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Searching for Hif1-α interacting proteins in renal cell carcinoma

V. Medina Villaamil[1], G. Aparicio Gallego[1], I. Santamarina Caínzos[1], M. Valladares-Ayerbes[1,2], L. M. Antón Aparicio[2,3]

1. INIBIC, CHU, A Coruña, A Coruña, Spain
2. Medical Oncology Service, CHU A Coruña, A Coruña, Spain
3. Medical Department, UDC, A Coruña, Spain

Abstract
Introduction. Kidney tumours are frequently characterised by hypoxic conditions due to a local imbalance between oxygen (O_2) supply and consumption. Hif1-α regulates angiogenesis, tumour growth, tumour progression, metastatic spread, and glucose metabolism by acting as a transcription factor for relevant genes. Here, we describe an immunohistochemical study of Hif1-α, a comprehensive computational study of Hif1-α interacting proteins (HIPs), an analysis correlating expression levels of Hif1-α with upstream and downstream proteins, and an analysis of the utility of Hif1-α for prognosis in a cohort of patients with renal cell carcinoma.
Materials and methods. The patient cohort included 80 patients. For immunohistochemistry evaluation, tissue microarrays were constructed. The IntAct, MINT, and BOND databases were used for the HIP approach. The Kruskal–Wallis test was used for comparing protein expression with pathology measurements. Correlation was expressed as the Pearson coefficient.
Results. Hif1-α expression correlates significantly with the "clear" histological subtype of renal cell carcinoma ($p < 0.01$). The samples with the worst prognoses related to the pathological variables analysed showed the highest levels of Hif1-α expression. Significant correlations were found with Bcl-2, CAIX, C-kit, EGFR, TGF-β, proteins of the VEGF family, proteins related to differentiation (such as Notch1 and Notch3) and certain metabolic enzymes. Bioinformatic analysis suggested 45 evidence-based HIPs and 4 complexes involving protein Hif1-α.
Conclusions. This work summarises the multifaceted role of Hif1-α in the pathology of renal cell carcinomas, and it identifies HIPs that could help provide mechanistic explanations for the different behaviours seen in tumours.

Keywords
Angiogenesis; Biomarkers; Hif1-α; Protein interactions; Renal tumours

Introduction

Renal cancer accounts for 3 % of all malignant tumours. Carcinomas arising from the renal epithelium account for approximately 85 % of renal tumours. The major risk factors include smoking (responsible for 24–30 % of all cases of renal cell carcinoma), obesity, and various environmental and occupational factors.

Renal cell carcinoma (RCC) is a heterogeneous group of cancers, but 60–80 % of cases belong to the "clear" histological subtype. Other histology subtypes include the "papillary" and "chromophobe" subtypes.

These different subtypes represent very different diseases that are not strictly variants of RCC. Understanding histological subtypes and associated gene alterations has allowed the development of targeted therapeutic agents.

Much research has focused on finding reliable indicators of the underlying biology of RCC. With the advancement of technologies that allow probing of the genetic and molecular underpinnings of this cancer, many discoveries have led to major innovations in RCC treatment, including a panel of molecularly targeted drug therapies.

Like many solid tumours, kidney tumours are frequently characterised by hypoxic conditions, which result from a local imbalance between oxygen (O_2) supply and consumption [1]. Indeed, hypoxia and compensatory hyperactivation of angiogenesis are thought to be particularly important in RCC compared to other tumour types, given the highly vascularised nature of kidney tumours and the specific association of mutation in Von Hippel-Lindau (VHL), a critical regulator of the hypoxic response, with onset of RCC [2].

Hif1-α regulates angiogenesis, tumour growth, progression, metastatic spread, and glucose metabolism by acting as a transcription factor for crucial proteins such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), epidermal growth factor receptor (EGFR), insulin-like growth factor (IGF), glucose transporters (GLUT-1), chemokine receptors (CXCRs), and carbonic anhydrase IX and XII (CAIX, CAXII). In addition, Hif1-α plays an important role in regulating the cell cycle and apoptosis [3, 4].

Although a relatively infrequent malignancy, kidney cancer has no satisfactory treatment options. It would be of great help to be able to diagnose patients on the basis of easily accessible biomarkers, potentially identifying the disease early, before it becomes metastatic and while the tumour is still resectable.

