a viability assay using crystal violet.

Plasmid constructions and mutagenesis. HnRNP-K constructs (27) and prostate-specific antigen (PSA)-Luc reporter (p61-Luc) and AR 5' untranslated region (5'-UTR)-Luc (16) were described. HnRNP-K binding site mutants 414 and 478 were constructed by site-directed mutagenesis according to the QuikChange II Mutagenesis Kit (Stratagene). Primers used to exchange nucleotides were 5'-CTCACCACCTTCTCAAACCCGCC-CACCCGCC-3'/5'-GGGCGGGTGGGCGGGTTTGGAGAAGGGTGGTGGAG-3' for 414 and 5'-TGCAGAGAGGTAACCTCAATTTGGCTGCGAGCGGG-3'/5'-CCCGCTCGCAGCAAATTGAGTTACCTCTCTGCA-3' for 478. A combination of 414/478 (double mutants) was achieved using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocol.

Nuclear extract preparation. Cells were suspended in 1 mL of cold buffer [20 mmol/L HEPES (pH 7.4), 10 mmol/L KCl, 2 mmol/L MgCl₂, 0.5% IGEPAL, 1 mmol/L DTT, 100 μ mol/L vanadate, and a cocktail of protease inhibitors] and placed on ice for 10 min. The 1,500 \times g supernatant was centrifuged again at 13,000 \times g for 15 min to collect the cytoplasmic fraction. The pellet, after washing, suspended in the above buffer with 0.5 mol/L NaCl. Lysates were centrifuged for 15 min at 13,000 \times g at 4°C, and supernatants (nuclear extract) were collected.

Electroporation. Nucleofector (Amaxa, Inc.) was used to electroporate 2 μ g of human hnRNP-K plasmids or 100 nmol/L of each siRNA into LNCaP cells according to the manufacturer's protocols in the presence and absence of R1881.

Transient transfections and luciferase assays. Transient transfections were performed with Lipofectamine 2000 (Invitrogen) as described (28). Plasmid DNA (0.5–1 μ g) was used and R1881 (1 nmol/L), rapamycin (100 nmol/L), or the respective vehicles (ethanol/bovine serum albumin) were added for 20 h in serum-free medium.

Luciferase activity was measured using a dual luciferase assay kit as described (28). Either Renilla or total protein was used to normalize the relative luciferase activity. Background luciferase activity (pGL3 basic for p61PSA-Luc) was also measured for comparison. All experiments were carried out in triplicate and repeated thrice using different preparations of plasmids.

RNA silencing. ON-TARGETplus SMART pool siRNA oligonucleotides (Dharmacon) were designed to target different splice variants of hnRNP-K. Controls were scrambled sequences.

Immunoprecipitation and Western blot analysis. Triton X-100 soluble and insoluble lipid raft proteins were prepared essentially as described (29). Protein (400 μ g) was immunoprecipitated with either polyclonal hnRNP-K or monoclonal Akt antibody with protein A-Sepharose beads. Bead-bound complexes were immunodetected as described (28). Dilutions for primary antibodies were 1:1,000 for AR, HA, and Akt; 1:2,000 for EGFR and KDR; and 1:10,000 for β -actin. Horseradish peroxidase-conjugated secondary anti-

bodies were used at a dilution of 1:5,000. Immunoreactive bands were visualized by chemiluminescence.

Glutathione S-transferase pull-down. Bacterially expressed glutathione S-transferase (GST)-hnRNP-K fusion proteins were adsorbed to glutathione-Sepharose beads (GE-Healthcare BioSciences AB), incubated with purified recombinant Akt1 (Upstate Biotechnology) in binding buffer [20 mmol/L Tris (pH 8.0), 137 mmol/L NaCl, 10% glycerol, 1% NP40] for 60 min at 4°C, and analyzed by Western blotting using Akt-specific antibody (Cell Signaling).

RNA gel mobility shift. Nonradioactive RNA gel mobility shift was performed with biotinylated AR 5'-UTR RNA probe and purified GST-hnRNP-K fusion protein. The biotinylated probe was prepared using Biotin-16-UTP and *Xba*I digested 5'-UTR AR construct using the AmpliScribe T7 and T3-Flash transcription kits. The binding reaction was performed using the LightShift Chemiluminescent EMSA kit. The RNase T1 step (10 min at 22°C) and heparin (5 mg/mL) addition step (10 min at 22°C) was performed before incubation with a labeled riboprobe. For competition assays, unlabeled RNA (25 and 100 ng) was added for 10 min before the incubation with labeled probe. The binding reaction, gel electrophoresis, and the electrophoretic transfer to nylon membrane were as described in the Pierce protocol. Samples were fixed on the membrane by UV cross-linking for 3 min (UV Stratalinker 2400, Stratagene) and detected by chemiluminescence.

In vitro translation. T7-hAR constructs (16) were transcribed and translated in a coupled rabbit reticulocyte lysate system (Promega) in the absence and presence of recombinant GST-hnRNP-K fusion protein. Reactions were performed at 30°C for 90 min either in the presence of [³⁵S]methionine for radioactive exposure or of unlabeled methionine. Translation of luciferase was used as a control.

Immunoprecipitation/reverse transcription-PCR. Cells were lysed in a buffer containing 10 mmol/L HEPES (pH 7.6), 3 mmol/L MgCl₂, 40 mmol/L KCl, 5% glycerol, 0.2% NP40, 1 mmol/L DTT, standard protease inhibitors, and 50 units of RNaseOUT, and were incubated with either IgG or hnRNP-K antibodies for 45 min at 4°C. A mixture of protein-A and protein-G beads was added and incubated for 30 min. RNA was extracted and the One Step reverse transcription-PCR (RT-PCR) procedure (Invitrogen) was followed using the primers 5'-GCATGCCAGAGTGCCTATC-3' and 5'-TCCCAGAGT-CATCCCTGCCTCAT-3' for AR.

