HAVCR/KIM-1 Activates the IL-6/STAT-3 Pathway in Clear Cell Renal Cell Carcinoma and Determines Tumor Progression and Patient Outcome

Thaís Cuadros1, Enric Trilla3, Eduard Sarró1, Maya R. Viïl1, Jordi Vilardell5, Mayte Salcedo4, Joan López-Hellín1, Alex Sánchez2, Santiago Ramón y Cajal6, Emilio Itarte5, Juan Morote5, and Anna Meseguer1,6,7

Abstract
Renal cell carcinoma (RCC), the third most prevalent urological cancer, claims more than 100,000 lives/year worldwide. The clear cell variant (ccRCC) is the most common and aggressive subtype of this disease. While commonly asymptomatic, more than 30% of ccRCC are diagnosed when already metastatic, resulting in a 95% mortality rate. Notably, nearly one-third of organ-confined cancers treated by nephrectomy develop metastasis during follow-up care. At present, diagnostic and prognostic biomarkers to screen, diagnose, and monitor renal cancers are clearly needed. The gene encoding the cell surface molecule HAVCR1/KIM-1 is a suggested susceptibility gene for ccRCC and ectodomain shedding of this molecule may be a predictive biomarker of tumor progression. Microarray analysis of 769-P ccRCC-derived cells where HAVCR/KIM-1 levels have been upregulated or silenced revealed relevant HAVCR/KIM-1–related targets, some of which were further analyzed in a cohort of 98 ccRCC patients with 100 month follow-up. We found that HAVCR/KIM-1 activates the IL-6/STAT-3/HIF-1α axis in ccRCC-derived cell lines, which depends on HAVCR/KIM-1 shedding. Moreover, we found that pSTAT-3 S727 levels represented an independent prognostic factor for ccRCC patients. Our results suggest that HAVCR/KIM-1 upregulation in tumors might represent a novel mechanism to activate tumor growth and angiogenesis and that pSTAT-3 S727 is an independent prognostic factor for ccRCC. Cancer Res; 74(5); 1416–28. ©2014 AACR.

Introduction
Renal cell carcinoma (RCC) is the third most prevalent urological cancer. It accounts for approximately 3% of all new cancer cases and the incidence rates for all stages have been rising steadily over the last 3 decades (1–3). The clear cell RCC (ccRCC) is the most common subtype and accounts for approximately 80% of all renal cancers. Commonly asymptomatic, one third of ccRCCs are diagnosed when they are already metastatic, resulting in a 95% mortality rate. Moreover, one third of organ-confined cancers treated by nephrectomy develop metastasis during the follow-up (4). Although ccRCC is resistant to chemotherapy, development of new therapies has improved the median survival period of patients with advanced ccRCC, which is about 26 months now (5).

Hepatitis A virus receptor/kidney injury molecule 1 (HAVCR/KIM-1) is overexpressed in ccRCC tumors and blocks expression of epithelial differentiation markers when over-expressed in 769-P cells (6). The HAVCR1 gene codes for a type I transmembrane glycoprotein that contains an extracellular immunoglobulin-like domain topped a long mucin-like sequence, which is shed and released by metalloproteinases. HAVCR, initially described in primate kidney cells (7), represents the founding member of the HAVCR/KIM/TIM family (8, 9). HAVCR/KIM-1 ectodomain detection in urine represents a diagnostic marker for acute kidney injury (10, 11) and early detection of ccRCC (12, 13). HAVCR/KIM-1 ectodomain shedding has been recently correlated with a more invasive phenotype in vitro and more aggressive tumors in vivo (14).

To examine the biologic function of HAVCR/KIM-1 effects in ccRCC, microarray assays on 769-P ccRCC–derived cells, with upregulated or silenced HAVCR/KIM-1 levels, were conducted and relevant HAVCR/KIM-1 targets further analyzed in patients with ccRCC. Results presented in this article provide relevant data to better understand the role of HAVCR/KIM-1 on renal cancer development and progression.

Patients and Methods
A complete description is given in Supplementary Patients and Methods.