So there is a need to study the associations between the marker of hypoxia Hif1-α and proteins related to the main pathways which feed RCC: angiogenic [(VEGF, vascular endothelial growth factor receptor 2 (FLK1) and VHL], apoptotic [tumour protein suppressor p53, B cell lymphoma 2 protein (BCL-2), Bcl-2-associated X protein (BAX), murine doble minute 2 (MDM2) and baculoviral inhibitor of apoptosis repeat-containing 5 or BIRC5 (SURVIVIN)], differentiation (notch receptors referred to NOTCH1-4, and notch ligands DLL1, DLL3-4 and JAGGED1), metabolic (GLUT1-5 and CAIX), proliferation and/or invasiveness [epidermal growth factor receptor (EGFR), PDGFR-α, PDGFR-β, proto-oncogene c-Kit (C-KIT) and transforming growth factor (TGF-α)] and proteins related to the inflammatory process as TGF-β.

In this report, we present an immunohistochemical (IHC) study of Hif1-α, undertake a comprehensive computational study of Hif1-α interacting proteins (HIPs), correlate expression levels of HIPs with upstream and downstream proteins, and evaluate the utility of Hif1-α for prognosis in a cohort of RCC patients.

The analysis of HIPs is a good way to identify pathways and networks that are altered in RCC, which could be useful in the development of diagnostic tests for patients at increased risk for the disease, as well as in the development of novel therapeutic approaches for RCC.

Materials and methods

Patients

The patient cohort included 80 patients treated with a partial or radical nephrectomy for RCC, including chromophobe, papillary and clear cell variants, between 1996 and 2006. IHC studies were performed, and clinical data from an established kidney cancer database were reviewed. Patients were staged using radiographic studies and postoperative pathological data, according to the 1997 tumour-node-metastasis (TNM) criteria proposed by the American Joint Committee on Cancer (AJCC). Tumours were categorised using the Fuhrman grading scheme [5]. The patients consisted of 53 men and 27 women, aged 34–87 years, with a mean age of 64. There were 57 cases of clear cell type RCC (cRCC), 15 cases of chromophobe RCC (chRCC), 6 cases of papillary RCC (pRCC), 2 samples classified as "histological type not determined", and 11 healthy controls. Among the RCC patients, 50.6 % had tumours located on their right side, and 49.4 % had tumours on the left. Renal pelvic invasion was observed in 11 % of tumours. Only 2.5 % of the cases studied showed invasion of lymphatic vessels. We observed renal capsule rupture in 15 % of tumours ($n = 80$). Only one case showed invasion of the veins, being negative 98.8 % for this pathological parameter. Renal hilar invasion was seen in 7.3 % of tumours. Tumours ranged from 2 to 140 cm in length.

Tissue microarray construction

Archival tumour specimens from the cohort of 80 patients were obtained from the histopathological archives of the Pathology Department of the Modelo Hospital, A Coruña, Spain, with the approval of the institutional review board. Written informed consent was obtained from all patients.

For IHC evaluation, tissue microarrays (TMA) were constructed using a manual tissue arrayer with accessories provided by Durviz. Representative haematoxylin–eosin-stained tumours were reviewed to assess the histological type and grade and to identify viable, morphologically interesting areas of the specimen. Three cylindrical core biopsies (2 mm in diameter) were taken from different sites of each tumour and precisely arrayed in a recipient paraffin tissue microarray block to construct a TMA containing 48 samples in a 6×8 grid. To serve as controls, 11 specimens from healthy kidney were also analysed. One pathologist (JM Rois) blinded to the pathological data, evaluated immunoreactivity of the TMA.

Immunohistochemistry and evaluation of expression

The primary antibodies used are listed in Table 1. IHC technique was carried out as previously described [6].