Polysome analysis. Polyribosome preparation was performed as described (30). In brief, LNCaP cells were treated with cycloheximide (50 μ g/mL) and in some cases with 200 μ mol/L puromycin for 60 min before collection. The cell pellet was resuspended in 300 μ L low-salt buffer [10 mmol/L Tris (pH 7.4), 15 mmol/L NaCl, 12.5 mmol/L MgCl₂, 500 μ g/mL heparin, 50 μ g/mL cycloheximide] and 250 μ L of lysis buffer [25 mmol/L Tris (pH 7.4), 15 mmol/L NaCl, 12.5 mmol/L MgCl₂, 1.2% NP40, 500 μ g/mL heparin, 5% (w/v) sucrose] was added. The 1,000 \times g extract was loaded onto a linear sucrose gradient [15–45% (w/w) sucrose] in 10 mmol/L Tris buffer (pH 7.4), 200 mmol/L NaCl, 10 mmol/L MgCl₂, 200 μ g heparin with a SW 28 rotor and centrifuged at 126,000 \times g at 4°C for 80 min. Twelve gradient fractions were collected (0.5 mL each) and the polysome profile (*A*₂₆₀) was determined. RNA was extracted by the Trizol reagent (Invitrogen) and RT-PCR was performed using the primers 5'-CACCCGT-CTTCAGGGCTTCTTGGTTT-3' and 5'-CATTTCCACCATCTGGTTGGC-TGGCTC-3'.

Tissue microarray immunohistochemistry. The human prostate tissue microarray (TMA) consists of benign and prostate tumor tissues from the Brigham and Women's Hospital (Boston, MA), University of Michigan (Ann Arbor, MI), and the University of Ulm (Ulm, Germany) and is composed of benign prostate (*n* = 18), localized prostate cancer (*n* = 36), and metastases (*n* = 36). The metastatic cohort consists of 18 lymph node hormone naïve and 18 hormone (castrate)-resistant distant metastases. One pathologist (D.D.V.) excluded cases if adequate paraffin-embedded tumor tissue was not available; consequently, a total of 14 benign, 24 organ-confined tumor, and 24 metastases were included. Immunohistochemistry for hnRNP-K was performed on 5- μ m sections using the avidin-biotin procedure and analyzed semiquantitatively by the ACIS System (ChromaVision Medical System, Inc.) as described (18, 31). The obtained values were analyzed alone and in