Case selection
Clinical and pathologic data from 168 patients with renal masses treated with radical or partial nephrectomy for RCC...
between January 2002 and December 2005 at Vall d’Hebron Hospital were reviewed. Standard tumor registry data and outcome information were retrospectively collected by tumor registrars for only ccRCC (n = 98). Pathologic information was based on primary tumor histology on re-review of surgical samples to determine histologic subtype using the World Health Organization (WHO) 2004 criteria. Anatomic extent of the tumor was classified using the 2010 tumor-node-metastasis system, and clinical and follow-up information was based on physician reports. Prognostic stratification of patients affected by ccRCC was scored using the University of California, Los Angeles (UCLA) Integrated Staging System—Union Internationale Contre le Cancer (UICC) nomogram.

**Tumor microarray design**

Tissue microarray (TMA) was constructed from a total of 98 ccRCC, as described in ref. 14.

**Immunohistochemistry**

Immunohistochemistry was performed as described in ref. 14 using mouse monoclonal antibody (mAb) against pSTAT-3 Y705 and rabbit mAb against pSTAT-3 S727 (Cell Signaling Technology). Two pathologists, blinded to clinicopathologic variables, evaluated TMA sections.

**Statistical analyses**

Associations between pSTAT-3 S727 and pSTAT-3 Y705 expression and clinicopathologic parameters were evaluated with the nonparametric Mann–Whitney U test. Disease-free survival was calculated as the date of surgery to the date of loco-regional or distant recurrence. Subsequently, Kaplan–Meier survival estimates were compared using the log-rank test. Multivariate analysis was performed using a Cox regression model to estimate the independent prognostic importance of clinicopathologic parameters. Statistical analysis was performed with the Statistical Package for Social Sciences, version 12 software (SPSS).

**Cell culture**

Human renal adenocarcinoma cell lines, 769-P(CRL-1933) and 786-O (CRL-1932), and the human embryonic kidney cell line, HEK293T (CRL-11268), obtained from American Type Culture Collection, were cultured as described in ref. 14. The 769-P and 786-O cell lines were authenticated by LGC Standards.

**Establishment of stable cell lines**

For HAVCR/KIM-1- and interleukin (IL)-6–silenced cell lines, three short hairpin RNAs (shRNA) of the human HAVCR or IL-6 genes were used (MISSIONshRNA; Sigma) and introduced into the 769-P cell line by lentiviral infection. Transfection with a MISSION nontarget shRNA control vector was used as negative control. For HAVCR/KIM-1 overexpression in 769-P cell line, HAVCR/KIM-1 cDNA (ID: 26762) was cloned into the pBGI-2r vector (kindly provided by Dr. J. Madrenas, University of Western Ontario, London, Ontario, Canada) including an human influenza hemagglutinin (HA) epitope and stably transfected in 769-P cells using Lipofectamine and Plus reagent (Invitrogen). Overexpression of HAVCR and its shedding mutants (FUW 246-274 and FUW 267-278) in the 786-O cell line was previously described (14).

**Cell lysate preparation and Western blot analysis**

Cell lysates from HAVCR/KIM-1–overexpressing or HAVCR/KIM-1–silenced 769-P cells and Western blot assays were performed as described in ref. 14.

**Proliferation assay**

Cell proliferation assays were carried out by labeling 769-P cells with the amine reactive fluorescent probe 5(6)-Carboxy-fluorescein diacetate N-succinimidyl ester (CFSE; Sigma-Aldrich). Fluorescence was measured using FACSCalibur (Becton Dickinson). Data were analyzed using the FCS Express 4 software. Parent cells correspond to cells labeled and analyzed immediately after labeling.

**Wound healing**

769-P cells were cultured to confluence and artificial wound was created by scratching cells with a 200-μl pipette tip. Closure-denuded area was monitored using the Live Cell-R Station (Olympus). Digital images were obtained every 30 minutes. Width of wounds at every time point was measured using the ImageJ software. Results are representative of three independent experiments.

**Microarray expression analysis**

Total RNA was extracted from cells either overexpressing HAVCR/KIM-1 or with HAVCR/KIM-1 silenced from three separate experiments. Twelve independent microarrays were performed using the Human Exon 1.0 arrays (Affymetrix–Genechip array) at Vall d’Hebron Institute of Research Genomics facility. Statistical microarray analyses are described in Supplementary Patients and Methods. Minimum Information About a Microarray Gene Experiment (MIAME) guidelines have been followed. Accession number for datasets is E-MTAB-2071.

