von Hippel-Lindau tumor suppressor gene-dependent mRNA stabilization of the survival factor parathyroid hormone-related protein in human renal cell carcinoma by the RNA-binding protein HuR

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We have shown that parathyroid hormone-related protein (PTHrP) is a survival factor for human renal cell carcinoma (RCC) and that its expression is negatively regulated by the von Hippel-Lindau (VHL) tumor suppressor gene at the level of messenger RNA (mRNA) stability, as observed for tumor growth factors (TGFs). Our goals were to analyze the alternative splicing of PTHrP mRNA in human RCC and from these results to identify VHL/hypoxia-induced factor (HIF) system-regulated mRNA-binding proteins involved in PTHrP mRNA stability. We used: (i) a panel of human RCC cells expressing or not VHL; (ii) VHL-deficient 786-0 cells transfected with active or inactive VHL and (iii) human RCC samples and corresponding normal tissues. By quantitative real-time reverse transcription–polymerase chain reaction analysis, the 141 PTHrP mRNA isoform was found to be predominant in all cells and tumors (80%). In cells transfected with VHL, the expressions of all isoforms were decreased by 50%. Eight proteins with molecular weights ranging from 20 to 75 kDa were found to bind to biotinylated transcripts spanning the 141 PTHrP mRNA AU-rich 3′-untranslated region whose abundance was dependent on VHL expression. The protein having an apparent molecular weight of 30 kDa was identified by western blot as HuR, a RNA-binding protein with stabilizing functions on various mRNA coding for proteins important in malignant transformation including vascular endothelial growth factor and TGF-β. PTHrP expression studies confirmed the involvement of HuR in PTHrP upregulation in this disease. Common mRNA-binding proteins regulated by the VHL/HIF system may constitute new therapeutic opportunities against human RCC that remains refractory to therapies.

Introduction

Renal cell carcinoma (RCC) represents a heterogenous group of tumors accounting for 3% of adult malignancy and results in over 100 000 deaths worldwide annually (1,2). Its incidence increases steadily by ~3% per year. RCC is the most lethal urologic tumor and the sixth leading cause of cancer deaths in western countries. Conventional renal cell carcinoma (CRCC) is the main subtype of RCC representing ~80% of all RCC cases. The 5 year survival rate is 70% for localized RCC, but decreases to only 5% in the metastatic group. RCC represents a radiation- and chemotherapy-resistant tumor. Immunotherapeutic strategies with interleukin-2 and/or interferons, which has been for long the unique therapeutic options for selected patients, appears to be effective in only 10–20% of patients (3). New therapeutic strategies have recently emerged with inhibitors of tyrosine kinase receptors, especially sunitinib and sorafenib. However, although the time to progression is increased with these compounds, there does not seem to be an overall survival benefit. New therapeutic options for this refractory disease have thus still to be uncovered.

Inherited and sporadic forms of CRCC are associated with mutations, deletion or silencing of the von Hippel-Lindau (VHL) tumor suppressor gene (4). The VHL gene products (pVHL) are found in a multiprotein complex and are involved in the degradation, in normoxic conditions, of HIF target genes including angiogenic, metabolic and growth factors, such as vascular endothelial growth factor (VEGF), erythropoietin, glucose transporters and tumor growth factor (TGFs) (5), that are all involved in malignant transformation.

Parathyroid hormone-related protein (PTHrP) is a polypeptide responsible for humoral hypercalcemia of malignancy, a paraneoplastic syndrome associated to various cancers (6). However, PTHrP also possesses complex growth factor-like properties and is involved in the growth and invasion of multiple cancers including breast, prostate and lung malignancies (7,8). We have recently shown that PTHrP is over-expressed in human CRCC and that it is critically involved in tumor growth in vitro and in vivo through inhibition of tumor cell apoptosis. In nude mice bearing human CRCC tumors treated with PTHrP-neutralizing antibodies, we observed antitumor effects ranging from inhibition of tumor progression to complete tumor regression (9). Furthermore, more recently we showed that the survival properties of PTHrP was obtained through stimulation of the phosphoinositide-3-kinase/Akt/NF-kappaB-signaling pathways, PTHrP appearing as one of the major factor responsible for the constitutive activation of this pathway in this cancer type (10).