Table 1. Antibody panel

Antibody	Company	Concentration	Antigen retrieval	Positive control
Hif1-α	Abcam	1:1500	Citrate	Squamous cell carcinoma
VHL	ABR	Ready to use	Citrate	Healthy kidney
VEGF	Sta Cruz	1:100	Citrate	Healthy kidney
Flk-1	Sta Cruz	1:50	Citrate	Healthy kidney
CAIX	Abcam	1:500	Citrate	Renal cell carcinoma
EGFR	Dako	1:25	Proteinase K	Squamous cell carcinoma
PDGFR-α	Sta Cruz	1:200	Citrate	Ovarian carcinoma
PDGFR-β	Serotec	1:40	EDTA	Breast carcinoma
TGF-α	LabVision	Ready to use	Citrate	Anterior pituitary
TGF-β	Millipore	1:500	Citrate	Colon carcinoma
C-KIT	Dako	1:50	No required	GIST
NOTCH1	Abcam	3 ug/ml	Citrate	Healthy kidney
NOTCH2	Lifespan	1:250	Citrate	Healthy kidney
NOTCH3	Sta Cruz	1:50	Citrate	Healthy kidney
NOTCH4	Sta Cruz	1:100	Citrate	Healthy kidney
JAGGED1	Sta Cruz	1:50	EDTA	Astrocytoma
DLL1	Serotec	10 ug/ml	EDTA	Healthy kidney
DLL3	Aviva	15 ug/ml	Citrate	Healthy kidney
DLL4	Serotec	10 ug/ml	Citrate	Healthy kidney
p53	Dako	1:50	EDTA	Breast carcinoma
BAX	Abcam	Ready to use	EDTA	Amygdala
MDM2	Abcam	1.5 ug/ml	Citrate	Squamous cell carcinoma
Survivin	Sta Cruz	1:100	Citrate	Breast carcinoma
BCL2	Dako	1:75	EDTA	Amygdala
Glut-1	Abcam	Ready to use	Citrate	Healthy esophagus
Glut-2	Sta Cruz	1:50	Citrate	Liver
Glut-3	Abcam	1:25	Citrate	Placenta
Glut-4	Abcam	1:250	Citrate	Hearth
Glut-5	Abcam	1:250	Citrate	Healthy small intestine

Antibody expression was evaluated in a blinded fashion to validate the diagnostic morphology of each array spot. The evaluation of expression involved determining the site and degree of reactivity. The site of reactivity included evaluation of the relevant histological subtype, as well as the subcellular localisation. Degree of reactivity included evaluation of maximal staining intensity using a 0–3 scale (0, negative; 1, weak; 2, moderate; 3, strong), as well as the percentage of positive cells at each stated intensity.

Bioinformatics tools

We made use of two databases belonging to The International Molecular Exchange (IMEx) consortium, which provides non-redundant, manually annotated data. The number of databases providing data may vary, depending on the status of their services. Only those that are active are used in this query to study HIPs:

– IntAct: an open-source, open data molecular interaction database and toolkit. Data are abstracted from the literature or from direct data depositions by expert curators following a deep annotation model providing a high level of detail [7]

- MINT: one of the major public repositories for molecular interactions reported in peer-reviewed journals [8]. The MINT scoring system reflects the quantity and quality of independent supporting evidence stored in the database. The function "Cumulative Evidence" is defined as the sum of all supporting evidence weighted by coefficients that reflects the confidence in the specific approach. The resulting scores range between 0 and 1, with only well supported interactions obtaining a value close to 1. More details and updates are available at http://mint.bio.uniroma2.it/mint/doc/MINT-confidence-score.html.
- BOND: The Biomolecular Object Network Databank is the first open access search resource to integrate sequence and interaction information. It serves the interests of the developing global interactome effort encompassing the genomic, proteomic and metabolomic research communities. It integrates data from several component databases including GenBank and BIND (Biomolecular Interaction Network Database) [9].

Statistical analysis

The Kruskal–Wallis test was used for comparing expression among pathological variables. Correlation was assessed with the Pearson coefficient. A significance level of 0.05 was used for all statistical tests. The statistical package SPSS version 18 was used for the analyses.

Results

Immunohistochemical staining of Hif1-α and its correlation with pathological variables

Anti-HIF1-α is a mouse monoclonal antibody (Abcam), partial recombinant HIF1-α protein. A 46.3 % of the samples showed a maximum score (+++ average expression cell/tissue from 80 %) for this antibody. 27 % of the samples showed a moderate positivity (++). We visualised cytoplasm localisation in 100 % of positive samples (see Fig. 1 to visualise IHQ expression profile of those proteins with significative statistically association with Hif1-α). Among the pathological variables studied, statistical analysis showed that the expression of Hif1-α is related significantly with the "clear" histological subtype ($p < 0.01$). The highest scores occurred in samples from tumours with pelvic invasion, broken capsule, and hilar invasion ($p = 0.010$, $p = 0.018$, and $p = 0.014$, respectively). In addition, we found a significant relationship between those cases showing Hif1-α high expression and were been diagnosed with high node invasion (N) ($p < 0.01$). We found no statistical support to associate this protein with other pathological variables.

