Role of the RNA-binding protein HuR in human renal cell carcinoma

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Human conventional renal cell carcinoma (CRCC) remains resistant to therapy. The RNA-binding protein HuR regulates the stability and/or translation of multiple messenger RNAs involved in malignant transformation. In this study, we aimed to evaluate the potential role of HuR in this pathology. Using seven human CRCC cell lines expressing or not the von Hippel-Lindau (VHL) tumor suppressor gene as well as 15 normal/renal cell carcinoma tumor pairs, we showed that HuR is overexpressed in all tumors independently of the VHL status. Futhermore, HuR cytoplasmic presence appears to be more common in early tumor stages, suggesting a role in tumor promotion. We then assessed the effect of HuR knockdown using small interfering RNA in cultured cell and of the hypoxia-inducible transcription factor-2

Introduction

Renal cell carcinoma (RCC) represents a heterogeneous group of tumors accounting for 3% of adult malignancy and results in over 100,000 deaths worldwide annually (1,2). It 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 ~75% of all cases. The 5 year survival rate is 70% for localized RCC, but decreases to only 5% in the metastatic group, mainly because RCC is a radiation- and chemotherapy-resistant tumor and because <15% of patients respond to immunotherapy that has been the only therapeutic approach for RCC for long (3). Recent advances have led to novel targeted therapeutic approaches with small molecule tyrosine kinase receptor inhibitors such as sunitinib or sorafenib. These two compounds, that have been approved for the treatment of advanced kidney cancer by the Food and Drug Administration and European Union in 2006 and 2007, respectively, improve progression-free survival but do not appear to have an effect on overall survival (1,4,5). In addition, this effect is limited in time due to the development of tumor resistance and they cause clinically significant toxic effects.

Abbreviations: CRC, Conventional renal cell carcinoma; HIF, hypoxia-induced transcription factor; MAPK, mitogen-activated protein kinase; mRNA, messenger RNA; NF-κB, nuclear factor-kappaB; PI3K, phosphoinositide 3-kinase; PTHR, parathyroid hormone-related protein; RCC, renal cell carcinoma; sRNA, small interfering RNA; TGF, tumor growth factor; VEGF, vascular endothelium growth factor, VHL, von Hippel-Lindau.

It remains urgent to develop new therapeutic options, including combination regimens, for this refractory disease. Inherited and sporadic forms of CRCC are associated with mutations, deletion or silencing of the von Hippel-Lindau (VHL) tumor suppressor gene (6,7). The VHL gene products (pVHL) are part of an E3-ubiquitin–ligase complex that degrades hypoxia-induced transcription factors (HIFs)–α in normoxic conditions. This leads to the downregulation of several HIF-target genes including angiogenic, metabolic and growth factors, such as vascular endothelium growth factor (VEGF), tumor growth factors (TGFs), parathyroid hormone-related protein (PTHrP), erythropoietin, glucose transporters and transferrin (2,7), all shown to contribute to CRCC tumorigenesis.

HuR (Hu antigen R or ELAVL1) belongs to the Drosophila-like embryonic lethal abnormal vision family of RNA-binding proteins. All members of this family, HuB, HuC and HuD, primarily found in the nervous system, and the ubiquitously expressed HuR, participate in the posttranscriptional regulation of messenger RNAs (mRNAs) bearing U- and AU-rich elements (8,9). These particular sequences are known to be intimately involved in mRNA stability and thus in gene regulation (10–11). HuR, like other embryonic lethal abnormal vision family members, contains three RNA recognition motifs through which it binds to specific mRNAs and regulates their stability and/or their translation. HuR is predominantly present in the nucleus in unstimulated cells but upon cell stimulation by, for example growth or stress factors, HuR translocates to the cytoplasm where it binds target mRNAs and in most cases prevents their decay (8,9). Target mRNAs include those encoding proteins important for cell growth (such as epidermal growth factor and various cyclins), survival (such as Bcl-2 and Mcl-1) and invasion (such as matrix metalloproteinase-9) as well as for angiogenesis (such as VEGF and HIF-1α) and evasion of immune recognition (such as galectin-1) (8,9,13). Moreover, and interestingly, we recently identified a novel target for HuR, the CRCC survival factor PTHrP, the mRNA of which is stabilized by HuR in a VHL-dependent manner (14). Thus, HuR influences all the acquired phenotypic traits of tumor cells and accordingly, it has been shown to be involved for example in the malignant transformation of breast, lung and colon cancers (15–19).