In our former studies, we also identify PTHrP as a new target of the VHL/HIF system. Indeed, the reintroduction of the VHL gene in RCC cells deficient in VHL decreased substantially PTHrP expression. This effect was not the consequence of a decrease in PTHrP transcription rate but was obtained through the decrease of PTHrP messenger RNA (mRNA) stability (11). The specific effect of VHL on PTHrP mRNA stability is similar to what has been shown for the other VHL/HIF targets TGF-α and TGF-β. In addition, many, if not all, of the factors whose expression is controlled at the transcriptional level by the VHL/HIF system, such as VEGF or erythropoietin (12,13), are also largely regulated at the level of mRNA stability by this system. Interestingly, mRNA-binding stabilizing proteins with similar molecular weights have been shown to bind to the mRNA of these factors. These proteins are also expressed in hypoxic conditions and dependently on the VHL status of the cells, including RCC cells. These observations led us to hypothesize that common mRNA-binding proteins involved in mRNA stabilization exist that are controlled by the VHL/HIF system. This hypothesis is further argued by the observations that an inactive C-terminal-truncated mutant of VHL, VHL (1-115), that does not possess binding domain to elongin C, is unable to repress the stabilization of the mRNA of these factors (14).

In the present studies, we quantitatively investigated the expression of total PTHrP mRNA and of each of the three PTHrP mRNA isoforms in human CRCC in cultured cells expressing or not the VHL gene either spontaneously or following gene transfer and in freshly harvested tumors. Second, we performed ultraviolet (UV) cross-linking of the most abundantly expressed target PTHrP mRNA isoform.
fragment corresponding to the 3′-untranslated region (UTR) with cytoplasmic proteins from CRCC cells expressing or not the VHL gene followed by gel analysis. Our main result is the identification of the RNA-binding protein HuR, which has been shown by others to stabilize various factors including VEGF and TGFs, two VHL/HIF-regulated genes that are involved in human kidney tumorigenesis. Accordingly, in addition to PTHrP, the expressions of VEGF and TGFs were also substantially decreased by blocking the expression of HuR, suggesting that these targets share common mRNA-binding proteins.

Materials and methods

Cells, cell culture and stable transfection

Human CRCC cells line either deficient in normal VHL gene expression (786-0, A498, UOK-126 and UOK-128) or expressing wild-type VHL (ACHN, Caki-1 and Caki-2) were used. All cell lines were obtained from American Type Culture Collection (Manassas, VA; local distributor LGC-Promochem, Teddington, UK) except UOK cell lines that were generously given by Dr P. Anglard (Institut National de la Santé et de la Recherche Médicale U575, Center de Neurochimie, Strasbourg, France). Cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen, Cergy-Pontoise, France) supplemented with 10% fetal bovine serum and used at 80% confluence unless otherwise specified. We also used 786-0 cells (786-0 wt) that were stably transfected with PCR3.1-Uni vector alone (786-0 V), vector containing the von Hippel-Lindau complementary DNA (cDNA) hemagglutinin-tagged at the C-terminus (786-0 VHL) or inactive C-terminal truncated von Hippel-Lindau (1-111) (786-0 ΔVHL), using Lipofectamine (Invitrogen). Stable clones were selected using 6418 (500 μg/ml). Clones obtained by transfection were described previously (11).

Human RCC biopsies

The tumor and normal corresponding tissues of 10 patients (four pT1a, three pT1b and three pT3b) with sporadic CRCC were obtained from the Urology Department of the University Hospital of Strasbourg. Informed consent was obtained from all patients.

Quantitative analysis of PTHrP mRNA isoforms in cells and tumors

Total RNAs were extracted from cells and tissues using the Trizol method according to the manufacturer’s protocol (Invitrogen). Five micrograms of total RNA were reverse transcribed in the following reaction buffer: reverse transcriptase (400 U), RNAase inhibitor (10 U), deoxyribonucleotide triphosphate mix (1 mM each) (Invitrogen) and non-specific primer pTD15 (1 μM) (Roche Diagnostics, Meylan, France), at 37°C for 1 h.

cDNAs specific for each PTHrP mRNA isoforms (139, 141 and 173 isoforms) and specific for total PTHrP mRNA (Figure 1A) were amplified using the LightCycler-FastStart DNA Master SYBR (syr) Green kit (Roche Diagnostics). Sense and antisense primers used are depicted in Table I (supplementary Table I is available at Carcinogenesis Online).

Standard curves were obtained for each mRNA target by serial dilution of mixed cDNA samples according to one of the protocol preconized by the kit manufacturer for quantitative analysis of gene expression. Negative control was systematically performed by replacing cDNA with polymerase chain reaction (PCR)-quality sterile water. Each sample was analyzed three times and quantified with the analysis software for LightCycler (Roche Diagnostics).

Each amplicon was verified on a 2% agarose gel (Figure 1B). All PTHrP amplicons were then amplified by PCR using the same primer pairs and the same PCR conditions and used at known concentrations to serve as calibrator in each reaction. 100 μg of the protein extract was incubated with 30 μl of purified biotinylated RNA in 100 μl of binding buffer containing (final concentration in mM): N′-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid buffer (20), MgCl2 (3), KCl (40), dithiothreitol (1) and glycerol 5%. After incubation at room temperature for 15 min, heparin (Sigma–Aldrich) at 5 mg/ml in diethyl pyrocarbonate-treated water was added to the reaction mixtures that were incubated again at room temperature for 15 min.