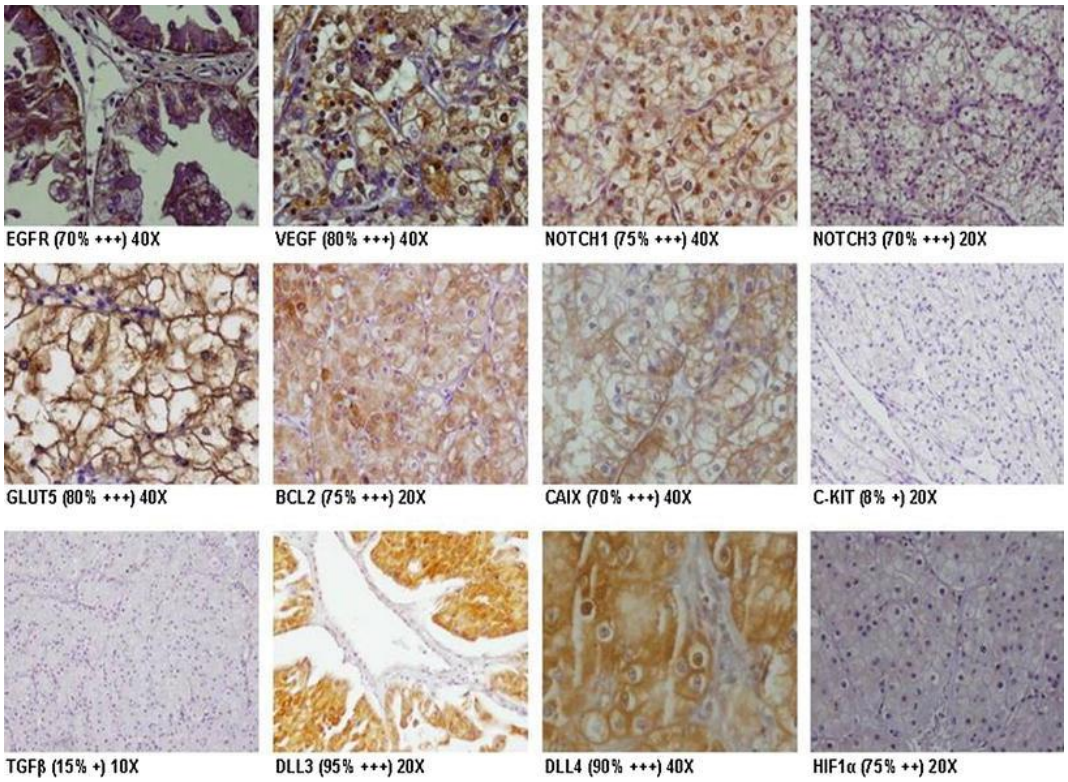


Fig. 1. Immunohistochemistry expression profile for those proteins with Hif1-α statistically association in clear cell histological type where Hif1-α shows more expression in a significative statistically fashion ($p < 0.01$)

Association of Hif1-α protein with other markers

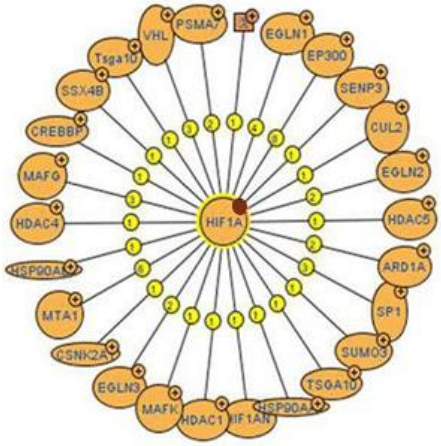
Statistical significant values of associations between molecular variables are listed in Table 2. Analysis of the relationship between Hif1-α and a panel of relevant RCC tumour markers (see Table 1) revealed a significant correlation with Bcl-2, CAIX, C-kit, EGFR, TGF-β, proteins of the VEGF family, proteins related to differentiation, such as Notch1 and 3 and DLL3 and 4, and proteins related to fructose uptake such as Glut5.

Table 2. Statistically significant correlations between Hif1-α and other biomarkers using Pearson's correlation coefficient test

	FLK1	VEGF	CAIX	EGFR	CKIT	TGFB	NOTCH1	NOTCH3	DLL3	DLL4	GLUT5	BCL2
HIF1-α Pearson's correlation $N = 80$	-, 448	,277	,287	,291	-,429	,668	-,529	,365	,268	,329	,521	-,664
	$P < 0.01$	$P = 0.014$	$P = 0.010$	$P = 0.009$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P = 0.001$	$P = 0.016$	$P = 0.003$	$P < 0.01$	$P < 0.01$

