PATIENTS AND METHODS

Case Selection

Clinical and pathologic data from 168 patients with renal masses treated with radical or partial nephrectomy for renal cell carcinoma between January 2002 and December 2005 at Vall d’Hebron Hospital were retrospectively reviewed. Standard tumor registry data including date of diagnosis, demographic information, stage, Fuhrman grade, nodal and metastatic spread at time of presentation, initial therapy information, follow-up information, subsequent therapy information, and outcome information were retrospective collected by tumor registrars for only clear cell renal cell carcinomas (n=98). pSTAT3 S727 and pSTAT3 Y705 levels were evaluated in 84 clear cell carcinomas. Primary tumor histology was recorded from surgical resections and pathologic information was based on re-review of all surgical samples to determine histologic subtype using the WHO 2004 criteria. Anatomic extent of the tumor was classified using the 2010 TNM system, and clinical and follow-up information was based on physician reports. Prognostic stratification of patients affected by ccRCC was scored using the UCLA Integrated Staging System-UICC nomogram.

TMA Design

A kidney cancer tissue microarray (TMA) was constructed from a total of 98 clear cell renal cell carcinomas. Formalin-fixed, paraffin-embedded kidney cancer specimens were provided by the Department of Pathology, Vall d’Hebron Hospital, Barcelona, Spain. Hematoxylin- and eosin-stained sections were reviewed for all cases and targeted areas (normal, transitional and tumor) were selected on the corresponding paraffin blocks. TMAs were constructed using a semi-automated tissue arrayer (Chemicon International Inc., Billerica, MA, USA). For each surgically-removed specimen, 1.2-mm diameter cores from 3 benign areas far from the tumor,
3 transitional areas limiting normal and tumor tissues, and 3 representative areas of the carcinoma were obtained and embedded in recipient paraffin blocks as described (15). Consent for the use of all human tissues used in this study was obtained following the rules of the Spanish Biomedical Research Law and approved by the Ethics Committee of the Hospital.


Immunohistochemistry

For immunohistochemical analysis, sections (4 μm) of the resulting tumour tissue microarray block (as described in Ref 15) were subjected to antigen retrieval by microwaving for 15 min in 10mM sodium citrate buffer, pH 6.0. Endogenous peroxidase activity was quenched by incubating in a 3% hydrogen peroxide solution for 10 min. After blocking, sections were incubated with primary antibodies (mouse mAb against pSTAT3 Y705 and rabbit mAb against pSTAT3 S727 (Cell Signaling)) overnight at 4°C, followed by 1h incubation with biotinylated secondary antibodies and 30 min incubation with the avidin-biotin complex at room temperature. Sections were visualized with diaminobenzidine tetrahydrochloride substrate and hematoxylin counterstain. TMA sections were evaluated by two expert pathologists blinded to clinicopathological variables using optical microscope at x400 magnification as described (15).

Statistical analyses

Associations between pSTAT3 S727 and p STAT3 Y705 expression and clinical-pathologic parameters were evaluated with the non-parametric 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 clinical-pathologic parameters. Statistical analysis was performed with the Statistical Package for Social Sciences, version 12, software (SPSS, Chicago, Ill).

Cell culture

The 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 ATCC, were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% FBS (Biological Industries), 1% MEM EAGLE Non-essential amino acids solution 100X, 1% L-Glutamine solution 200mM, 1% Sodium Pyruvate solution 100mM (Biological Industries), and 1% Antibiotic-antimycotic solution 100mM (GIBCO) and maintained at 37°C in 5% CO₂. 769-P and 786-O cell lines were authenticated by LGC Standards (Teddington, Middlesex, UK) by the PowerPlex 16 HS 16 Loci Service method, in October 2013. According to their results, 769-P cell line matches all of the 8 reference STR loci (plus Amelogenin) available from ATCC for CRL-1933. As for cell line 786-O, it matches 7 of the 8 reference STR loci (plus Amelogenin) available from ATCC for CRL-1932. Both cell lines profile display good characteristics.