**Reverse transcriptase quantitative PCR**

We used the 7500 Real-Time PCR System (Applied Biosystems) with the following TaqMan probes SNAI2 (Hs00950344_m1), SMARCA1 (Hs00161922_m1), ANPEP (Hs00174265_m1), IL-6 (Hs00985639_m1), HIF-1A (Hs0015153_m1), SLC2A1 (Hs00892681_m1), VEGFA (Hs00900055_m1), and PPIA (Hs00999904_m1) genes. PPIA probe was used as endogenous control. Triplicate PCR amplifications were performed for each sample.

**IL-6 ELISA**

IL-6 levels in cell culture supernatants were measured using the commercially available quantitative assay from R&D Systems (cat# D6050).

**Indirect immunofluorescence assay**

Cells grown on 24-well chamber slides from Nunc (Fisher Scientific) were processed at confluence, as described in ref. 14. Antibodies used include mouse mAb HAVCR/KIM-1 antibody (R&D Systems), rabbit mAb gp130 (Santa Cruz Biotechnology), and influenza hemagglutinin (HA) epitope.
HAVCR/KIM-1 Activates the IL-6/STAT-3 Pathway

HAVCR/KIM-1 overexpression or silencing was studied in 769-P cells because they exhibit endogenous HAVCR/KIM-1 levels allowing overexpression or downregulation in the same cell context. HAVCR/KIM-1 levels in 769-P cells overexpressing HAVCR/KIM-1 (HK-16R) or control cells (CR4) are shown in Fig. 1A. For HAVCR/KIM-1 silencing, 769-P stable cell pools obtained from three different si-HAVCR/KIM-1 RNAs and their corresponding siRNA control cells were tested for HAVCR/KIM-1 protein expression (Fig. 1B). From them, sh851 showed to be the most effective and was chosen for further experiments. Immunocytochemistry showed that subcellular location of HAVCR/KIM-1 is not affected by its overexpression or silencing (Fig. 1C). Effects of HAVCR/KIM-1 in proliferation and migration were analyzed in 769-P cells. We observed that HAVCR/KIM-1 overexpression or silencing promotes statistically significant augmented or decreased cell proliferation index, respectively (Fig. 1D), indicating that HAVCR/KIM-1 induces cell growth. Cell migration assays performed by wound-healing experiments showed that HAVCR/KIM-1 overexpression induced a slight delay on migration at short times that was nonstatistically significant at later times. Contrarily, cells migrated faster than controls when HAVCR/KIM-1 levels were below constitutive levels (Fig. 1E). Overall, results suggest that tumor cells might control cell growth and migration by modulating HAVCR/KIM-1/1 expression levels.

HAVCR/KIM-1 overexpression or silencing affects gene expression in ccRCC cells: microarray assays

To explore the effects of HAVCR/KIM-1 up or downregulation on global gene expression, cDNA microarray analyses were performed using the 769-P-modified cell lines from above. A gene was called differentially expressed if the adjusted P value was less than 0.01 and the log-fold change was at least 1 (fold change of at least 2). These analyses result in two sets of genes that are represented in volcano plots as those modulated by HAVCR/KIM-1 silencing (Fig. 2A, left) or overexpression (Fig. 2A, right). From 466 genes significantly regulated by HAVCR/KIM-1 overexpression and 236 genes regulated by HAVCR/KIM-1 silencing, 112 genes were altered in both situations. Indeed, all of the 112 genes that were commonly regulated in both experiments underwent changes in opposite directions (Fig. 2B). Figure 2C depicts a heatmap of expression profiles of differentially expressed genes. Ingenuity Pathway Analysis (IPA) was used to identify pathways that were affected by the genes selected and also to represent the relations appearing between genes. These analyses revealed highly significant alterations mainly in cancer but also in gastrointestinal, hematologic, infectious, and cardiovascular disease–related molecules, upon HAVCR/KIM-1 overexpression or silencing. Gene expression, cell death and survival, cell proliferation, cell development, and cell cycle were the most significant molecular and cellular functions correlating with HAVCR/KIM-1 expression levels. Reverse transcriptase quantitative PCR (RT-qPCR) data of randomly selected genes confirmed and validated the microarray results (Fig. 2D).