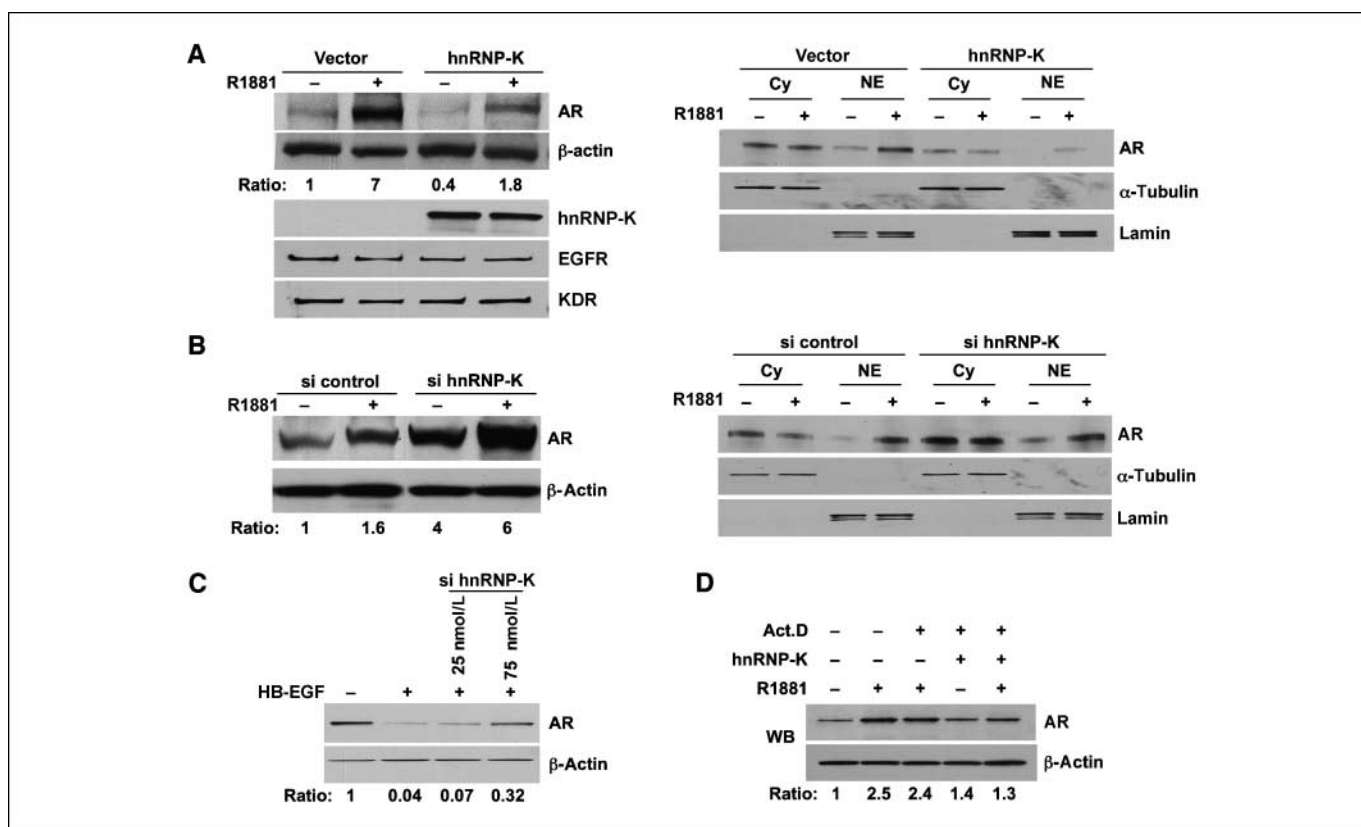


Figure 1. hnRNP-K down-regulates AR expression in the presence and absence of androgen. *A, left*, LNCaP cells were transiently transfected with either hnRNP-K or vector constructs using Nucleofection (Amaxa). Protein was transferred to nitrocellulose and blotted either with AR, HA (for hnRNP-K), EGFR, KDR, or β -actin antibody. *Right*, fractionation of LNCaP cells before and after hnRNP-K transfection using Lipofectamine 2000 (Cy, cytoplasmic fraction and NE, nuclear fraction); α -tubulin and lamin were used as cytoplasmic and nuclear markers, respectively. *B, left*, knockdown of hnRNP-K increased AR expression in the presence and absence of 1 nmol/L R1881 (using Nucleofection as transfection method). *Right*, nuclear and cytoplasmic fractionation of LNCaP cells after knockdown of endogenous hnRNP-K. *C*, knockdown of endogenous hnRNP-K reverts HB-EGF (EGFR)-mediated down-regulation of AR. *D*, steady-state AR protein level in the presence and absence of actinomycin D (5 μ mol/L for 24 h) and R1881 (1 nmol/L) after hnRNP-K transfection. In each case, data were quantified using NIH Image 1.63 software and represented as the ratio of AR and β -actin levels.

combination with serial sections stained with anti-AR antibody.⁶ The resulting intensity expression values (range from 0 to 255) for each TMA core were transformed, mean centered, and SD set to 1 to standardize the variables to the same scale. Quadruplicate data points of each case were averaged after assessment of intracase expression homogeneity with respect to intradiagnostic group variability (32). Expression level differentiation among diagnostic groups was assessed by *t* test for unpaired data. To assess potential correlations, intensity mean values were dichotomized and Fisher's exact test was applied on contingency tables. All *P* values were considered two-tailed and 0.05 was used as upper threshold for statistical significance. Origin 8 software (OriginLab Corporation) was used for statistical analysis. Specimens positive for hnRNP-K were classified as expressing (a) nuclear (N) or (b) nuclear + cytosolic (N+C) signal and the number of samples with cytoplasmic localization were counted and statistically analyzed across the diagnostic groups using a *t* test for unpaired data.

Results

hnRNP-K down-regulates AR protein levels. LNCaP cells stably engineered to express myristoylated Akt1 (Myr-Akt1; ref. 29), and HEK293 cells transiently transfected with Myr-Akt1 were used to generate Akt1 immune complexes for analysis by tandem mass

spectrometry (LC-MS/MS). Lipid raft-enriched fractions were used for this purpose (29). hnRNP-K was identified by LC-MS/MS in these immunoprecipitates in multiple independent trials (not shown). We verified by coimmunoprecipitation that endogenous hnRNP-K forms a complex with Myr-Akt1 preferentially in lipid raft fractions (Supplementary Fig. S1A). GST-hnRNP-K also formed a complex with purified Akt *in vitro* (Supplementary Fig. S1B), verifying the mass spectrometry data.

hnRNP-K has been shown to regulate the translation of a number of mRNAs (33–36), and the protein lies downstream from the EGFR in some contexts (37). Consequently, hnRNP-K is a logical intermediate in the previously demonstrated EGFR→mTOR pathway in which AR protein levels are down-regulated at the level of mRNA translation (16).

To test the potential involvement of hnRNP-K in this mechanism, we first examined the effect of hnRNP-K on AR expression in LNCaP cells. Enforced expression of hnRNP-K lowered AR protein levels in both the presence and absence of the synthetic androgen, R1881 (1 nmol/L), without any effect on the levels of the signaling receptors EGFR and KDR or on β -actin (Fig. 1A). To determine whether hnRNP-K alters translocation of AR to the nucleus, we evaluated the extent of accumulation of AR in cytoplasmic versus nuclear fractions after enforced expression of hnRNP-K. We found a similar lowering of AR in nuclei and cytosol (Fig. 1A, right),

⁶M.P. Lisanti, unpublished observations.