The fact that the expression of the transcription factor HIF-1α has been shown to be increased by HuR through mRNA stabilization and/or translation is of particular interest in human CRCC (13,20). Indeed, as presented above, HIF-1α is constitutively expressed in the majority of human CRCC due to the VHL deficiency. HuR, by its stabilizing effect on HIF-1α mRNA, might then sustain the proliferative, survival and angiogenic pressure initiated by HIF-1α. In addition, VHL has recently been shown through its elongin-binding domain to interact specifically with one of the RNA-binding domains of HuR. This interaction inhibited the stabilizing function of HuR: a sequestering effect that was dependent on oxygen tension since hypoxia decreased the association of HuR with VHL (21). Overall, these findings suggest, in conditions of VHL deficiency or hypoxia and in addition to its stabilizing effects on numerous mRNAs, that HuR might critically be involved in renal tumorigenesis. The observation by Lopez de Silanes et al. (19) that HuR is preferably cytoplasmic in nine RCC samples studied compared with corresponding normal tissues is in accordance with this hypothesis. However, to our knowledge, no data are available concerning a potential role of HuR in the growth of CRCC neither in vitro nor in vivo.

In this study, we have observed that HuR is overexpressed in human CRCC and subjected to nucleocytoplasmic shuttling in early tumor stages. In addition, we showed that HuR is involved in tumor growth and in the posttranscriptional control of growth and angiogenic signaling pathways that are critical for human CRCC tumorigenesis. Thus, HuR might constitute an important candidate for targeted therapy in this refractory disease.
Materials and methods

Cells, cell culture and stable transfection
Human CRCC cell lines either deficient in VHL gene expression (786-0, A498, 786-0-D 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 Sante et de la Recherche Medicale U575, Centre 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 human Von Hippel-Lindau complementary DNA hemagglutinin-tagged at the C-terminus (786-0 VHL) or inactive C-terminal truncated human Von Hippel-Lindau (1-111) (ΔVHL) that we have described previously (22).

Human CRCC biopsies
Paired tissue samples of CRCC and adjacent normal tissue of 15 patients with sporadic CRCC were obtained from the Urology department (University Hospital of Strasbourg). Informed consent was obtained from all patients. Immediately after surgical resection, tissues were fresh frozen and kept in liquid nitrogen until protein expression analysis by western blot.

siRNA transfection
small interfering RNA (siRNA) duplexes specific for human HuR and control non-silencing siRNA were obtained from Applied Biosystems (Ambion local distributor, Courtaboeuf, France). Transfections were performed using Lipofectamine following the manufacturer’s instructions.

Immunoblotting
Whole cell lysates or tumor tissues and corresponding normal tissue lysates were prepared as described (22–26). In addition, cytoplasmic and nuclear protein fractions were obtained using a nuclear extraction kit and as preconized by the manufacturer (Millipore, Billerica, MA). Protein concentrations were determined according to the method of Lowry et al. (27). Western blot and all antibodies used are described in supplementary Materials and Methods, available at Carcinogenesis Online.