Identification of Hif1-α interacting proteins with bioinformatics tools

The IntAct website allows searches to be performed using the Molecular Interaction Query Language (MIQL). This provides a set of predefined fields that can be combined, allowing complex queries using Boolean logic. Once a query has been submitted to the website, the "Interactions" tab automatically opens and presents the list of interactions matching the query. We used the field for Advanced Search to submit the query "HIF1A AND Homo sapiens". We obtained 0 binary interactions in IntAct, but our query matched 55 interaction entries from 1 other IMEx database called MINT (Fig. 2) and 1,215 interaction evidences from 6 other databases: APID (134), BIND (13), Drug Bank (3), iRefIndex (596) and STRING (469).



protein	evidences	score	direct	ass.	coloc.	enz.	complex.	H.T.
× EP300 Homo sapiens (Q09472)	8	0.85	4	4				
× EGLN1 Homo sapiens (Q9GZT9)	4	0.81		2		2		
× MAFG Homo sapiens (Q9BRP3)	3	0.67	1	1	1			
× Tsga10 Mus musculus(Q6NY15)	1	0.64		1				
× EGLN2 Homo sapiens (Q96KS0)	2	0.59		1		1		
× EGLN3 Homo sapiens (Q9H6Z9)	2	0.59		1		1		
× MTA1 Homo sapiens (Q13330)	6	0.59		5	1			
× VHL Homo sapiens (P40337)	3	0.59		3			1	
× ARD1A Homo sapiens (P41227)	2	0.55	1	1				
× PSMA7 Homo sapiens (O14818)	2	0.55	1	1				
× CREBBPHomo sapiens(Q92793)	1	0.43	1					
× CSNK2A1Homo sapiens(P68400)	1	0.43				1		
× HSP90AA1Homo sapiens(P07900)	1	0.43	1					
× HSP90AB1Homo sapiens(P08238)	1	0.43	1					
× MAFK Homo sapiens (A5PLQ5)	1	0.43	1					
SP1 Homo sapiens (P08047)	3	0.43		3				
CUL2 Homo sapiens (Q13617)	1	0.28		1			1	
× HDAC1 Homo sapiens (Q13547)	1	0.28		1				
× HDAC4 Homo sapiens (P56524)	1	0.28		1				
× HDAC5 Homo sapiens (Q9UQL6)	1	0.28		1				
× HIF1AN Homo sapiens (Q9NWT6)	1	0.28		1				
SSX4B Homo sapiens (O60224)	1	0.28		1				1
SUMO3 Homo sapiens (P55854)	1	0.28		1				
× X Hepatitis B virus (Q7TDY3)	1	0.28		1				
SENP3 Homo sapiens (Q9H4L4)	1	0.17			1			
× TSGA10 Homo sapiens (Q9BZW7)	1	0.17			1			

Fig. 2. List of proteins discharged by the database MINT after submitting the query "HIF1A AND Homo sapiens". X (*red*) identifies proteins linked to a disease. *direct*. Direct interaction, *ass.* physical association, *coloc.* colocalization, *enz.* enzymatic reaction, *complex.* interaction with more participants, and *HT.* high throughput experiments (more than 50 interactions)

The list of proteins from the program output, the scoring system, based on both, experimental data and literature citation is shown in Fig. 2. It contains 25 human proteins and a protein from Hepatitis B virus. The protein orthologue for Tsga10 in Homo sapiens is TSGA10 (Q9BZW7), which prevents nuclear localisation of Hif1-α [10]. Those that show more evidence of interaction have scores closer to 1. In our case, the highest scoring entries are histone acetyltransferase p300 (EP300, score = 0.85) and Egl nine homologue 1 (EGLN1, score = 0.81). EP300 functions as a histone acetyltransferase and regulates transcription via chromatin remodelling. It acetylates all of the four core histones found in nucleosomes. Histone acetylation serves as an epigenetic tag for transcriptional activation. It also binds to and may be involved in the transforming capacity of the adenovirus E1A protein. It mediates cAMP gene regulation by binding specifically to phosphorylated CREB protein. In the case of HIV-1 infection, it is recruited by the viral protein Tat and regulates Tat's transactivating activity, and it may help to induce chromatin remodelling