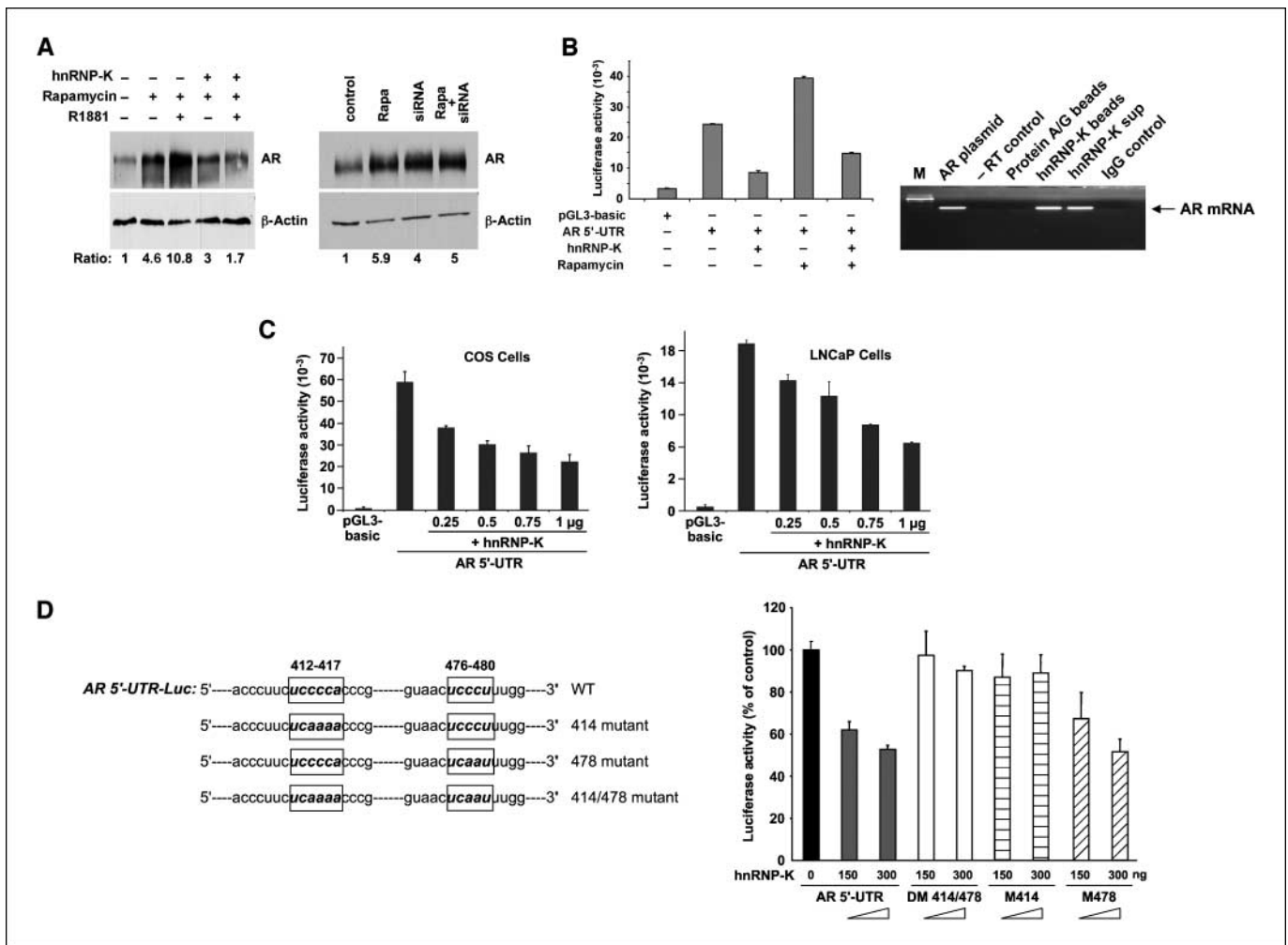


Figure 2. hnRNP-K regulates AR expression at the AR 5'-UTR. *A, left*, LNCaP cells were transfected with hnRNP-K expression plasmid and treated with either 100 nmol/L rapamycin and/or 1 nmol/L R1881. *Right*, determination of AR level in the presence and absence of hnRNP-K siRNA and 100 nmol/L rapamycin alone or in combination. All data were analyzed by NIH Image 1.63 and represented as the ratio of AR and β -actin. *B, left*, effect of hnRNP-K on AR 5'-UTR luciferase activity in the presence and absence of rapamycin. AR 5'-UTR luciferase plasmid (500 ng) or the control plasmid (500 ng) were cotransfected with 500 ng of hnRNP-K in LNCaP cells. Lysates were assayed for luciferase activity and protein content. *Right*, to verify that hnRNP-K binds to AR mRNA, LNCaP cells were immunoprecipitated with either IgG or hnRNP-K antibody and RNA was extracted and subjected to RT-PCR analysis using AR-specific primers. Negative controls included no added reverse transcriptase (*-RT*), beads (protein A/G) alone, and IgG immunoprecipitate. Specific PCR band in extracts of LNCaP cells was used as a positive control. *C*, COS and LNCaP cells were transfected with control plasmid (pIR-Luc) or plasmid containing the AR 5'-UTR in the presence of varying amounts of hnRNP-K. *D, left*, schematic representation of AR 5'-UTR-Luc constructs highlighting the conserved hnRNP-K binding motifs (UCCCCA and UCCCU) in human AR mRNA. These two motifs were altered to UCAAAA (414 mutant) and UCAAU (478 mutant). *Right*, either AR 5'-UTR-Luc wild-type or single (M414 and M478) and double mutants (DM 414/478) were tested for activity in the presence of varying doses of hnRNP-K.

indicating that hnRNP-K does not influence AR transit from cytoplasm to the nucleus, whereas it alters steady-state levels of AR. Conversely, knockdown of endogenous hnRNP-K by RNA interference (Fig. 1B) increased AR levels, suggesting that endogenous hnRNP-K is conferring the same function as the overexpressed protein. hnRNP-K siRNA also attenuated the suppressive effect of HB-EGF on AR mRNA levels (Fig. 1C; Supplementary Fig. S2), suggesting that hnRNP-K controls AR levels downstream from the EGFR.

To assess whether the effect of hnRNP-K on AR is confined to LNCaP cells, AR-negative HeLa cells were transiently cotransfected with hnRNP-K along with human AR expression constructs and the effect of enforced expression of hnRNP-K on AR levels was monitored. Our results showed a significant decrease of AR expression in the presence of hnRNP-K in the presence and

absence of androgen (Supplementary Fig. S3), consistent with the findings in the LNCaP cell background.

Posttranscriptional regulation of AR by hnRNP-K. The experiments with HeLa cells suggested that the effect on AR was unlikely to be a result of changes in transcription of AR mRNA. Consistent with this hypothesis, we observed no changes in AR mRNA following enforced expression of hnRNP-K in LNCaP cells (Supplementary Fig. S4A). hnRNP-K overexpression also did not affect AR mRNA levels when cells were treated with the transcription inhibitor, actinomycin D, for up to 20 h (Supplementary Fig. S4B). Actinomycin D similarly did not alter the observed hnRNP-K effects on AR protein levels (Fig. 1D).

The selective mTOR inhibitor, rapamycin, increased AR expression in LNCaP cells, consistent with published results (ref. 16; Fig. 2A). hnRNP-K siRNA evoked a comparable, but not additive,

Table 1. Consensus hnRNP-K[U(C)nA/U] binding sites in human AR mRNA

	Position	Sequence
5'-UTR	412-417	UCCCA
	476-480	UCCU
	664-668	UCCCA
ORF	1403-1408	UCCCCA
	1510-1514	UCCCA
	1608-1612	UCCU
	2255-2266	UCCCCA-UCCCCA
	2656-2665	UCCCA-UCCCA
3'-UTR	3887-3901	UCCCCA
	4090-4094	UCCCA
	4115-4119	UCCCA

effect on AR protein levels, suggesting that hnRNP-K may act upstream of mTOR (Fig. 2A, right). Rapamycin exerts regulatory effects on cap-dependent mRNAs (38). EGFR activation was previously shown to attenuate AR protein levels in a rapamycin-sensitive manner, at least partly through the AR mRNA 5'-UTR (16). Thus, we analyzed the effect of enforced expression of hnRNP-K on