UV cross-linking reactions were performed in a spectrophotometer XL-1000 UV cross-linker (Spectronics Corporation, Lincoln, NE) for 5 min at the maximal setting.

RNA–protein complexes purification

The biotinylated RNA–protein complexes were purified using a separation technique with paramagnetic spheres coupled to streptavidin (Dynal). The spheres are first washed three times in 300 μl of standard sodium citrate buffer 0.5× containing NaCl (75 mM) and sodium citrate (dehydrated trisodique salt) (7.5 mM) in diethyl pyrocarbonate-treated water, pH 7.2. The spheres are then resuspended in 60 μl of standard sodium citrate buffer 0.5× and added to each cross-linking reaction. After a 10 min incubation at room temperature with frequent gentle mixing, the spheres, which are then linked to the biotinylated RNA–protein complexes, are again isolated and washed four times in standard sodium citrate buffer 0.1×. After the final wash, the spheres are suspended in 200 μl of RNA-binding buffer containing RNase A/T1 and incubated at 37°C for 30 min. The supernatant free of spheres is then harvested and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

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SDS–PAGE analysis of RNA-bound proteins

Proteins are denatured by incubation in Laemmli buffer, at 100°C for 6 min and resolved by SDS–PAGE (10%). Proteins were then revealed by silver nitrate using the Proteo Silver Plus Stain kit (Sigma–Aldrich) exactly according to the manufacturer’s protocol.

Western blot analysis

The samples were prepared as for silver nitrate assay above and subjected to SDS–PAGE (10%). Western blot analysis was performed as detailed previously (16) with mouse monoclonal anti-human HuR antibody (3A2, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1000 and monoclonal mouse anti-HuR antiserum (Sigma–Aldrich) diluted 1:5000 for visualization of protein gel loading. Immunoreactivity was visualized, after secondary antibody treatment, with the Immobilon western blotting detection kit (Millipore, St Quentin en Yvelines, France).

Stability of HuR expression by cycloheximide treatment

Untransfected and transfected 786-0 cells and clones (wt, V, VHL and AVHL) were seeded in 24-well plates (20 000 cells per well), grown for 48 h and then treated with cycloheximide at 20 μg/ml for 0–48 h as indicated in the corresponding figure. The level of expression of HuR was evaluated by western blot analysis, as presented above.

PTHrP, VEGF and TGF-β mRNA expression in HuR-depleted cells

Untransfected 786-0 cells were transiently transfected with siRNA duplexes specific for human HuR (HuR siRNA) and control non-silencing siRNA (Ctl siRNA) (Ambion) or siRNA diluent. Cells were transiently transfected with Lipofectamine RNAiMax (Invitrogen) for 24–96 h with HuR control siRNAs,
according to the manufacturer’s instructions. Total RNA was isolated and total PThrP, VEGF and TGF-β1 mRNAs expression analyzed by real-time reverse transcription–PCR using human glyceraldehyde phosphate dehydrogenase (GAPDH) for normalization. Primer pairs are depicted in Table I (supplementary Table 1 is available at Carcinogenesis Online).

Actinomycin D experiments in HuR-depleted CRCC cells
Untransfected 786-O cells were transiently transfected with HuR siRNA and Ctrl siRNA (Ambion) for 0–72 h and exposed to actinomycin D at 5 μg/ml. Total RNA was isolated and the 141 PTHrP mRNA isoform stability analyzed by real-time quantitative reverse transcription–PCR using human GAPDH for normalization of PCRs as described above. The relative abundance of the 141 PTHrP mRNA isoform in HuR-depleted cells was calculated and plotted as a percent of its abundance in Ctrl siRNA-transfected cells at each time point shown in the corresponding figure.

Cell proliferation measurements
Cell proliferation was analyzed by counting adherent cells and measurement of bromodeoxyuridine (BrdU) incorporation according to the manufacturer’s protocol (Roche Diagnostics). For cell counting, CRCC cells were seeded in 24-well plates (20 000 cells per well), grown for 48 h and then transiently transfected with 100 nM HuR siRNA or control siRNA. Diluent of siRNA also served as control. The number of cells was counted 24, 48, 72 and 96 h post-transfection. For BrdU incorporation studies, CRCC cells were seeded in 96-well plates (20 000 cells per well), treated as above for cell counting and then assayed for BrdU incorporation at 24, 48, 72 and 96 h post-transfection, as described previously (11).