of proviral genes. Defects in EP300 may play a role in epithelial cancer. The interactions provided by the MINT website include 4 based on direct interaction between Hif1-α and EP300, and the other 4 evidence interactions indicate physical association between these proteins. EGLN1 catalyses the post-translational formation of 4-hydroxyproline in hypoxia-inducible factor (HIF) alpha proteins. It hydroxylates Hif1-α at 'Pro-402' and 'Pro-564', and it hydroxylates prolines in Hif-2α. It functions as a cellular oxygen sensor and, under normoxic conditions, targets Hif through hydroxylation for proteasomal degradation via the von Hippel-Lindau ubiquitination complex. Defects in EGLN1 are the cause of erythrocytosis familial type 3 (ECYT3). For this protein we found 4 supported interactions based on 2 physical associations and 2 enzymatic reactions. The list also highlights proteins related to tumour diseases, such as MTA1, a component of the chromatin remodelling complex. MTA1 is upregulated in many carcinomas [11]. VHL also appears on the list; somatic mutations in renal cell carcinomas occur in the VHL gene. Von Hippel-Lindau syndrome is a dominantly inherited familial cancer syndrome predisposing carriers to a variety of malignant and benign neoplasms, most frequently retinal, cerebellar, and spinal haemangioblastoma, RCC, pheochromocytoma, and pancreatic tumours. The evolutionarily conserved VHL gene encodes 2 protein products, a 30-kD full-length form (p30) and a 19-kD form (p19). The protein products of the VHL gene play a role in the oxygen-sensing pathway, in microtubule stability and orientation, tumour suppression, cilia formation, regulation of senescence, cytokine signalling, collagen IV regulation, and assembly of a normal extracellular fibronectin matrix [12]. ARD1A is a protein acetyltransferase in mammalian cells that acts by binding Hif1-α to regulate its stability. Also, ARD1A-mediated acetylation enhances the interaction of Hif1-α with VHL and Hif1-α ubiquitination, suggesting that the acetylation of Hif1-α by ARD1A is critical to proteasomal degradation. The role of ARD1A in the acetylation of Hif1-α provides a key regulatory mechanism underlying Hif1-α stability [13]. CREBBP is another protein on the list; when cellular levels of cAMP increase, a cascade of events leads to the induction of genes that contain cis-regulatory elements called cAMP-response elements (CREs). Elevated cAMP levels cause stimulation and nuclear translocation of protein kinase A, which activates the transcription factor CREB (CRE-binding protein) by phosphorylating it at a single residue, serine-133. The recurring translocation t(11; 16) (q23; p13.3) has been observed only in cases of acute leukaemia or myelodysplasia secondary to therapy with drugs targeting DNA topoisomerase II [14]. HSP90AA1 and HSP90AB1 are highly conserved molecular chaperones that have key roles in signal transduction, protein folding, protein degradation, and morphological evolution. HSP90 proteins normally associate with other co-chaperones and play important roles in folding newly synthesised proteins or stabilising and refolding denatured proteins after stress. There are 2 major cytosolic HSP90 proteins: HSP90AA1, an inducible form, and HSP90AB1, a constitutive form. HSP90 is a molecular chaperone that plays a key role in the conformational maturation of oncogenic signalling proteins, including HER2/ERBB2, AKT, RAF1, BCR-ABL, and mutated p53. Tumour cells could contain HSP90 complexes in an activated, high-affinity conformation that facilitates malignant progression, which may represent a unique target for cancer therapeutics [15, 16]. The transcription factor SP1 is a DNA-binding protein that interacts with a variety of gene promoters containing GC-box elements. Overexpression of human SP1 induced apoptosis in all mouse and hamster cell lines tested. The apoptotic pathways induced by SP1 overexpression were cell type specific and required the SP1 DNA-binding domain [17]. CUL2 specifically associates with the trimeric VHL-elongin B-elongin C (VBC) complex in vitro and in vivo. This association was disrupted by mutations in VHL that disrupt elongin binding. Nearly 70 % of the naturally occurring cancer-disposing mutations in VHL abrogate elongin binding, suggesting that binding to elongin-CUL2 complexes contributes to the ability of VHL to suppress tumour growth in vivo. CUL2 is suggested as a candidate tumour-suppressor gene [18]. $HDAC_{1,4,5}$ histones, nuclear proteins that bind DNA and form nucleosomes, are directly involved in both the packaging of DNA into chromosomes and the regulation of transcription. Histone acetylation/deacetylation is a major factor in regulating chromatin structural dynamics during transcription. HDAC1 physically interacts and cooperates with RB1 (retinoblastoma tumour-suppressor protein). Deacetylation of p53 is mediated by a HDAC1-containing complex. The HDAC1/HDAC2 complex is associated with cancer-causing chromosomal translocations [19]. HDAC1 is a direct target of miR449A, which regulates cell growth and viability of prostate cancer cells in part by repressing expression of HDAC1 [20]. EST database analysis suggested that HIF1AN is expressed in multiple cell types. HIF1AN interacts with Hif-1-α and VHL to mediate repression of Hif-1 transcriptional activity [21]. SSX4B, fusion genes identified in synovial sarcoma represent a situation in which several members of a gene family have fused to a single partner gene and associated with the same malignant disease. The t(X; 18) (p11.2; q11.2)

translocation is closely associated with the development of synovial sarcoma. SUMO proteins, such as SUMO3, and ubiquitin post-translationally modify numerous cellular proteins and affect their metabolism and function. However, unlike ubiquitination, which targets proteins for degradation, sumoylation participates in a number of cellular processes, such as nuclear transport, transcriptional regulation, apoptosis, and protein stability.