a 570-nt fragment of the AR 5'-UTR, which contains several *cis*-acting elements and cap- and stem-loop secondary structures and was shown previously to be rapamycin sensitive (16). To do this, we used a luciferase plasmid system in which the 5'-UTR fragment regulates the CMV promoter and that reports effects on cap-dependent mRNA translation (16). Consistent with published findings (16), rapamycin induced luciferase expression from this plasmid construct (Fig. 2B, left). In the presence and absence of rapamycin, transient expression of hnRNP-K in LNCaP cells inhibited luciferase expression. These data strongly suggest that hnRNP-K exerts its attenuating effects on AR expression at the level of mRNA translation. Consistent with this interpretation, we verified the direct binding of hnRNP-K with AR 5'-UTR RNA corresponding to this same UTR region by RNA gel mobility shift in concert with purified GST-hnRNP-K (Supplementary Fig. S5), and also with endogenous AR mRNA in whole cells using immunoprecipitation/RT-PCR (Fig. 2B, right). The inhibitory effect of hnRNP-K on expression driven by the AR 5'-UTR (Fig. 2B) does not require the AR, because similar findings were obtained in AR-negative COS cells (Fig. 2C, left).

Inspection of the 570-nt rapamycin-sensitive region in the AR 5'-UTR using the consensus sequence U(C)nA/U (39) revealed two potential hnRNP-K binding sites at nt 412 to 417 (UCCCCA) and 476 to 480 (UCCU). To evaluate whether either of these UC-rich

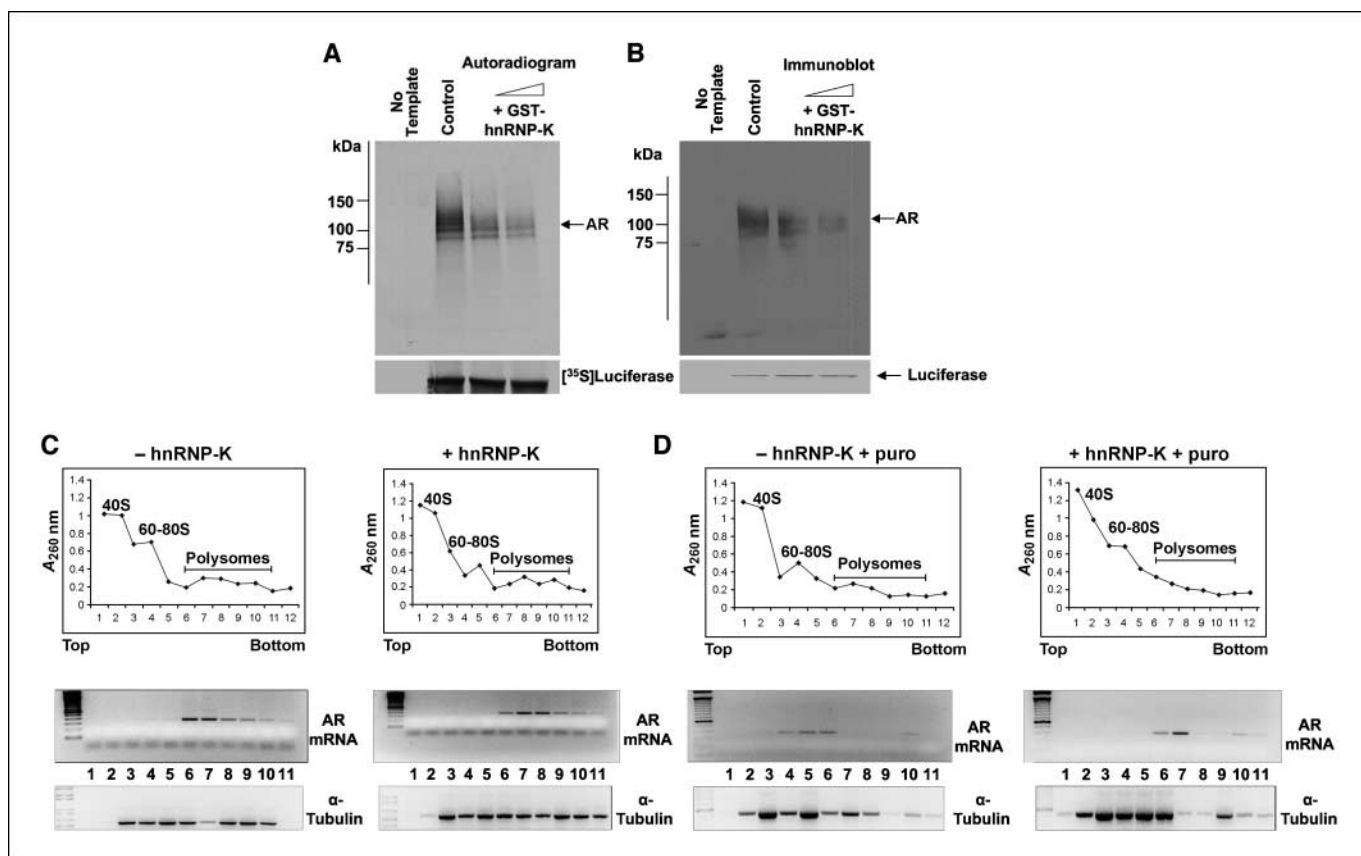
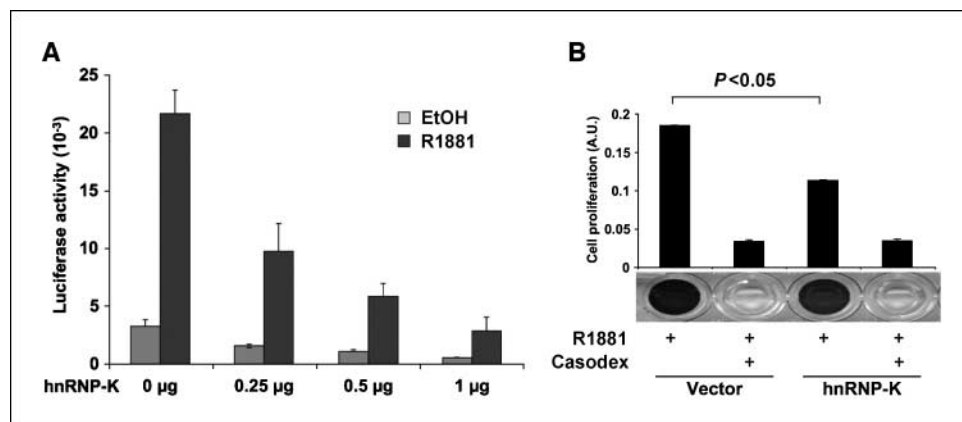