HuR–RNA immunoprecipitation assay
To determine whether HuR binds directly to HIF-2α mRNA in human CRCC cells, we used the RNA-Binding Immunoprecipitation MagnaRIP Kit (Millipore). Experiments were performed following exactly the manufacturer’s protocol. Briefly, 786-0 cells were lysed in lysis buffer. The HuR–RNA complexes present in cell extracts were then immunoprecipitated using either the HuR antibody (detailed in supplementary Materials and Methods, available at Carcinogenesis Online) or the negative control normal mouse IgG with protein A/G magnetic beads. The magnetic beads bound complexes were then immobilized using a magnet followed by serial washings to wash out unbound materials. Immunoprecipitated RNAs were then extracted and analyzed by real-time reverse transcription–polymerase chain reaction for HIF-2α mRNA presence (indicating effective HuR–HIF-2α mRNA binding) again according exactly to the manufacturer’s protocol, using the ‘LightCycler–FastStart DNA Master SYBR (syber) Green’ kit (Roche Diagnostics, Meylan, France) according exactly to the manufacturer’s protocol, using the ‘LightCycler–FastStart DNA Master SYBR (syber) Green’ kit (Roche Diagnostics, Meylan, France). Sense and antisense primers for human HIF-2α were respectively: 5′-CAACAGAAGCCGTACCTGTC-3′ and 5′-TCTCGATTTGGTTCACACAT-3′. Each sample was analyzed three times and quantified with the analysis software for LightCycler (Roche Diagnostics).

Cell proliferation measurements
Cell density. CRCC cells were seeded in 24-well plates (20 000 cells/ml), grown for 48 h and then transiently transfected with 100 nM siHuR or siCtrl. The number of cells was counted 24, 48, 72 and 96 h posttransfection.

Bromodeoxyuridine incorporation studies. CRCC cells were seeded in 96-well plates (20 000 cells/ml), grown for 48 h in the presence of 10% fetal bovine serum and then in serum-free medium for 48 h prior to transient transfection with 100 nM siHuR or siCtrl. The incorporation of bromodeoxyuridine (Merck Chemicals Ltd, Nottingham, UK) was measured 96 h posttransfection, as described (22).

Assessment of cell death
CRCC cell death was analyzed by fluourescent-activated cell sorting. CRCC cells were seeded in 25 cm² plates (20 000 cells/ml) and treated as above for cell proliferation measurements. Fluorescent-activated cell sorting analysis was performed using annexin V-fluorescein isothiocyanate and propidium iodide as described (23–25).

Results

Immunoblotting analysis of HuR expression and subcellular localization in human CRCC cells and tissues
By immunoblot analysis, total HuR was shown to be expressed in all CRCC cell lines tested (Figure 1A). No difference was observed as a function of VHL expression. However, since VHL has been shown to bind and sequester HuR (21), we used the VHL-deficient 786-0 cells either non-transfected (wild-type, wt) or transfected with native VHL (786-0 VHL), truncated inactive VHL (786-0 AVLH) or with the vector alone (786-0 V) that we previously engineered (22) to assess whether VHL influences HuR expression and subcellular localization.

In our hand, and accordingly to the study of Datta et al. (21), no differences in total and subcellular expressions of HuR were observed in these cells and cellular clones (Figure 1B). The loading and purity of the extracts were followed using lamin (nuclear protein) and β-actin (cytoplasmic protein) (Figure 1B).

The total and subcellular expression of HuR were also assessed by immunoblot in freshly harvested pT1 and pT3 tumors and normal corresponding tissue specimens. Pairwise comparisons revealed a marked increase in HuR expression in 86% of pT1 tumors (six of seven samples) and in 100% of pT3 tumors (eight of eight samples) compared with corresponding non-malignant tissues (Figure 2A and B). In pT1 tumors, HuR presence was increased in the nucleus in all tumors versus corresponding non-malignant tissues and its cytoplasmic presence was increased in five tumors (72%), equal in one (14%) and lower in one tumor (14%) (Figure 2A). However, in pT3 tumors, while the nuclear presence of HuR still remained increased in 88% cases (seven of eight samples and one tumor equal to normal), its cytoplasmic presence was equal in five tumors (62.5%) and lower in three tumors (37.5%) compared with corresponding non-malignant tissues (Figure 2B). We have quantified the cytoplasmic presence of HuR in pT1 versus pT3. The relative expression intensity (tumor/normal intensity ratios, with N normalized to 1) was significantly higher in pT3 tumors (6.30 ± 2.11, P < 0.01 from 1) was significantly higher in pT3 tumors (6.30 ± 2.11, P < 0.01 from 1) was significantly higher in pT3 tumors (6.30 ± 2.11, P < 0.01 from 1) was significantly higher in pT3 tumors (6.30 ± 2.11, P < 0.01 from 1) was statistically significant.