Statistical analysis
All values are expressed as mean ± SEM. Values were compared using multifactorial analysis of variance followed by the Student–Newman–Keul’s test for multiple comparisons. P < 0.05 was considered significant.

Results
The 141 PThrP mRNA isoform is predominantly expressed in tumor cells
The organization of the human PTHrP gene is presented in Figure 1A. Expression of total PThrP mRNA expressed in femtomolar (fM) was
higher in cells deficient in VHL expression compared with cells expressing wild-type VHL (data not shown). The 141 PTHrP mRNA isoform was preponderant in all cell lines (~70%), followed by the 173 PTHrP mRNA isoform, representing ~20%, and by the 139 PTHrP mRNA isoform that was barely detectable (~10%), again independently of VHL expression (Figure 1C).

**VHL expression does not modify the PTHrP mRNA isoform expression in tumor cells**

We previously engineered and characterized 786-0 cell clones expressing wild-type human VHL (1-213) in pCR3-Uni vector and clones transfected with vector alone (11). In addition to these clones, we have here constructed 786-0 cell clones transfected with inactive truncated VHL, 786-0 ΔVHL, to use as control, as detailed. Two clones of each construct were used here.

Total PTHrP mRNA expression and the expression of all PTHrP mRNA isoforms were measured in these clones and again in 786-0 wt cells. When expressed in fm, the expression of all PTHrP mRNA isoforms was diminished by 50% in clones transfected with VHL compared with untransfected, vector alone-transfected or ΔVHL 786-0 cells and clones (Figure 2A and B). The same was observed for total PTHrP mRNA expression (Figure 2B). The expression profile of all isoforms was similar in all cells and clones, with a clear preponderance of the 141 PTHrP mRNA isoform (Figure 2A and B).

**The 141 PTHrP mRNA isoform is also predominantly expressed in tumors**

We analyzed total PTHrP mRNA expression and the expression of all three isoforms in 10 tumor samples (seven pT1 and three pT3) and normal corresponding tissues. In pT1 samples, total PTHrP mRNA expression as expressed in fm was quite different from sample to sample, but was higher in five of seven cases compared with corresponding normal tissues (data not shown). Again, the profile of the expression of the PTHrP mRNA isoforms was similar to what we observed in vitro with a near predominance of the 141 PTHrP mRNA isoform, in all cases in tumors as well as in normal tissues, except for patient 3 (Figure 3A and B, pT1a and pT1b, respectively). In pT3 samples, total PTHrP mRNA expression as expressed in fm was also quite different in the three samples, but was higher in all tumors compared with normal corresponding tissues (data not shown). Again, the 141 PTHrP mRNA isoform was predominant in all samples except for one normal tissue, patient 8, where this isoform was equal to the 173 PTHrP mRNA isoform (Figure 3C).

**Eight proteins bind to the 3'-UTR of the predominant 141 PTHrP mRNA isoform**

Because of the large predominance of the 141 PTHrP mRNA isoform independently of VHL expression in vitro and in vivo, we focalized the search of RNA-binding proteins on that particular isoform in the subsequent experiments.

For that, we prepared three overlapping biotinylated RNA probes corresponding to the 3'-UTR of exon 9 specific for the 141 PTHrP mRNA isoform as well as a probe corresponding to the whole 3'-UTR (Figure 4A). The 3'-UTR of exon 9 is particularly rich in AU sequences and contains 5'-AUUUA-3' sequence, known to be important in mRNA turnover (17). After a first UV cross-linking reaction set with protein extracts from vector alone- or VHL-transfected 786-0 clones, bound protein isolation, gel electrophoresis and revelation with silver nitrate, eight bands were observed with apparent molecular weight ranging from 20 to 75 kDa that were or not differentially expressed depending on the VHL status of the protein cell extract (Figure 4B).

However, these bands were only observed with the probe corresponding to the whole 3’-UTR sequence. Indeed, with the other three probes, we were unable to get relevant and reproducible results (data not shown). Among these bands, we were interested in the band having an apparent molecular weight of ~30 kDa, which was most abundant in vector alone-transfected cells compared with VHL-transfected cells, since it could correspond to HuR, a RNA-binding protein with well-known stabilizing and/or translational activation functions (18).

We thus next examined this possibility by immunoblotting. For that, a second set of UV cross-linking reactions were performed with protein extracts from untransfected 786-0 cells and two clones of vector alone, VHL, and AVHL cells with the biotinylated RNA probe corresponding to the whole 3’-UTR sequence of the 141 PTHrP mRNA isoform. Proteins bound to RNAs were isolated and subjected to western blot for HuR detection. HuR was detected in all samples but was substantially more abundant in untransfected 786-0 cells and in 786-0 V and 786-0 ΔVHL clones compared with 786-0 VHL clones (Figure 4C).