Figure 3. hnRNP-K inhibits the translation of AR mRNA. *A*, effect of purified GST-hnRNP-K on translation of AR mRNA as determined by coupled *in vitro* transcription/translation using [³⁵S]methionine. *B*, Western blot analysis with monoclonal AR antibody after *in vitro* translation with unlabeled methionine. Luciferase (bottom) was used as a translation control in *A* and *B*. *C*, LNCaP cells were treated with R1881 in the presence and absence of hnRNP-K for 24 h. A cytoplasmic extract was prepared and centrifuged through a linear sucrose gradient to isolate polysomes. Total RNA was extracted from each of the 12 sucrose gradient fractions. RT-PCR was performed using AR and β -tubulin primers. PCR products were separated on ethidium bromide-stained 1% agarose gels. The polysome profile was determined by measurement of the A_{260} for each fraction. *D*, polysome profile (A_{260}) and polysome analyses were performed in the presence of 200 μ M puromycin. LNCaP cells were treated with indicated amount of puromycin for 30 min before sucrose gradient fractionation.

Figure 4. hnRNP-K inhibits AR-dependent gene expression and LNCaP cell growth. *A*, enforced expression of hnRNP-K dose dependently inhibits a luciferase reporter driven by the PSA promoter. *B*, enforced expression of hnRNP-K inhibits proliferation of androgen-dependent LNCaP cells. The AR inhibitor Casodex (bicalutamide) was used at a concentration of 10 $\mu\text{mol/L}$.



regions mediates some or all of the inhibitory effects of hnRNP-K on the translation reporter, we generated single and double mutants within the rapamycin-sensitive fragment (Fig. 2*D*, left). Alteration of the consensus sequence (by mutating UCCCCA to UCAAAA) at the 412 to 417 site, but not alteration of the 476 to 480 site, abolished the inhibitory effects of hnRNP-K on reporter expression (Fig. 2*D*, right), confirming the involvement of the 412 to 417 site within the AR 5'-UTR in the regulatory effect we observed with hnRNP-K.

hnRNP-K inhibits translation of AR mRNA. hnRNP-K regulates translation of the L2 capsid protein of human papillomavirus type 16 by binding within the open reading frame of the coding region (35). Inspection of the entire AR mRNA indicated that the message may harbor as many as nine other potential hnRNP-K binding sites in the coding region and in the 3'-UTR (Table 1), suggesting the possibility that hnRNP-K may also regulate AR expression through one or more of these additional sites. To address this question, we examined the effect of hnRNP-K on translation of AR mRNA using a construct containing only the AR coding region, which contains six potential hnRNP-K binding sites (Table 1). AR was synthesized with a coupled transcription/translation system, using either [³⁵S]methionine for radioactive detection or unlabeled methionine for Western blot identification. Addition of purified GST-hnRNP-K dose dependently decreased the synthesis of the 100- and 110-kDa translated products (Fig. 3). In contrast, no translation inhibition was observed on the internal control luciferase mRNA (bottom panels). Results with cold methionine (Fig. 3*B*) confirmed the identity of the translation products as AR by immunoblotting. In a complementary experiment, enforced expression of hnRNP-K in HeLa cells reduced the expression of AR expressed from the same hAR construct used in the coupled transcription-translation experiments (Supplementary Fig. S3). These findings indicate that hnRNP-K uses one or more binding sites within the coding region in a manner that is functionally compatible with the single binding site we identified in the rapamycin-sensitive region of the 5'-UTR.

In an independent test of the ability of hnRNP-K to act as a regulator of AR translation, we probed the distribution of AR mRNA in polysomal fractions isolated from LNCaP cells under conditions where hnRNP-K expression was enforced. If hnRNP-K acts as an inhibitor of AR mRNA translation at the level of initiation, then its forced expression would result in the dissociation of AR mRNA from actively translating ribosomes and a repartitioning of the mRNA toward the lighter fractions of

the gradient. As indicated in Fig. 3*C* (left and right), hnRNP-K had no such effect on the distribution of AR mRNA in gradient fractions, suggesting that the protein does not inhibit translation initiation of AR mRNA. hnRNP-K had no effect on the distribution of tubulin mRNA (Fig. 3*C*, bottom), as expected. Next, we tested the possibility that the AR mRNA under conditions of enforced hnRNP-K expression was associated with translationally inactive ribosomes, an event generally associated with a defect in peptide elongation. To test this, we used puromycin, which causes premature termination and release of all actively elongating mRNAs from polysomes. If an mRNA species is not being actively translated, puromycin will not be incorporated into the peptide chain and mRNA release from polysomes will not occur. In the absence of elevated hnRNP-K, puromycin caused a release of tubulin and AR mRNAs from the polysomal fractions (compare Fig. 3*C*, left, with *D*, left, particularly lanes 8 to 11, which contain AR and tubulin mRNAs associated with large polysomes). However, when hnRNP-K expression was enforced, AR mRNA was retained in heavy polysomal fractions, even after puromycin treatment (compare Fig. 3*C* and *D*), indicating that hnRNP-K evokes a reduction in rate of AR mRNA chain elongation.

hnRNP-K inhibits PSA promoter activity and LNCaP cell proliferation. To determine whether AR down-regulation by hnRNP-K has functional consequences in prostate cancer cells, we assessed the effect of enforced hnRNP-K expression on the androgen-responsive human PSA promoter and on LNCaP cell growth. Androgen-induced activation of the reporter gene was strongly and dose-dependently inhibited by hnRNP-K (Fig. 4*A*), indicating that hnRNP-K antagonizes androgenic signaling, consistent with a role as an AR antagonist. Similarly, hnRNP-K overexpression suppressed LNCaP cell proliferation (Fig. 4*B*), a result consistent with the PSA promoter-reporter data.