HAVCR/KIM-1 protein levels and ectodomain shedding control IL-6 mRNA levels in ccRCC-derived cell lines

Among the 112 genes altered by both up or downregulation of HAVCR/KIM-1, we decided to study IL-6 and its downstream pathway because it has been described that the majority of patients with metastatic RCC and poor survival exhibit increased IL-6 serum levels. Moreover, it was observed that primary ccRCC cultures express IL-6 (15–18). IPA diagrams represented in Fig. 3A show that essential components of the IL-6 signaling pathway are altered by HAVCR/KIM-1 overexpression (left) or silencing (right). RT-qPCR assays confirmed that 769-P cells overexpressing HAVCR/KIM-1 exhibited increased mRNA IL-6 levels, whereas HAVCR/KIM-1–silenced 769-P cells showed diminished IL-6 levels (Fig. 3B, left). RT-qPCR performed in independent HAVCR/KIM-1–...
Figure 2. Microarray assay results in 769-P cells with HAVCR/KIM-1 overexpression or silencing. A, volcano maps indicate fold change in gene expression in HAVCR/KIM-1-overexpressed (right) or HAVCR/KIM-1-silenced (left) 769-P cells, compared with control cells. Genes with an adjusted $P$ value less than 0.01 and fold change of at least 2 were considered differentially expressed. B, Venn diagrams showing the number of genes up- or downregulated by HAVCR/KIM-1 overexpression (right circle) or HAVCR/KIM-1 interference (left circle). Genes modified in both situations are placed in the intersection. C, heatmap analyses reveal a global view of genes up- and downregulated in overexpressed (HK16) or silenced (sh851) in relation to their respective controls (CR4 and shCV). Triplicates for each experimental situation were performed. D, RT-qPCR of random selected genes to validate the microarray results (top). $^*$, $P < 0.05$; $^{**}$, $P < 0.01$. Log$_2$ fold-change values from microarrays are indicated in each case (bottom).
overexpressing 769-P clones produced the same results (Supplementary Fig. S1). Augmented IL-6 mRNA levels correlated with increased IL-6 in conditioned media of HAVCR/KIM-1–overexpressing 769-P cells (Fig. 3B, right). To investigate the implication of HAVCR/KIM-1 shedding on IL-6 production, we used 786-O cells that show barely detectable endogenous HAVCR/KIM-1 levels. As in 769-P cells, overexpression of HAVCR/KIM-1 in 786-O induced IL-6 expression (Fig. 3C, left). Moreover, expression of the HAVCR/KIM-1 mutant with constitutive shedding (FUW 246-274) resulted in increased IL-6 mRNA levels, whereas shedding-defective (FUW 267-268) was unable to induce IL-6 expression (Fig. 3C, right).

HAVCR/KIM-1 activates the IL-6/STAT-3 signaling pathway in 769-P cells

IL-6 exerts its biologic effects through binding to its ligand-binding receptor gp80 and thereupon to the transducing receptor gp130 (19). Phosphorylated tyrosines in gp130 form

Figure 3. Augmented IL-6 production by HAVCR/KIM-1 and effects of HAVCR/KIM-1 shedding impairment. A, IPA pathways showing up- and downregulation of the IL-6 pathway in HAVCR/KIM-1–overexpressed (left) and HAVCR/KIM-1–silenced cells (right). IL-6 and gp130 are encircled on each diagram. Red and green indicate up- and downregulation, respectively. B, RT-qPCR to determine IL-6 levels in 769-P cells with HAVCR/KIM-1 overexpression (HK16R) or silencing (sh551; left) and CR4 and shCV control cells. Concentrations of IL-6 in cell culture supernatants of HAVCR/KIM-1 overexpression and control cell lines (right). C, RT-qPCR to determine IL-6 levels in 786-O cells with HAVCR/KIM-1 overexpression (FUW hHAVcr-1) and 786-O control cell line (FUW; left). Effect of HAVCR/KIM-1 shedding defective mutant (FUW 267-278) on IL-6 mRNA levels (right). *, P < 0.05; **, P < 0.01.
Figure 4. The IL-6/gp130/STAT-3 pathway is activated by HAVCR/KIM-1. A, effects of HAVCR/KIM-1 overexpression (HK16R) and HAVCR/KIM-1 silencing (sh851) in gp130, pSTAT-3 Y705, and pSTAT-3 S727 levels in 769-P cells when compared with CR4 and shCV control cells (right and left, respectively).