**Since controversy exists concerning HuR expression level as a function of VHL status, we also performed HuR expression level test by immunoblot in 786-0 wt, V, VHL and AVHL cells, and to western blot for HuR detection.**

**HuR knockdown decreases HuR, PTHrP, VEGF and TGF-β mRNAs expression**

The transient transfection with HuR siRNA decreased HuR expression by up to 80% after 48 h (Figure 6A). The expressions of PTHrP, VEGF and TGF-β were significantly decreased in cells transfected with HuR siRNA (Figure 6B).

**HuR knockdown decreases the 141 PTHrP mRNA isoform stability**

The 141 PTHrP mRNA isoform stability was decreased as a function of time and by ~80% in cells transfected with HuR siRNA after 72 h, with a profile similar to what we observed in Figure 5B and C.

**HuR knockdown decreases human CRCC cell proliferation**

We then explored the effects of HuR gene silencing on cell proliferation in human 786-0 cells. As shown in Figure 6A, HuR knockdown induced a time-dependent decrease in cell density of up to 60%, the effect being maximal 96 h post-transfection. These effects were confirmed by BrdU incorporation studies (Figure 6B). No effects were observed after transfection with Ct siRNA for both cell counting and BrdU assay.

**Discussion**

In part of our former studies, we identified PTHrP as a new target for the VHL/HIF system. The regulation of PTHrP expression by this

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<th>Target</th>
<th>Sense primer (5’–3’)</th>
<th>Antisense primer (5’–3’)</th>
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<tr>
<td>Common human PTHrP</td>
<td>CGGGTTTCTCTTGCTGAGCTA</td>
<td>TGCCAGATGGTGAAAGGA</td>
</tr>
<tr>
<td>139 Human PTHrP isoform</td>
<td>TCTCAGCGCGCGCGCTCATC</td>
<td>AGAGAAGCGTGTATTACG</td>
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<tr>
<td>141 Human PTHrP isoform</td>
<td>ACTGCTGCTGCTGCTGTA</td>
<td>CAAGGCTGCTGATGTTACG</td>
</tr>
<tr>
<td>173 Human PTHrP isoform</td>
<td>GGAATAGATAGAAGCTCACAG</td>
<td>GACAAATGAGGAGGCCCTG</td>
</tr>
<tr>
<td>Human VEGF</td>
<td>AAGGCTGAGAAGGAGAGA</td>
<td>TTTCTGCGCTTTCGTTTCTT</td>
</tr>
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<td>Human TGF-β</td>
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<tr>
<td>Human GAPDH</td>
<td>GGAAGGGTGAAAGGGCAAGTC</td>
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system was taking place specifically at the post-transcriptional level, the PTHrP mRNA half-life as well as the expression of the protein itself being decreased by ~50% by the reintroduction by gene transfer of the human VHL gene in CRCC cells deficient in VHL expression (11). Although many of the VHL/HIF system target genes are regulated at the transcriptional level by HIF, it should be stressed that the majority of them are also controlled at the level of mRNA stability by this system. It is now well accepted that increasing the stability of mRNA lead to a higher level of expression of the protein into consideration than its transcriptional control (19). Most importantly, among the known VHL/HIF target genes, PTHrP belongs to a particular group of targets being specifically controlled at the level of mRNA stability, to which TGF-α and TGF-β also belong (20). From all these above-mentioned studies, we have hypothesized that common mRNA-binding proteins may exist that are under the control of the VHL/HIF system and thus involved in the overall regulation of target genes expression.

The human PTHrP gene consists of nine exons and is the subject of intense regulations giving rise, through alternative splicing and through the control by three different promoters (two TATA boxes and one GC box), to up to 12 transcripts and three initial translation products containing 139, 141 and 173 amino acids in a tissue-specific manner (21). This latter isoform is specific to humans. We have developed herein a technic to quantify the expression of each PTHrP mRNA isoform along with that of total PTHrP mRNA in human CRCC. We show that all three isoforms are expressed in vitro and in vivo at various concentrations but with a similar expression profile revealing a large predominance of the 141 PTHrP mRNA expression. In the panel of human CRCC cell lines tested, total PTHrP mRNA expression was higher in cells deficient in VHL compared with cells expressing VHL, which is in accordance with the negative regulatory effect of the VHL/HIF system on PTHrP expression we observed in the present study and also in our recent studies in which we measured immunoreactive PTHrP level in the same cell lines (11). However, it would be probably necessary to test more cell lines to definitively settle down such results. Our results also indicate that the expression profile of PTHrP mRNAs is not affected by VHL. However, one interesting observation is that the reintroduction of VHL determines a similar decrease in the expression of all three PTHrP isoforms and of total PTHrP mRNA expression, suggesting