Overall, these results strongly suggest that HuR is overexpressed in CRCC tumors compared with normal tissue and that it is significantly more abundant in the cytoplasm of pT1 tumor cells than in pT3 tumor cells suggesting a role for HuR in tumor promotion. Finally, its expression does not appear to be influenced by VHL.
Effects of HuR knockdown on human CRCC cell proliferation and death in vitro

In order to assess the significance of HuR in human carcinogenesis, we knocked down HuR expression through gene silencing technology using specific HuR siRNA.

The transient transfection of human CRCC cells with siHuR led to a time-dependent decrease in HuR expression, which was almost complete 96 h posttransfection (Figure 3A).

We then explored the effects of HuR gene silencing on cell proliferation in human CRCC. HuR knockdown induced a time-dependent decrease in cell density of up to 60%, the effect being maximal 96 h posttransfection (Figure 3B). The studies of bromodeoxyuridine incorporation in quiescent cells showed that at least part of this effect was due to inhibition of cell proliferation (Figure 3C).

In addition to regulate the expression of genes involved in cell cycle progression, HuR also controls various genes involved in cell protection to apoptosis. We therefore analyzed whether HuR knockdown might induce cell apoptosis in human CRCC, and for that, we performed fluorescent-activated cell sorting analysis. The treatment of CRCC cells with siHuR significantly increased tumor cell apoptosis, and no evidence of necrosis was observed (Figure 3D).

Consequently, in addition to be a mediator of cell proliferation, HuR is also involved in cell growth through induction of cell survival in human CRCC.

Effectiveness of intratumor HuR silencing against human CRCC in vivo

To assess the in vivo relevance of our results obtained in culture cells, nude mice were separated in two groups receiving intratumor injection of siHuR or siCtl. Intratumor injection of siHuR inhibited significantly tumor growth by ~60% compared with tumors which received injection of siCtl (Figure 4A and B). Importantly, in some mice, partial regression was observed following the treatment. All the mice maintained body weight and no difference were observed between groups (data not shown).

Western blot analysis of HuR expression in tumors harvested from each group showed that HuR is substantially downregulated in siHuR-treated tumors compared with siCtl-treated tumors indicating accurate targeting of the siRNAs in vivo (Figure 4A, top left).

By immunohistochemistry analysis, we observed that the proliferative index (Ki67 staining) was decreased by 38% and the apoptotic index (TUNEL staining) increased by 92% in siCtl-treated tumors versus siHuR-treated tumors (supplementary Figure 1A and B is available at Carcinogenesis Online). Concerning neovascularization (factor VIII staining), neither total number of vessels nor number of vessel intersecting points showed a difference between the two groups (supplementary Figure 1C is available at Carcinogenesis Online).

These results strongly indicate that HuR is critically involved in the growth of human CRCC in vivo as well and that it acts through stimulation of cell proliferation and death, as observed in cultured cells, but not through an increase in tumor neovascularization. These results strongly indicate that HuR is critically involved in the growth of human CRCC.

Effects of HuR knockdown on the expression of downstream targets in vitro and in vivo

We measured the expression of VEGF and TGF-β, two well-known HuR targets, the mRNA of which is stabilized through HuR binding, in the conditioned medium of 786-0 cells transfected with siCTL or with siHuR 96 h posttransfection as well as in xenografted tumors treated with siCtl or with siHuR. The expressions of VEGF and of TGF-β were substantially and significantly decreased by 40% and 50%, respectively, in cells transfected with siHuR 96 h posttransfection compared with cells transfected in control (Figure 5A and B). Similarly, the intratumor injection of siHuR also leads to a significant decrease in the expression of both targets (Figure 5C and D).