B, 769-P cells expressing wild-type HAVCR/KIM-1 (HK16R) and plasmid control (CR4) were subjected to indirect immunofluorescence using anti-gp130 (right), anti-pSTAT-3 Y705 (left), and anti-pSTAT-3 S727 antibodies (bottom). Cells were stained with Hoescht 3333 dye (middle) and observed by confocal fluorescence microscopy.

C, pSTAT-3 Y705 and pSTAT-3 S727 levels in IL-6–silenced HAVCR/KIM-1 overexpressing 769-P cells (HK16RshIL6) compared with cells carrying silencing empty vector overexpressing HAVCR/KIM-1 (HK16RshCV) measured by Western blot (left) and immunohistochemistry (pSTAT-3 S727; right).
HAVCR/KIM-1 Activates the IL-6/STAT-3 Pathway

docking sites for activator of transcription STAT-3 (20), which becomes activated through phosphorylation at tyrosine 705 (21). As for IL-6, HAVCR/KIM-1 overexpression in the HK16R clone correlated with increased gp130, pSTAT-3 Y705, and pSTAT-3 S727 levels (Fig. 4A). Immunohistochemistry also showed the presence of pSTAT-3 Y705 and pS727 in the nucleus of HAVCR/KIM-1–overexpressing cells (Fig. 4B, left and bottom). The relationship between pSTAT3 S727 and HAVCR/KIM-1 levels was further demonstrated in 786-O cells (Supplementary Fig. S2). Moreover, knocking-down IL-6 expression in cells overexpressing HAVCR/KIM-1 prevented HAVCR/KIM-1–induced STAT3 Y705 and S727 phosphorylation (Fig. 4C). Overall, these results indicate that upregulation of IL-6 levels by HAVCR/KIM-1 favors phosphorylation of STAT-3 at Y705 and S727 residues in ccRCC-derived cells.

The IL-6/STAT-3 activation by HAVCR/KIM-1 controls HIF-1A mRNA and protein expression

STAT-3 transcriptionally regulates genes involved in tumor proliferation, apoptosis inhibition, and angiogenesis. Among those genes, HIF-1A is a key element in promoting hypoxia-induced angiogenesis (22). Results from the microarray assays indicated that HIF-1A was one of the top 10 upregulated genes by HAVCR/KIM-1 overexpression [log2 fold change (FC), 3.29; adj. P, 0.001–12] and one of the most downregulated when HAVCR/KIM-1 is silenced (log2 FC, −2.14; adj. P, 6.27E−07). IPA diagrams show that components of the HIF-1A signaling pathway are also altered by HAVCR/KIM-1 overexpression (Fig. 5A, left) or silencing (Fig. 5A, right). RT-qPCR assays confirmed that 769-P cells overexpressing HAVCR/KIM-1 exhibited increased mRNA HIF-1A levels, whereas HAVCR/KIM-1–silenced 769-P cells showed diminished HIF-1A levels (Fig. 5B, left). Accordingly, HIF-1A protein levels were also increased in HK16R1 cells (Fig. 5B, right). Because 769-P cells express barely detectable levels of HIF-1A, the effect of HAVCR/KIM-1 silencing on HIF-1A protein was less apparent. To explore whether HIF-1A protein levels correlated with expression of HIF-1A target genes regulated by KIM-1 overexpression, we analyzed mRNA levels of two classic HIF-1A–regulated genes, GLUT-1 and VEGFA, and found a good correlation of GLUT-1 with HAVCR/KIM-1 overexpression (Fig. 5C, right), but not with VEGFA (Fig. 5C, left). These data fit well with results obtained in the microarray, in which statistical significance increments were obtained for GLUT-1 (SLC2A1; log2 FC, 0.91; adj. P, 0.003), but not for VEGFA (log2 FC, −0.22; adj. P, 0.449) upon HAVCR/KIM-1 overexpression.