Fig. 2. The PTHrP mRNA expression profile is not dependent on the VHL status. Expression in fM of all three PTHrP mRNA isoforms in untransfected 786-0 cells (786-0 wt) and in 786-0 cellular clones transfected with vector alone (786-0 V), with human VHL (786-0 VHL) and with truncated inactive VHL (786-0 ΔVHL). Results are shown as mean ± SEM, n = 4; *P < 0.05 expression of PTHrP mRNA isoforms in 786-0 VHL versus in 786-0 wt, 786-0 V and 786-0 ΔVHL. The expressions of each PTHrP mRNA isoform were not different in 786-0 wt, 786-0 V and ΔVHL (A). Same results as in (A) but plotted individually for total PTHrP mRNA and for the three PTHrP mRNA isoforms in 786-0 wt and transfected clones. Results are shown as mean ± SEM, n = 4; *P < 0.05 expression in 786-0 VHL versus in 786-0 wt, 786-0 V and 786-0 ΔVHL (B).
that the VHL/HIF system acts on the expression of the three isoforms.

Pairwise comparisons between cancer and normal tissues also revealed a similar PTHrP isoforms expression profile suggesting that tumors did not modify qualitatively PTHrP expression. PTHrP expression was variable from sample to sample and was overexpressed in the majority of tumors compared with the corresponding normal tissues, which is in accordance with the two studies dealing with such aspect in human CRCC (22). Interestingly, PTHrP mRNA concentrations were lower in pT3 samples compared with pT1b samples that were also lower compared with pT1a samples suggesting that PTHrP might be more important at early tumor stages or that the tumor needs for PTHrP decreases with tumor stage. The difference in PTHrP expression in the various samples could be dependent on the VHL status of the tumor, but VHL status was not explored in this study. Again, more pairwise comparisons between cancer and normal tissues would be necessary to confirm such assumption.

The three PTHrP mRNA isoforms share the majority of the coding region of $\text{PTHrP}$ but have a unique 3′-UTR. The biological significance of these regions has not been defined yet, but could intervene in the selection of the isoforms produced by the tissue, not only depending on the environmental conditions but also depending on the

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**Fig. 3.** The 141 PTHrP mRNA isoform is preponderantly expressed in pT1 and pT3 tumors. Expression in fM of all three PTHrP mRNA isoforms in pT1a tumors (T1, 2, 3 and 4) and normal corresponding tissues (N1, 2, 3 and 4). Results are shown as mean ± SEM, $n = 4$; *$P < 0.05$ expression of all PTHrP mRNA isoforms in tumors versus normal corresponding tissues (A). Expression in fM of all three PTHrP mRNA isoforms in pT1b tumors (T5, 6 and 7) and normal corresponding tissues (N5, 6 and 7). Results are shown as mean ± SEM, $n = 4$; *$P < 0.05$ and **$P < 0.01$ expression of all PTHrP mRNA isoforms in tumors versus normal corresponding tissues (B). Expression in fM of all three PTHrP mRNA isoforms in pT3 tumors (T8, 9 and 10) and normal corresponding tissues (N8, 9 and 10). Results are shown as mean ± SEM, $n = 4$; *$P < 0.05$ and **$P < 0.01$ expression of total PTHrP mRNA in tumors versus normal corresponding tissues (C).
phenotype, normal versus tumoral. According to that, various concentrations of these diverse isoforms have been demonstrated to be expressed in a cell- or tissue-dependent manner in normal and pathologic conditions such as cancer (21,23). In addition to that, cell line-specific utilization of the promoters and the 3\(^\#\) alternative splicing pathways was detected among various tissues in humans including bone, breast, kidney and lung cell lines, and each cell line could potentially produce the three PTHrP isoforms (23). The observations that cell transformation is not at the origin of a preferential expression of one or more PTHrP mRNA isoforms in human CRCC contrasts with distinct studies obtained in other tumors. For example, Sellers et al. (21) have shown that the 141 PTHrP mRNA isoform was the exclusive isoform expressed in normal human lung cells, whereas lung tumor cells expressed all three isoforms with a clear predominance of the 139 PTHrP mRNA isoform followed by the 141 and the 173 PTHrP mRNA isoforms (23). Similar observations were made by the same authors in immortalized human keratinocytes. In their studies, Richard et al. (23) have shown through a quantitative approach in the 786-0 human CRCC cell line, i.e. one of the cell line we used herein, that these cells expressed almost exclusively the 139 PTHrP mRNA isoform (>90%), whereas the 141 PTHrP mRNA isoform is expressed at low level (<10%). These results are in contradiction with the results we report in the present study not only in this cell line but also in a panel of other human CRCC cell lines and in freshly harvested tumors and non-malignant corresponding tissues. Although we do not have clear explanations for such a discrepancy, it should be stressed that in their publication these authors (same group than reference 23 above) also reported that the expression profile of PTHrP mRNAs in normal lung tissue was similar to the tumor lung tissue, with an expression of the three PTHrP mRNA isoforms and even a preponderant expression of the 141 PTHrP mRNA isoform (50%), results that contrast with their subsequent studies cited above.