Establishment of stable cell lines
For HAVCR/KIM-1 and IL-6 silenced cell lines, five shRNAs targeting different exons of the human HAVcr-1 or IL-6 genes were designed (Mission Sigma shRNA) and introduced into the 769-P cell line by lentiviral infection. Transfection with a MISSION® Non-Target shRNA Control Vector (shCV, SHC002), served as a negative control.

Viruses were generated by simultaneous transfection of HEK293T cells, using polyethylenimine (PEI, PolysciencesInc) transfection reagent (stock 1 mg/mL) with four plasmids providing vector, gag-pol and env functions at a ratio of 1:1:3:5 (VSVG:RTR2:PKGPIR:Transfer vector(encoding for different s shRNAs)). A total of 6 µg DNA was combined with 30µL of PEI in 600µL of NaCl 150mM solution. The suspension was incubated for 20 minutes at room temperature and added drop-wise to the HEK293T cells. Transfected cells were incubated o/n at 33°C in 5% CO₂ in a humidified incubator; transfection supernatants were removed and 769-P cells were overlaid with 5 mL of fresh 10% FBS DMEM for 48 and 72h harvests of viral vector supernatants. Transfection supernatants were filtered through a 0.22 µm-pore-size filter (Millipore, Bedford MA, USA) and supplemented with 10% FBS, polybrene up to 8µg/mL and Non Essential Amino Acids (NEAA) up to 1X (stock at 100X). Transformants were selected 5 days later by the addition of Puromycin (Sigma-Aldrich) at 1µg/mL to the cell culture medium.

For HAVCR/KIM-1 overexpression in 769-P cell line, the entire coding sequence of human HAVCR/KIM-1 gene (ID: 26762) was amplified from pIRES HK vector (6) and cloned into a BamHI-Spal site in the pBIG-2r vector (kindly provided by Dr. Madrenas) including an HA epitope. All constructs were sequenced to verify the correct reading frame. Plasmid-DNA complexes were stably transfected into 769-P cells using Lipofectamine and Plus reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendations. Control cells were transfected with the empty pBIG2r vector. Cells were selected with Hygromycin (450 µg/ml).
The overexpression of hHAVcr-1 and its shedding mutants (FUW 246-274 and FUW 267-278) in the 786-O cell line, have been previously described in Cuadros et al. (15).

Cell lysate preparation and western blot analysis

HAVCR/KIM-1 overexpressing or HAVCR/KIM-1 silenced 769-P cells and their corresponding control cells were washed with PBS and lysed in whole-cell lysis buffer (1% Triton X-100, 20 mM HEPES [pH 7.4], 2 mM EGTA, 1mM DTT, 50 mM β-glycerophosphate, 10% glycerol, 1mM NaVO₃) with proteinase inhibitors. Western blot analysis were performed as follows: samples were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and blocked in Tris-buffered saline/0.1% Tween-20 containing 5% BSA. Primary antibodies used for Western blot were, murine mAb against HAVCR/KIM-1 (R&D systems), rat mAb against HA (ROCHE), rabbit mAb against β-actin (Sigma-Aldrich), rabbit mAb against gp130 (Santa Cruz biotech), mouse mAb against pSTAT3 Y705 (Cell Signaling), mouse mAb against ϕSTAT3 (Cell Signaling), mouse mAb against HIF-1A (H1alpha67) from Novus Biologicals (Cambridge, UK) and mouse mAb against α–tubulin (Sigma-Aldrich) diluted in blocking solution. Horseradish peroxidase-conjugated secondary antibodies were obtained from DAKO (Glostrup, Denmark). Blots were developed with the enhanced chemiluminescence substrate ECL plus Western blotting detection system (GE Healthcare UK, Buckinghsmshire, UK) and detected with AmershamHyperfilm ECL (GE Healthcare Limited, Buckinghsmshire, UK).