The expression of the transcription factor HIF-1α, which plays a critical role in human CRCC, has been shown to be increased by HuR through mRNA stabilization and/or translation (13,20). Thus, we also investigated whether this is true in our system. However, since 786-0 cells express HIF-2α, we analyzed HIF-2α expression levels in cells transfected with siCTL or with siHuR and found its expression also decreased in siHuR-transfected cells compared with siCTL-transfected cells in both cells and tissues (Figure 6A, left). To go further in this investigation, we have analyzed whether HuR interacts directly with HIF-2α mRNA. For that, we used a RNA-binding protein immunoprecipitation assay in 786-0 cells extracts, as detailed in Materials and Methods. By quantitative reverse transcription–polymerase chain reaction on immunoprecipitated HuR–mRNA complexes with specific human HIF-2α primers, the results show that HuR binds the human HIF-2α mRNA (Figure 6A, right). The fold enrichment was 65 in HuR-immunoprecipitated lysates compared with control IgG-immunoprecipitated lysates (Figure 6A, right).

We and others have shown previously that the survival pathways phosphoinositide 3-kinase (PI3K)/Akt, NF-kB and mitogen-activated protein kinase (MAPK) are critically involved in human CRCC tumorigenesis (24,25,28). Since HuR stabilizes and/or increase the translation of genes involved in the activation of these pathways, including VEGF and TGF-β (8,9) through their respective tyrosine kinase receptors and PTHrP (14), we have analyzed the phosphorylation, and thus the activation, of Akt, NF-kB and extracellular signal-regulated kinase 1/2 MAPK in cells and xenografted tumors treated with siCtl or siHuR. Both 786-0 and Caki-1 cells were used for these experiments to ensure that the results obtained are not cell specific.
Very interestingly, we observed that the activation of each of these signaling pathways is significantly decreased in HuR-depleted cells except the NF-κB pathway in both cell types and in tumor tissues (Figure 6B).

These results not only validate our experimental models but also extend the list of HuR targets to HIF-2α as well. In addition, HuR appears as a regulator of the activation state of critical signaling pathways involved in human CRCC growth, probably through the regulation of the expression of receptors and ligands acting on these pathways.

Discussion

The AU-rich elements of labile mRNAs are potent stimulators, through decapping and deadenylation, of mRNA turnover in vertebrate cells. A broad range of AU-binding proteins exists, the majority of them being turned toward mRNA destabilization and degradation, such as tristetrapolin and AUΦ-1, whereas others, and predominantly HuR, stabilize the mRNA of target proteins and/or increase their translation (8,9). Interestingly, HuR, as the other embryonic lethal abnormal vision proteins, is predominantly localized in the nucleus but undergoes a stimulus-dependent shuttling between the nucleus and the cytoplasm through a specialized HNS domain present in its hinge region (29). The nucleocytoplasmic shuttling of HuR has been linked to its mRNA stabilizing function and mRNA translation modulation (8,9) and so far its cytoplasmic translocation has been shown to be regulated by at least four signaling pathways including MAPK, adenosine monophosphate-activated protein kinase, protein kinase C and PI3K/Akt (30–35). The involvement of the PI3K/Akt survival pathway is particularly interesting knowing our previous findings that this pathway is constitutively activated in human CRCC and turn toward tumor cell growth in this disease (25). Similarly, protein kinase C and MAPK have also been shown to be activated in human CRCC and to participate in the growth of this tumor type (28,36). Although the mechanistic details of the nucleocytoplasmic shuttling is still not completely understood, recent data support the notion that this event is controlled at least by phosphorylation of the HuR protein by protein kinase C and Chk2 and probably other kinases (8,29). The mechanism of the mRNA stabilizing effects of HuR is not completely understood but could be related to the protection of mRNAs from the degradation machinery or to the displacement of mRNA-binding proteins with destabilizing functions.