**Fig. 4.** Eight proteins bind to the 3\(^\#\)-UTR of the 141 PTHrP mRNA isoform. The 3\(^\#\)-UTR of the 141 PTHrP mRNA isoform was divided in three regions or not divided. The corresponding in vitro transcribed RNAs, shown on the agarose gel (right), were subjected to UV cross-linking with S100 protein extract (A). Eight proteins of vector alone- or VHL-transfected clones were cross-linked to the biotinylated RNA probe corresponding to the whole 3\(^\#\)-UTR of the 141 PTHrP mRNA isoform. One of the predominant protein had a molecular weight of ~30–35 kDa. VHL, S100 extract from 786-0 VHL; V, S100 extract from 786-0 V; w/o Prot, lane without protein. The silver nitrate-stained gel shown is representative of at least three independent experiments (B). Immunoblots for HuR after UV cross-linking of protein extracts of 786-0 wt and 786-0 V, VHL and ΔVHL clones with the biotinylated RNA probe corresponding to the whole 3\(^\#\)-UTR of the 141 PTHrP mRNA isoform. Shown is a representative gel of at least three independent experiments (C). Immunoblots for HuR (left) and corresponding β-actin (right) in untransfected 786-0 cells (wt) and in clones transfected with vector alone (clone V3), VHL (clone VHL2) and ΔVHL (clone ΔVHL3) treated with cycloheximide for 0–48 h, as indicated. Shown are representative immunoblots of at least three independent experiments (D).
Evidence of altered regulation of PTHrP mRNAs has also been evidenced in other cancer types including breast and prostate cancers (24,25), although the pre-mRNA processing of PTHrP has not yet been the subject of intense studies. However, and importantly, an increase of the expression of the 139 PTHrP mRNA isoform and corresponding protein have been correlated to the risk of bone metastasis in breast cancer (26). In addition, differential expression of PTHrP mRNAs is probably to be involved in the pathogenesis of humoral hypercalcemia of malignancy. Alternative splicing is an important mechanism of gene regulation allowing to increase the coding capacity of the genome and thus protein diversity (27), generally in a cell- or tissue-specific manner. Alternative splicing and post-translational processing can serve as a regulatory mechanism, whereby developmental programming and environmental factors and stimuli affect biological activities of translated proteins. Multiple examples exist in the literature of such proteins including cyclooxygenase-1 and -2 and calcitonin gene-related peptide of insulin-like growth factor-1 leading to the synthesis of multiple proteins with distinct biological functions (28–30). Many of the transcriptional products of these proteins contain AU-rich sequences in their 5′ and/or 3′ end, including the consensus 5′-AUUUA-3′. These sequences are known to be involved in the rapid degradation of labile mRNAs (31), generally through the binding of destabilizing mRNA-binding proteins. Two copies of this sequence are retrieved in the 3′-UTR of the 139 and 173 PTHrP mRNA isoforms and four copies in the 3′-UTR of the 141 PTHrP mRNA isoform. Interestingly, more recent findings also indicate that AU-rich sequences could be the sites of binding of mRNA-stabilizing proteins (32,33).

The presence of multiple copies of the 5′-AUUUA-3′ sequence in the 3′-UTR of the 141 PTHrP mRNA, the fact that this region is rich in A and U residues, along with the observation that this isoform is largely preponderant in human CRCC led us to further analyze the mechanism of PTHrP mRNA stability on this particular isoform. The mechanisms of the post-transcriptional control of gene expression by the VHL/HIF system still remain unknown, although the majority, if not all, of the target genes of this system are regulated, at least partially, at the level of mRNA stability. Various mechanisms may account for mRNA stabilization/destabilization such as the expression and/or activity of mRNA-degrading enzymes (34,35) or the binding of specific proteins. A broad range of AU-binding proteins exist, the majority of them being turned toward mRNA destabilization and degradation, such as tristetrapolin and AU-rich element/poly(U)-binding/degradation factor-1, whereas others, and predominantly HuR, stabilize the mRNA of target proteins and/or increase their translation (36–39). In other studies, a few proteins with mobilities of 30–100 kDa have been shown to bind to the 3′-UTR of the 139 and 141 PTHrP mRNA isoforms (40,41). These proteins were not identified but their binding, and consequently the PTHrP isoforms stabilities, was shown to be regulated by epidermal growth factor or TGF-β. One of the proteins we identified had an apparent mobility of ~30 kDa, corresponding to the molecular weight of the mRNA-binding protein HuR, that was