To investigate whether HIF-1A upregulation by HAVCR/KIM-1 depends on IL-6–induced STAT-3 activation, we silenced IL-6 in HK16R1 cells (Fig. 5D, left) and found that IL-6 knockdown diminishes HIF-1A mRNA induced by HAVCR/KIM-1 (Fig. 5D, right).

pSTAT-3 is activated in patients with ccRCC and pSTAT-3 S727 H-score constitutes a novel independent prognostic factor for patient outcome

The relevance of the pSTAT-3 activation in patients with ccRCC was studied in the same TMA previously used to determine expression of HAVCR/KIM-1 (14). It includes 98 patients with ccRCC with the following characteristics: 57.1% men and 42.9% women; median age, 64 years; range, 25 to 86. Tumor was on the right side in 59.1% and left in 40.8% of the cases. Incidental presentation was found in 52.5% of the patients and 46.9% were symptomatic. Of note, 87.9% of the patients presented with localized tumors and 11.1% with metastasis. A total of 92.9% patients underwent radical nephrectomy, whereas 6 underwent nephron-sparing surgery. Median tumor size was 6.4 cm (range, 1.5–16). Fuhrman grade was 1 in 20.4%, 2 in 41.8%, 3 in 24.4%, and 4 in 13.2% of the patients. The most frequently observed pathologic classification of primary tumor size (pT) stages were pT1a in 26.5% and pT1b in 27.5%. Lymphovascular invasion was present in only 5.1% of patients. Finally, the UICC risk group was 1 in 72.4% and 2 in 27.5% of the patients studied.

Representative images of ccRCC tumors and unaffected normal kidney counterparts stained with specific antibodies against pSTAT-3 Y705 and pSTAT-3 S727 are shown in Fig. 6A. Tumors showed positive staining for both pSTAT-3 Y705 and pSTAT-3 S727 mainly located in the nuclear compartment that was not found in normal tissues. Upon signal intensity evaluation using H-score, results were correlated with clinical outcome. H-score value of 100 was arbitrarily set up as the threshold value to separate patients with high or low pSTAT-3 staining. Kaplan–Meier diagrams showing specific survival rates in relation to patient follow-up (months) showed no significant correlation with pSTAT-3 Y705 H-score (not shown). However, Kaplan–Meier estimates of mortality showed statistically significant differences in overall survival rates between patients with high versus low pSTAT-3 S727 H-score among individuals included in the same clinic and pathologic group (Fig. 6B–D).

In this study, we found significant statistical association between pSTAT-3 S727 and recurrence-free survival and progression. Univariate [P = 0.015; OR, 5; 95% confidence intervals (CI), 1.3–19] and multivariate analyses [P = 0.016; OR, 3.132; 95% CI, 1.247–7.870] indicate that expression levels of pSTAT-3 S727 represent an independent prognostic factor with respect to classical clinicopathologic features, which demonstrates that pSTAT-3 S727 is an independent factor that is clinically relevant.

Discussion

This work reveals a potent control of IL-6 expression by HAVCR/KIM-1 in ccRCC cell lines, mainly mediated by HAVCR/KIM-1 shedding. Increased IL-6 levels in serum and augmented expression of IL-6 receptor in tumors were described in patients with metastatic RCC and poor survival, indicating the presence of an IL-6 autocrine mechanism in RCC that correlates with malignancy (15–18).

The response of renal cancer cells to IL-6 is most likely mediated by STAT-3 activation, which is, in turn, triggered by IL-6–transducing receptor gp130 (23). Our results show that HAVCR/KIM-1 regulates gp130 levels and activates pSTAT-3 at Y705 and S727. pSTAT-3 activation in RCC, especially in metastatic disease, has been previously reported (24). pSTAT-3 Y705 has been associated with tumors that carry poor prognosis like ccRCC and papillary tumors but not with tumors with a benign outcome, like oncocytomas (25). This pattern of expression is the same as...
the one reported for HAVCR/KIM-1 (14). Therefore, it seems that effects of HAVCR/KIM-1 on pSTAT-3 Y705 observed in 769-P cells might also occur in vivo (14). Albeit the well-established association between pY705 and tumor malignancy, significant correlations between pY705 and patient outcome have not yet been described. No significant correlations were found between pY705 and clinical outcome but, for the first time, we described that STAT-3 S727 phosphorylation significantly correlates with prognosis and patient survival. Therefore, we propose pSTAT-3 S727 as a novel tissue biomarker for better stratification and follow-up of patients classified under the same stage and clinical score, assisting on clinical decisions.