Aberrant stabilization and/or translation of mRNA of oncogenes, growth factors, survival factors, angiogenic factors and proteins essential for cell migration and invasion is a critical step toward...
malignant transformation. In humans, HuR gene is localized on chromosome 19p13.2, a locus that has been shown to be associated with various translocations and oncogenic gains in tumors (37) but, to date, HuR gene mutations have never been reported in cancer. Nevertheless, since a role for HuR in antagonizing mRNA degradation has been uncovered 10 years ago, data have accumulated, which consistently showed clear correlations between HuR expression/cytoplasmic prominence and advanced malignant stages and patient survival in breast, lung, colon, gastric and ovary cancer (15–19).

HuR overexpression, activation and nucleocytoplasmic shuttling have been observed in various kidney stresses such as adenosine triphosphate depletion and metabolic acidosis (38,39). Here, we first showed that HuR is expressed in human CRCC both in vitro and in vivo as well as in normal renal tissues, consistent with its known ubiquitous expression (8,9). In all tumor stages, we observed a significantly higher total and nuclear expression of HuR in tumors versus non-malignant tissues. In the cytoplasm, a higher expression of HuR was observed in tumor cells compared with normal cells, suggesting that HuR expression is turn toward malignancy in human CRCC. However, such distribution was observed in early tumor stages but not in advanced stages suggesting that HuR activation could have important roles in the promotion of the malignant phenotype in human CRCC. Our results comfort and extend previous findings by Lopez de Silanes et al. (9) who have studied subcellular localization of HuR in 8–10 specimens of a large panel of human tumors (total 182 tumors and 135 corresponding normal tissues) including nine RCC samples. Indeed, by immunohistochemistry, they found that the tumor relative to normal HuR staining intensity was 0.8 in nuclei versus 1.6 in the

Fig. 3. HuR knockdown decreases human CRCC cell proliferation and induces cell death in vitro. (A) Western blot analysis of total HuR expression in 786-0 cell lysates that were transiently transfected with siCtl or siHuR for 0–96 h, as indicated in the figure, and incubated with antibodies against HuR and corresponding β-actin. Shown are representative radiographs of at least three independent experiments. (B and C) Effects of siCtl and siHuR transfection on human 786-0 cells proliferation. (B) Cells were transiently transfected with siCtl or siHuR for 0–96 h and adherent cells were counted at the time points indicated in the figure. Results are shown as mean ± SEM, n = 6; *P < 0.05; **P < 0.01 from siCtl. (C) Cells were transiently transfected with siCtl or siHuR for 0–96 h and subjected to bromodeoxyuridine incorporation studies. Results are shown as mean ± SEM, n = 4; *P < 0.05; **P < 0.01 from siCtl. (D) Fluorescent-activated cell sorting analysis of 786-0 transiently transfected with siCtl or siHuR for 72 or 96 h, as indicated in the figure. Examples of fluorescent-activated cell sorting analysis are illustrated on the right. No evidence of necrosis was observed in any cases. Results are shown as mean ± SEM, n = 3; **P < 0.01 from siCtl.
cytoplasm in RCC. However, in their study, the subtype of RCC tumors analyzed as well as the tumor stages were not precised. In addition, a case per case analysis of HuR distribution was not performed. Thus, the apparent slight cytoplasmic increase of HuR expression they report in the tumors is difficult to interpret but might reflect the analysis of a heterogeneous group of RCC. The important question of whether HuR might have prognostic relevance in human RCC, as in the other above-mentioned cancers, is currently under investigation in our laboratory using an extended tissue microarray built-up from 241 tumor samples and corresponding normal tissues. In human CRCC, HuR was shown to be involved in VHL-dependent elevation of p53 translation (40) and, more recently, VHL was shown to associate with the RNA recognition motif 1 of HuR inhibiting HuR-dependent stabilization of VEGF mRNA and thus VEGF expression (21). Both effects were observed in vitro and are in accordance with the tumor suppressor function of VHL in human CRCC. An increase in p53 translation through HuR binding to the 3' -untranslated region of p53 mRNA was also observed in RKO colorectal carcinoma cells exposed to UV light (41). Accordingly, UV-dependent HuR cytoplasmic presence has been demonstrated in different mammalian cells, suggesting a role for HuR in the regulation of the cellular response to UV damage through the stabilization of specific mRNAs such as the one encoding p21 (42). However, in these later studies, the link with VHL was not investigated. In the present study, we did not find any difference in total expression or subcellular presence of HuR depending on the VHL status of the cells neither in our panel of human CRCC nor in wild-type 786-0 cells compared with 786-0 cells transfected with native VHL or inactive ΔVHL. These results do not corroborate with the ones Galban et al. (40) obtained in 786-0 cells but are in accordance with those obtained by Datta et al. in the same cell type (20). Indeed, in their earlier study, Galban et al. (40) showed that the reintroduction of VHL by gene transfer in the VHL-deficient 786-0 cells compared with 786-0 cells transfected with native VHL or inactive ΔVHL. These results do not corroborate with the ones Galban et al. (40) obtained in 786-0 cells but are in accordance with those obtained by Datta et al. in the same cell type (20).