Proliferation assay

Cell proliferation assays were carried out by labeling 769-P cells with the amine reactive fluorescent probe 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE) (Sigma-Aldrich, St. Louis, MO). Briefly, cells were trypsinized, washed twice in PBS, and then re-suspended in
PBS containing 5 μM CFSE and incubated at 37°C for 10 min in the dark. To quench the labeling, cells were washed twice with complete culture medium. Labeled cells were seeded at 2000 cells/cm² in 21 cm² dishes and cultured for 4 days. After 96 hours of growth, cells were washed by PBS, trypsinized, washed in PBS and resuspended in complete medium. Fluorescence was measured on 10,000 cells/sample using a FACScalibur (Becton Dickinson, Mountain View, CA). Data were analyzed using FCS Express 4 Flow Research Edition. Parent cells correspond to cells labeled and analyzed immediately after the labeling step.

**Wound Healing**

769-P cells were cultured to confluence in 12-well plates. An artificial wound was created by scratching cell monolayer in a linear fashion with a sterile 200 uL pipette tip. Cells were gently washed with PBS and incubated with complete medium. The closure of the denuded area was monitored using the Live Cell Imaging Cell R Station (Olympus, Hamburg, Germany). Digital images were obtained every 30 minutes. The width of the wounds at every time point was measured using ImageJ software. The progression of migration was calculated by subtracting the width of the wound at every time point from that at 0 hours. Results are representative of three independent experiments.

**Microarray expression analyses**

Total cell RNA was extracted from cells by using RNeasy® Mini kit (QIAGEN, Valencia, California, USA) according to the manufacturer’s instructions. RNA quality was checked by using the Bioanalyzer (Agilent technologies). RNA samples representing three separate experiments from cells overexpressing HAVCR/KIM-1 and three experiments from cells treated with HAVCR/KIM-1 shRNA, along with appropriate controls, were submitted for analysis. Extracted total RNA was
used to synthesize double stranded cDNA using the One Cycle cDNA Synthesis Kit (Affymetrix, Inc.). Biotin-labeled antisense cRNA was obtained using the same kit starting with 5 g of total RNAs and the oligo dT primer 5’ GGCCAGTGA-ATTGTAATACGACTCACTATAGGGAGGCGG-(dT)24. cRNAs were purified using the columns from the GeneChip® Sample Cleanup Module (Affymetrix, Inc.) and then 20 microg. of cRNAs were fragmented at 94ºC for 30 minutes in 40 l of 40 mM Tris-acetate, pH 8.1, 100 mM KOAc, 30 mM Mg(OAc)2. The fragmented samples were added to a hybridization cocktail containing Control oligonucleotide B2 (50 pM) and Eukaryotic Hybridization controls (BioB, BioC, BioD, cre) at 1.5, 5, 25 and 100 pM final concentration respectively from the GeneChip Eukaryotic Hybridization Control Kit (Affymetrix, Inc.), herring sperm DNA (0.1 mg/ml) and acetylated BSA (0.5 mg/ml). Probe array was equilibrated to RT and prehybridized with 1x hybridization buffer (100mM MES, 1M [Na+], 20mM EDTA, 0.01% Tween 20) at 45ºC for 10 minutes with rotation. Two-hundred l of the mixture were used for hybridization to the Human Exon 1.0 arrays (Affymetrix Genechip® array; Santa clara, CA) at 45ºC for 16 hours with rotation. GeneChips were washed and marked with streptavidin phycoerythrin using the protocol EukGE-WS2-v5 provided by Affymetrix. Once washed and streptavidin phycoerythrin-marked the GeneChips were scanned in an Agilent G3000 GeneArray Scanner. The Human Exon 1.0 array allows a complete coverage of the Human Genome.

Preprocessing. Raw expression values obtained from .CEL files were preprocessed using the RMA method (16) which performs a three step process consisting of background correction, normalization and summarization at exon level. Following this process exon values corresponding to each gene were averaged to yield a unique gene (transcript) value. Conservative (low) thresholds were used to reduce possible false negatives.
Selection of differentially expressed genes. The selection was based on a linear model analysis with empirical Bayes moderation of the variance estimates as described in (17). In practice the method is similar to using a “t-test” with a better estimate of the variance. Genes with an adjusted p-value of less than 0.01 and a fold change of at least 2 were considered as differentially expressed. P-values adjustment was performed based on strong control of the False Discovery Rate (FDR) as provided by the Benjamini & Hochberg method (18). The display of differentially expressed genes, selected as explained in the methods section, was based on volcano plots, which plot a transformation of the minus-p-value (Y) vs the fold change (X) in a logarithmic scale. The display of differential profiles was based on heatmap plots which are rectangular plots of the expression of each selected gene (rows) in each sample (columns) using a colour scale which ranges from low to high expression values. Genes showing a similar behaviour between conditions are grouped in the plot yielding an image of groups of genes that may be simultaneously up or down-regulated and potentially co-regulated.