Figure 3 outlines HIF1-α binary interactions, as well as those of EP300. MINT does not show binary interactions in the case of EGLN1. The scoring system is an effective tool for filtering interactions. In Fig. 4 we have displayed all of the proteins that, according to the MINT database, interact with the proteins participating in the Hif1-α signalling pathway.

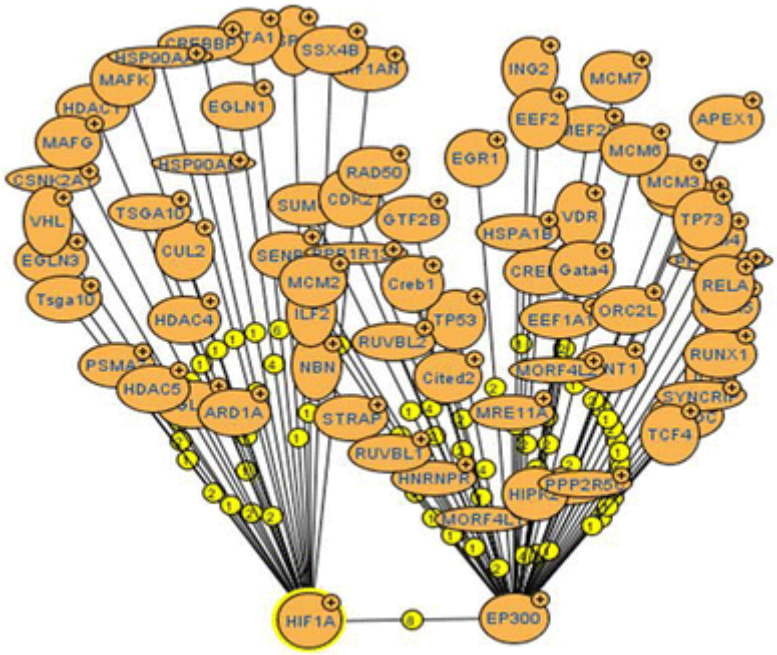


Fig. 3. Hif1-α and EP300 evidence-based binary interactions. The figure shows the complexity of the system since some of the proteins that are interacting with Hif1-α are in turn interacted with one another and so on