One of the most important finding herein is the demonstration that HuR participates in the overall growth of human CRCC. This was...
observed in vitro in cultured cells and in vivo in human CRCC-bearing nude mice. To our knowledge, this is the first study showing the antitumor effect of HuR knockdown in human CRCC not only in vitro but also in vivo thus pointing out the important role of HuR in renal carcinogenesis. Although this might be quite surprising knowing the biological properties of HuR, it should be stressed that the same statement could be made for other cancer types. Indeed, the large majority of studies dealing with HuR biological properties focussed on its ability to stabilize and/or translate mRNAs of proteins involved in tumorigenesis including those involved in cell proliferation, survival, migration, invasion, angiogenesis and immunosuppression (8,9), as well as on its ability to predict reduced patient survival in various cancer types (15–19), thus providing indirect evidence for its potential tumor-promoting effect. As a consequence, only a few studies are available describing posttranscriptional events involving HuR with a subsequent in vitro-based link to tumor cell proliferation, survival, migration or invasion and more especially in breast and colon cancers (19,43–46). In addition, to our knowledge, besides the study of Lopez de Silanes et al. (19) in colon cancer evoked above, the involvement of HuR in an in vivo model has not yet been described.

In their study, these authors found that RKO colorectal carcinoma cells overexpressing HuR by transfection proliferated more rapidly in vitro and produced larger tumors growing faster in nude mice, whereas cells transfected with HuR antisense or siHuR proliferated slower and produced smaller tumors in nude mice. In this later study, investigators used cells engineered by gene transfer before implantation into mice.

In the present study, we used specific siHuR to knockdown HuR in cultured cells and growing tumors. The antitumor effect of HuR knockdown was obtained at least through inhibition of cell proliferation and increase apoptosis. Although knocking down HuR decreases VEGF and TGF-β expressions as well as HIF-2α expression, we did not observe any effect on tumor neovascularization. This absence of effect is of course unexpected, but it could mean that these above-mentioned factors are preferentially turned toward tumor cell growth than tumor vascularization. According to that, it should be stressed that no consensus can be drawn from various studies dealing with the prognostic evaluation of neovascularization in human CRCC (2). In addition, in our previous work, we found induction of neovascularization despite tumor regression in nude mice bearing human CRCC treated either with an antibody against PTHrP or an inhibitor of the PI3K/Akt pathway (22,25).