hnRNP-K expression in human prostate cancer. Multiple data sets in the Oncomine cancer profiling database⁷ indicate that hnRNP-K is expressed in human prostate cancer. We analyzed a human prostate cancer TMA containing benign prostate tissues, organ-confined cancers, and metastases [hormone naive (HN) and hormone (castration) resistant (HR)] using a monospecific anti-hnRNP-K antibody. hnRNP-K was detected in the nucleus of the majority of benign and carcinoma samples at high levels, and expression was significantly increased in metastatic tumors in

⁷ <http://www.oncomine.org>

comparison with benign tissues and organ-confined tumors ($P = 0.0001$ and 0.0004 , respectively; Fig. 5A, left), a trend that was maintained when HR metastases were compared with HN metastases (Fig. 5A, right). Notably, hnRNP-K and AR levels were significantly inversely correlated in localized prostate cancer specimens ($P < 0.001$; Fig. 5B). Finally, hnRNP-K was cytosolic and nuclear localized in organ-confined tumors, but cytosolic expression was significantly decreased in the metastases (Fig. 5C and D).

Discussion

In this study, we show that the nucleic acid binding protein hnRNP-K is a physiologically relevant mediator of AR expression, which functions by the novel mechanism of repression of AR mRNA translation. We verified a previous observation that a rapamycin-sensitive region within the 5'-UTR of the AR message harbors regulatory information that modifies rates of AR translation (16); however, we now go on to show that hnRNP-K also uses information within the AR coding region independently of the 5'-UTR to regulate AR translation. We found that hnRNP-K acts to repress AR expression, androgen-regulated promoter

activity, and androgen-induced prostate cancer cell growth. Although AR expression is regulated by transcriptional and protein stability mechanisms (40–42), the potential for translational regulation of the AR mRNA is poorly studied and has not been fully established before this report.

Mizokami and Chang (43) implicated a ~0.6-kb region of the AR 5'-UTR as a component of the AR expression regulatory mechanism. In that study, this segment of the UTR was shown to regulate expression of a reporter gene in a transcription-independent manner, suggesting the possibility of the existence within this region of one or more *cis* elements that control the translational apparatus *in vivo*. A more recent study (16) identified this same region in the AR 5'-UTR as sensitive to the mTOR inhibitor rapamycin. This result is consistent with an earlier report demonstrating that continuous *in vivo* exposure of LNCaP xenografts to the EGFR activating ligand, HB-EGF, caused a stable reduction of AR levels in the tumors (17). Our identification of a functional hnRNP-K regulatory element at nt positions 412 to 417 within the rapamycin-sensitive element of the 5'-UTR is in agreement with these previous observations. The results we now describe indicate that this site is required to mediate attenuation of luciferase expression from a cap-dependent AR 5'-UTR translation