Because HAVCR/KIM-1 is not an independent factor of survival (14), no statistical association was found between HAVCR/KIM-1 levels and pSTAT3 S727 in tumors, in relation to recurrence-free survival and progression [univariate analyses; $P = 0.314$; risk ratio, $0.6$; 95% CI, 0.28–1.45], when analyzed in the same cohort of patients. Because our in vitro data correlate HAVCR/KIM-1 with pSTAT3 S727 levels only when IL-6 is produced and we have also demonstrated that IL-6 production depends on HAVCR/KIM-1 shedding, we postulate that, in patients with ccRCC, pSTAT3-3 S727 levels might correlate better with HAVCR/KIM-1 ectodomain levels in urine and IL-6 levels in plasma than with HAVCR/KIM-1 levels in tumors. Accordingly, we hypothesize that IL-6 and HAVCR/KIM-1 altogether might constitute useful surrogate biomarkers for tumor progression and patient follow-up assessment upon tumor removal.

STAT-3 is a potent regulator of HIF-1A activity (22). aberrantly enhanced HIF target genes, such as VEGF and GLUT1, linked to persistent activation of STAT-3 and HIF-1A upregulation, contribute to increased tumor growth and metastatic spread of renal carcinomas and correlate with worse prognosis in ccRCC (26, 27). In this report, we have proved that overexpression of HAVCR/KIM-1 activates STAT-3 and increases HIF-1A levels. Steady-state levels of HIF-1A protein might reflect the balance between transcriptional induction promoted by HAVCR/KIM-1 and posttranslational degradation of HIF-1A in normoxic conditions. HIF-1A protein levels correlated with GLUT1 upregulation but not VEGFA, which might require other transcription factors and/or coactivators likely not present in the experimental conditions used in this research.

Biologic implications of pSTAT-3 S727 in ccRCC, according to its correlation with poor patient outcome, might be related with enhanced transcriptional activation of a distinct subset of STAT-3 targets, which could be especially relevant in ccRCC progression. In this report, we demonstrate that HAVCR/KIM-1, first described as one of the most upregulated genes in ischemic rat kidney (8), is a potent activator of HIF-1A transcription, even in normoxic conditions. Therefore, constitutive expression of HAVCR/KIM-1 in normal parenchyma of patients with ccRCC, together with its further upregulation in tumors, might represent a novel mechanism to activate tumor growth and angiogenesis through activation of the IL-6/gp130/STAT-3/HIF-1A pathway.

HAVCR/KIM-1 has been mapped in 5q33.3 cytogenetic location (6). Because amplification of 5q chromosome is the second most frequent cytogenetic alterations in ccRCC tumors (67%; ref. 28), we postulated that HAVCR/KIM-1 overexpression observed in tumors could be related to the 5q amplification (6). Recently, The Cancer Genome Atlas (TCGA) Research Network refined the region of interest to the 5q35 region (28); thus, excluding the HAVCR/KIM-1 gene. This indicates that other mechanisms rather than gene amplification must account for HAVCR/KIM-1 overexpression in ccRCC. Pathway and integrated analyses performed by the TCGA Network highlighted the importance of the well-known VHL/HIF pathway, the newly emerging chromatin remodeling/histone methylation pathway, and the phosphoinositide 3-kinase/akt pathway in ccRCC (28). Because more than 60% of ccRCC tumors overexpress the HAVCR/KIM-1 gene and this results in augmented HIF-1A expression at mRNA and protein levels, it is apparent that the HAVCR/KIM-1 gene is involved in relevant pathways driving ccRCC development and progression.