**Fig. 5.** HuR knockdown decreases the expression of PTHrP, VEGF and TGF-β mRNAs and cell growth in human CRCC cell lines. Immunoblot for HuR and corresponding β-actin in 786-0 cells transiently transfected with HuR siRNA or control siRNA for 1–6 days. Shown is a representative immunoblot of at least three independent experiments (A). Effects of transient transfection with HuR siRNA, control siRNA (Ctl siRNA) or siRNA diluent for up to 72 h on HuR, PTHrP, VEGF and TGF-β mRNAs expression in 786-0 cells. Results are shown as mean ± SEM, n = 3; *P < 0.05; **P < 0.01 and ***P < 0.001 from corresponding control at 0 h (B). Stability of the 141 PTHrP mRNA isoform in 786-0 cells transiently transfected with HuR siRNA for up to 72 h as expressed as the stability from cells transiently transfected with Ctl siRNA. Results are shown as mean ± SEM, n = 3; *P < 0.05 and **P < 0.01 from Ctl siRNA-transfected cells (C).

**Fig. 6.** HuR knockdown decreases cell growth in human CRCC cell lines. Effects of HuR knockdown on cell density (A) and BrdU incorporation (B) in 786-0 cells. Results are shown as mean ± SEM, n = 4; **P < 0.01 from 786-0 wt, Ctl siRNA and siRNA diluent.
differentially bound as a function of VHL expression. HuR (Hu antigen R or ELAVL1) belongs to the Drosophila-like embryonic lethal abnormal vision family of RNA-binding proteins, which also includes HuB, HuC and HuD. HuR is ubiquitous, whereas the other members are primarily found in the nervous system (42). All members of this family participate in the post-transcriptional regulation of mRNAs bearing U- and AU-rich elements (42). HuR has crucial roles in mRNA stabilization/translation of numerous growth, motility and angiogenic factors including epidermal growth factor, VEGF, platelet-derived growth factor, TGF-β and HIF-1α, all shown to be important in renal tumorigenesis. In addition, HuR has been shown to be sequestered by VHL in human CRCC (43), suggesting that the level of available HuR protein would be decreased in the presence of VHL, although in their study, the total level of HuR was equal in cells expressing or not VHL. According to this, Galbán et al. (44) have shown in human CRCC that total expression of HuR was equal in cells expressing or not VHL, but that its cytoplasmic presence was higher in cells expressing VHL compared with cells not expressing it. So what could be the role of VHL in the regulation of available HuR levels in the nucleus and in the cytoplasm remains to be uncovered. These properties led us to analyze whether HuR accounts for the increase in PTHrP mRNA expression we previously observed in relation to the VHL status in human CRCC. As expected from its binding, we indeed observed a substantial and very similar decrease in PTHrP mRNA expression and in the stability of the main PTHrP mRNA isoform after HuR knockdown, still arguing the preponderance of the 141 PTHrP mRNA isoform. Similar effects were also observed on the total expression levels of VEGF and TGF-β1 mRNAs. In our hand, and according to the results obtained by Dutta et al. (43) and Galbán et al. (44), cycloheximide treatment did not affect the stability of HuR protein, regardless of the VHL status. The antitumor effect we observed on cultured cells after HuR depletion may thus reflect the downregulation of these, and probably additional, secreted growth factors. It will be interesting to determine the overall role of HuR in human CRCC growth not only in vitro as herein but also in vivo, and this particular point is currently under investigation in our laboratory.

What remains to be defined from our studies is the binding sequence of HuR in the 3’-UTR of the 141 PTHrP mRNA isoform. The 3’ end of this particular UTR is rich in A and U residues, shown to be important for HuR binding (45), and thus this sequence could contain the HuR-binding site. Unfortunately, we were unable to get relevant results with the three overlapping sequence of the 3’-UTR of the PTHrP mRNA isoform. Additional studies will be conducted to respond to this question. In addition, the identity of the additional binding proteins we observed herein remains unknown and will be the subject of mass spectral analysis. However, among the three and the four proteins with an apparent molecular weight below and above 30 kDA, respectively, some may correspond to what have been observed in other studies dealing with VEGF, erythropoietin, Glut-1 transporter and adenomdedullin in hypoxic conditions and PTHrP (46,47).

We show here that common mRNA-binding proteins regulated by the VHL/HIF system, such as HuR, leading to increased expression of a panel of tumorigenic factors do exist. Targeting specifically such proteins with mRNA stability properties might pave the way for the definition of new therapeutic options against human CRCC.

Supplementary material

Supplementary Table 1 can be found at http://carcin.oxfordjournals.org/

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References


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