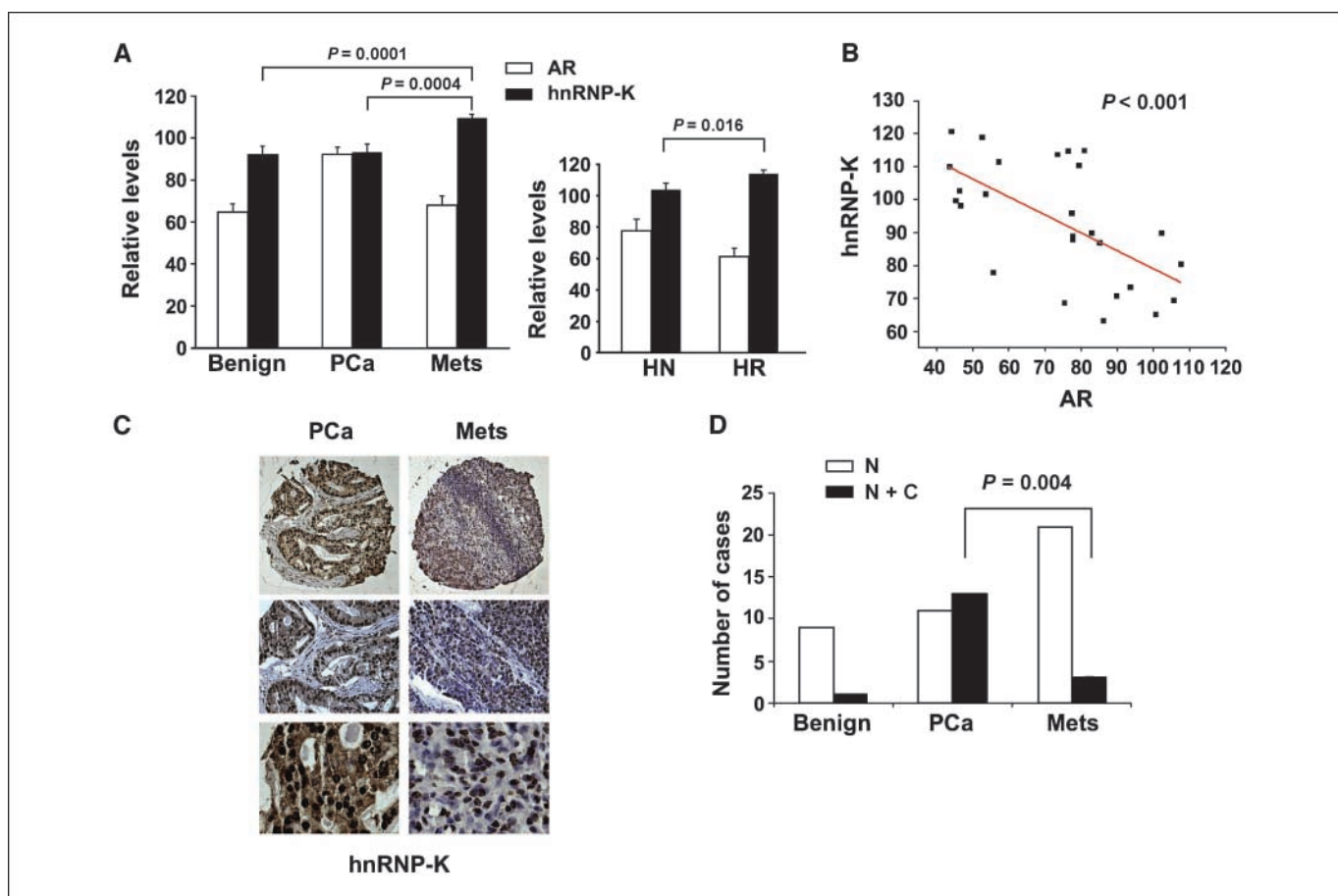


Figure 5. Quantitative analysis of hnRNP-K and AR levels in a human prostate TMA. *A*, relative levels of hnRNP-K and AR as assessed by ChromaVision analysis of TMA containing benign, local prostate cancer, and metastatic tumors (left). The right panel shows averaged values of hnRNP-K and AR in the metastatic subset (HN, hormone naïve; HR, hormone resistant). *B*, regression analysis showing a significant inverse correlation between hnRNP-K and AR in organ-confined tumors. *C*, representative micrographs showing hnRNP-K expression and subcellular localization. Note the loss of cytoplasmic staining in the metastatic tissue (bottom right). *D*, the graph shows the number of cases among the different diagnostic groups with predominant nuclear (N) or nuclear + cytosolic (N+C) staining. PCa, organ confined prostate cancer; Mets, metastases.

reporter when hnRNP-K expression is enforced (Fig. 2). Surprisingly, however, we found that this site is not required for the down-regulating effect of hnRNP-K on AR levels, pointing to redundancy in the mechanism controlling AR expression.

We have shown that enforced expression of hnRNP-K in LNCaP cells dramatically lowered AR protein levels, whereas siRNA knockdown of endogenous hnRNP-K elevated AR expression. hnRNP-K siRNA inhibited the ability of EGFR activation to reduce steady-state AR levels and also mimicked the AR-inducing effects of rapamycin seen in LNCaP cells. Similarly, hnRNP-K over-expression antagonized the ability of rapamycin to enhance AR expression. We confirmed that hnRNP-K binds to AR mRNA and showed that hnRNP-K binds and inhibits the activity of an AR 5'-UTR translation reporter. hnRNP-K inhibited AR translation in both *in vivo* and *in vitro* experimental formats. Finally, we showed that enforced expression of hnRNP-K in LNCaP cells redistributed AR mRNA to translationally inactive ribosomes in concert with the attenuation of androgen-dependent biological responses. Collectively, these data provide strong evidence that hnRNP-K is an endogenous regulator of AR translation in human cells.

Analysis of a human prostate cancer tissue microarray showed that hnRNP-K and AR levels were inversely correlated in local prostate cancer, and substantial loss of cytosolic staining was seen with progression to metastases. Thus, the presence of the AR inhibitor hnRNP-K correlates with reduced AR levels in a tumor subset. Furthermore, a reduction in cytosolic hnRNP-K, which presumably would be available to inhibit translation, is seen with progression to the lethal phenotype, where the AR is believed to remain active in the absence of normal levels of circulating androgen. If hnRNP-K serves to inhibit expression of AR *in vivo*, these data may reflect the regulatory process we identify here in the human disease.

Yeap and colleagues reported that several RNA binding proteins, including HuR, CP1, and CP2 bind AR mRNA at specific sites within the 3'-UTR (44), although the function of these interactions was not explored in that study. It is possible that dysregulation of multiple mechanisms affecting AR mRNA translation and/or mRNA stability may be operative in prostate cancer. Our data are compatible with this possibility. We observed here that metastatic tumors retain hnRNP-K in tumor cell nuclei, suggesting that the functional role of the protein may change with disease progression. Consistent with this idea, hnRNP-K was recently identified as a component of a transcriptional complex capable of regulating the AR gene in LNCaP cells (45). The possibility that hnRNP-K serves multiple functions in prostate and other tumor cells in a manner that can affect disease progression deserves further study.

A role for hnRNP-K in translation of other mRNAs has been identified (33–36, 46, 47), with the protein described as a translational regulator of c-myc (33), renin (34), human papillomavirus type 16 L2 capsid protein (35), and reticulocyte-15-

lipoxygenase (LOX; ref. 36) mRNAs. The premature expression of LOX in erythroid precursor cells is restricted by a translational silencing mechanism mediated by hnRNP-K binding to the differentiation control element in the LOX mRNA (36). In contrast to translational silencing of LOX, hnRNP-K promotes internal ribosome entry on c-myc mRNA (33), indicating that the protein can play stimulatory as well as inhibitory functions in translational regulation. Interestingly, Atsushi and colleagues recently reported that cytoplasmic accumulation of hnRNP-K mediates metastasis in human fibrosarcoma HT1080 cells (48). Because the AR is an important mediator of masculinization and other physiologic mechanisms in multiple cell types, including in nonreproductive tissues such as skeletal muscle and brain, it is possible that hnRNP-K regulates AR translation in other contexts. Furthermore, it cannot be assumed that the protein will always act to suppress AR. In fact, the literature suggests that a more complicated role for hnRNP-K in regulating AR expression in heterologous contexts is more likely. Because AR expression in breast cancer may be protective rather than tumor promoting (49), it would be interesting to compare the role of hnRNP-K in regulating the AR in this alternative context.

In summary, we have provided some of the first mechanistic evidence for a prominent role for mRNA translation in regulating AR protein expression. Because our data suggest that hnRNP-K is downstream from the EGFR, and most likely Akt, the protein may be important in the network of mechanisms that control AR expression and function in a variety of normal and pathologic conditions in which these signaling pathways have been implicated. In addition, given the prominent role of the AR in prostate cancer progression, including in castrate-resistant disease, these findings provide a rationale to consider chemotherapeutic agents directed against the protein synthesis machinery in prostate cancer treatment (50).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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