This study demonstrates that HAVCR/KIM-1 acts above the IL-6/gp130/STAT-3/HIF-1A axis, mediating, in part, the effects induced by hypoxia in this pathway. The hypothesized mechanism of action of HAVCR/KIM-1 is depicted in Fig. 7. Upon shedding by MMPs, HAVCR/KIM-1 ectodomain binds to putative HAVCR/KIM-1 receptors, triggers IL-6 production, and activates the gp130/STAT-3/HIF-1A pathway in ccRCC cells. Although MMP1 and MMP3 have been involved in HAVCR/KIM-1 shedding, mainly in situations inducing kidney injury (29), little is known on HAVCR/KIM-1–dependent MMPs in renal cancer. Besides this paracrine/autocrine effect on tumor cells, it shall be considered the possible impact of HAVCR/KIM-1 ectodomain on tumor-associated stromal cells, including endothelia, tumor-associated fibroblasts, T lymphocytes, and macrophages (30, 31). Soluble or surface HAVCR/KIM-1 expressed by renal epithelial cells upon injury has been proposed as the endogenous ligand for CD300lb/LMIR-5/CLM-7/miREM-3 receptors expressed in myeloid cells in an ischemia-reperfusion injury model (32).

In conclusion, by inducing IL-6 expression, HAVCR/KIM-1 activates STAT-3 and promotes expression of growth and angiogenic factors on tumor and likely in nontumor-associated cells that would help tumor growth and metastasis. The elucidation of HAVCR/KIM-1 downstream pathways has...
Figure 6. pSTAT-3 S727 and pSTAT-3 Y705 expression in tumor samples from patients with ccRCC and correlation with clinical outcome. A, representative examples of expression patterns in ccRCC, pSTAT-3 Y705 (top) and pSTAT-3 S727 (bottom) expression in ccRCC tumors (T) and normal counterparts (N). Original magnification, ×200; scale bar, 200 μm. B, Kaplan–Meier estimates of 100 months overall Führman grade 1 and 2 (left) and 3 and 4 (right) according to pSTAT-3 S727 expression level. C, Kaplan–Meier estimates of 100 months overall pT1-2 N0 M0 (left) and pT3-4 N0-1 M0-1 (right) according to pSTAT-3 S727 levels. D, Kaplan–Meier estimates of 100 months overall low risk (left) and high risk (right) according to pSTAT-3 S727 levels. Metastatic patients have been excluded. The intensity score was defined as follows: 0, no appreciable staining in cells; 1, weak-intensity cells; 2, intermediate intensity of staining; and 3, strong intensity of staining. [H-score = 1 × (% weak) + 2 × (% moderate) + 3 × (% intense) ranging from 0 to 300.] Correlation between pSTAT-3 S727 Histoscore expression level (blue line, HS above 100; green line, HS below 100) and mean survival of patients with ccRCC according to Führman grade (B), clinical stage (C), and the UICC risk group (D) are indicated.
provided with novel biomarkers for tumor prognosis, as well as with putative novel therapeutic targets to be further developed.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: T. Cuadros, M.R. Vilá, J. Morote, A. Meseguer
Development of methodology: T. Cuadros, I. de Torres, A. Meseguer
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Cuadros, E. Trilla, E. Sarri, M.R. Vilá, J. Vilardell, I. de Torres, S. Ramón y Cajal
Writing, review, and/or revision of the manuscript: T. Cuadros, E. Trilla, E. Sarri, M.R. Vilá, I. de Torres, M. Salcedo, E. Itarte, J. Morote, A. Meseguer
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E. Trilla, I. de Torres, J. Morote
Study supervision: E. Trilla, I. de Torres, S. Ramón y Cajal, A. Meseguer

Grant Support
This work was supported in part by the Fundació La Marató de TV3 (Ref: 052410 to A. Meseguer); the Ministerio de Ciencia e Innovación (BFU2009-10189, to E. Itarte; SAF2011-2950 and SAF2005-05167 to A. Meseguer); the Instituto de Salud Carlos III (FIS PI081351 and REDINREN 2.0 REF: RD12/0021/0013 to A. Meseguer); the Fundación Renal Iñigo Álvarez de Toledo (FRIAT to A. Meseguer), and the Fundación Senefro (SEN to A. Meseguer). A. Meseguer's research group holds the Quality Mention from the Generalitat de Catalunya (2009SGR).

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Received June 11, 2013; revised November 15, 2013; accepted December 4, 2013; published OnlineFirst January 3, 2014.

References