The effect of HuR knockdown on HIF-2α expression is of particular interest and has never been described to date. This finding extent the inhibitory effect of HuR previously observed on HIF-1α expression (13,20). Using a RNA-binding protein immunoprecipitation assay, we also show that HuR binds directly the human HIF-2α mRNA, again as observed previously for HIF-1α, thus extending the broad list of HuR target mRNAs to HIF-2α. HuR binds to the AU-rich 3′-untranslated region of HIF-1α (20). It will be thus interesting in the future to assess whether this is the same for HIF-2α. HIFs factors are important regulators of tumor’s oxygen and nutritional supply and are constitutively expressed in the majority of human CRCC as a consequence of the VHL deficiency (2). In this regard, CRCC tumor cells expressed either HIF-1α, HIF-2α or both (47). Thus, given the convergence of multiple cancer regulatory pathways to HIF-1α and HIF-2α, our finding is particularly significant. Remarkably, HuR depletion also decreased the constitutive activation state of oncogenic signaling pathways, i.e the PI3K/Akt and MAPK pathways in two different cell lines, i.e 786-O and SK-k1 cells arguing for a non-cell specific effect. However, HuR did not influence the activation state of the NF-kB pathway in both cell lines and in tumors, which has also been shown to be constitutively activated in this pathology. This finding is particularly important since these pathways have been shown to be critically involved in CRCC tumorigenesis (24,25,28). Although a clear explanation cannot be drawn from our data, it is possible that such effects might be due to the deregulation of growth and angiogenic factors, such as epidermal growth factor, platelet-derived growth factor and VEGF, and/or their receptors that are known to be involved in human CRCC growth. Indeed, HuR has been shown to be involved in the sustained expression of such factors in various pathologic conditions (8,9). Targeting HuR might then affect the tumoral phenotype not only through the decrease of the expression of specific target mRNAs but also through the decrease of the signaling pathways activated by the translation products of these target mRNAs.

Our results obtained through intratumoral injection of siHuR clearly show not only the feasibility of such approach but also its high efficiency for HuR targeting. In a therapeutic or clinical point of view, intratumor injection of siRNA might have limited application especially due to the mode of injection itself. In this context, and of particular interest, Meisner et al. (48) recently described low-molecular-weight inhibitors from microbial broths for HuR. These compounds, i.e dehydromutactin, MS-444 and okicenone, had already been described as antitumor agents through inhibition of other targets. Although this asks the question of the specificity of these compounds against HuR, as discussed also by Meisner et al., they may serve in the near future as tools for developing specific HuR-targeting drugs usable in therapy.
Taken together, our results provide direct evidence that the mRNA-binding protein HuR is critically associated with human CRCC tumorigenesis. Targeting HuR with therapeutic targeted in vivo-based siHuR or maybe more ideally with chemical compounds that might be available soon, either alone or in combination with other agents, should provide new hope to treat this refractory disease. The fact that MS-444 compound was shown to be safe in nude mice (48) in spite of the ubiquitous expression of HuR suggests that HuR is not essential to life in adults and consequently favorably pleads for HuR inhibition as an anticancer therapy.

Elucidating the molecular processes involved in kidney tumorigenesis and resistance to therapies is central to the development of improved therapies. Kidney cancer remains a challenge for the clinician and the researcher despite the development of targeted therapies. The identification of biomarkers of human CRCC progression still remain anecdotic, although they may all pave the way for innovative therapeutic and/or prognostic options. Here, we have identified the RNA-binding protein HuR as a new marker of human kidney tumorigenesis. The depletion in HuR of human CRCC cells or tumors showed that HuR is involved in tumor growth in vitro and in vivo not only by stimulating the expression of growth factors but also by being part of the activation process of the oncogenic HIF, PI3K/Akt and MAPK pathways. These regulatory mechanisms will ultimately result in sustained tumor growth. This report identifies HuR as an innovative and accessible therapeutic option for human CRCC.

Supplementary material
Supplementary Materials and Methods and Figure 1 can be found at http://carcin.oxfordjournals.org/

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