Analysis of Biological Significance. Gene Ontology Analysis (GOA) was based on an Overrepresentation Analysis (19) aiming at establishing if the genes that were found to be differentially expressed appeared to be more or less frequent than usual in some GO Categories that could be related to the biological processes involved in the analysis. A similar analysis (IPA, for Ingenuity Pathway Analysis) was used to establish which pathways are affected by the genes selected and also to represent the relations appearing between the genes.

Software used. All the statistical analysis, except IPA, were carried out using the free statistical language R and the libraries developed for microarray data analysis by the Bioconductor Project (http://www.bioconductor.org). The main methods and tools were used as described (20). IPA analysis was performed using Ingenuity Pathways Tool, available at http://www.ingenuity.com.
Standard guidelines for Minimum Information About a Microarray Gene Experiment (MIAME guidelines) have been followed. Accession number for datasets is E-MTAB-2071.

**RTqPCR**

For real-time RT-PCR experiments, we used the 7500 Real Time PCR System (Applied Biosystems, Fosters City, CA) according to the manufacturer’s instructions. The amplification reactions were performed in 20 μL final volume containing 10 μL of 2xTaqMan Universal PCR Master Mix (Applied Biosystems, Fosters City, CA), 1 μL of TaqMan probe (for JUP (Hs00158408_m1), 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 (Hs99999904_m1) genes), 4 μL of Rnase free water and 5 μL of cDNA. PPIA probe was used as endogenous control of the experiment. The conditions of the PCR were: 2 minutes at 50 ºC followed by 10 minutes at 95 ºC and then 40 cycles of 15 seconds at 95 ºC and 1 minute at 60 ºC. Triplicate PCR amplifications were performed for each sample.

**IL-6 ELISA**

Concentrations of IL-6 in cell culture supernatants of HAVCR/KIM-1 overexpression and control cell lines, were measured using the commercially available quantitative assay from R&D systems (R&D Cat# D6050, Minneapolis, MN) according to the manufacturer’s instructions. The absorbance was measured using a plate reader (BioTek Elx800) at 450 nm with an absorbance correction at 540 nm. The IL-6 concentration was calculated based on the standard curve and expressed in absolute terms (pg/mL). The sensitivity of the assay was 0.7 pg /mL.

**Indirect immunofluorescence assay**
Cells were grown on 24-well chamber slides from Nunc (Fisher scientific, Rochester, NY) at a density of $1.0 \times 10^5$ per well until they reached confluence. The cells were fixed with 4% formaldehyde in PBS for 20 min. After three washes with PBS, the plasma membrane was permeabilized with methanol for 10 min at 4°C. Cells were incubated with 5% normal mouse serum in PBS-0.5% Triton X-100 for 1h at room temperature before incubation with primary antibodies. Mouse mAb HAVCR/KIM-1 antibody (R&D systems) was used at a 1:250 dilution, rabbit mAb gp130 (Santa Cruz biotech.), rabbit mAb pSTAT3 S727 and mouse mAb pSTAT3 Y705 (Cell Signaling) were used at a 1:100 dilution. Secondary Alexa 468 anti-mouse (Invitrogen) was used at a 1:350 dilution and Alexa 488 anti-rabbit (Invitrogen) was used at a 1:300. The cells were then stained with Hoescht 33323 dye (Sigma-Aldrich) diluted 1:1000 with PBS for 10 minutes and after two washes with PBS the slides were mounted with Fluoromount-G (SouthernBiotech, AL, US). Cells were observed by confocal fluorescence microscopy.