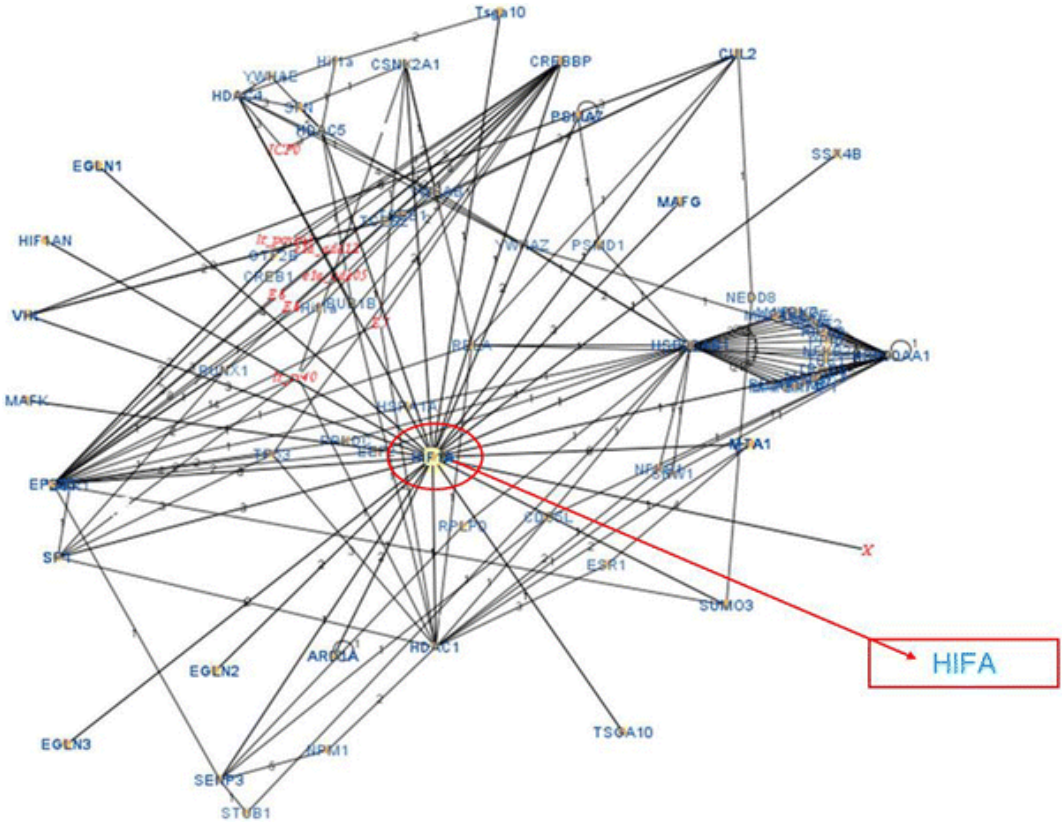


Fig. 4. Hif1-α signalling pathway obtained by MINT database

To confirm and extend the results obtained through the MINT database, we conducted a search through the Biomolecular Object Network Databank (BOND). The BOND interface provides a sophisticated way to perform cross-database queries of available sequence, interaction, complex, and pathway information. In the "Advanced Field Specific Search" section, we ran a query with the term "HIF1A AND Homo sapiens" which returned 41 interactions and 4 complexes. The new proteins that have evidence-based interactions with the protein Hif1-α are the following: *RB1* retinoblastoma 1, *EPO* erythropoietin precursor, *VEGF* vascular endothelial growth factor, *ATF2* activating transcription factor 2, *c-Jun* jun proto-oncogene, *HNF-4-α* hepatocyte nuclear factor 4, alpha, *MSH6* mutS homologue 6, *MSH2* mutS homologue 2, *CDKN1A* CDK-interacting protein 1, *ARNT* aryl hydrocarbon receptor nuclear translocator, *p53* tumour protein p53, *PHD2* egl nine homologue 1, *TAF1* transcription initiation factor TFIID subunit 1, *Jab1* COP9 constitutive photomorphogenic homologue subunit 5, *PHD3* egl nine homologue 3, *ARNT2* aryl hydrocarbon receptor nuclear translocator 2, *HSPCA* heat shock protein HSP 90-alpha, *p14ARF* cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4), *HPH-2* egl ninehomologue 1, and *OS-9* osteosarcoma amplified 9, endoplasmic reticulum lectin. With regard to the formation of complexes, the program showed us 4 complexes: (1) Hif1-α and ARNT bind to form a Hif1-α/ARNT heterodimer, which is present in increased amounts in hypoxia; (2) the Hif1-α-OS-9-PHD2 was isolated by a coimmunoprecipitation assay; (3) the Hif1-α-OS-9-PHD3 complex was isolated by a GST pull-down assay; and finally (4) Hif1-α and Hif1-β interact to form a complex.

Discussion

In this study of RCC, there are four main findings. First, the expression of Hif1-α is characteristic of the clear RCC histological subtype. Second, higher intracellular levels of Hif1-α are detected in cases with renal pelvis invasion, renal broken capsule and renal hilar invasion. Third, increases in the number of involved nodes (*N*) coincide with a greater cytoplasmic localisation of Hif1-α in tumour cells. Last, IHC shows that levels of Hif1-α protein are correlated with known markers consequence of its activity as transcriptional factor as CAIX, VEGF, and its receptor Flk-1; differentiation markers such as Notch1, 3 and their ligands DLL3 and DLL4; markers of invasiveness, proliferation or inflammation such as c-Kit, EGFR and TGF-β, markers associated with metabolic processes such as Glut5 and, finally, antiapoptotic markers such as Bcl-2.

Hif1-α expression is affected by oxygen concentration, VHL status, and the activity of interrelated molecular pathways such as the mammalian target of rapamycin pathway [22]. Hif1-α induces the transcription of several factors including VEGF, PDGF, CXCR, IGF-1, and CAIX [23].

Clear cell RCC had the highest expression of Hif1-α in a study that investigated Hif1-α expression in 66 clear cell, 20 papillary, and 6 chromophobe samples by western blot analysis [24]. No significant associations were observed between Hif1-α expression and tumour-node-metastasis stage, grade, tumour size, vein invasion, or DNA ploidy. In contrast with the study previously cited and in accordance with our results, other groups identified high Hif1-α expression as an adverse prognostic indicator [25, 26]. These disparate finding may be the result of different technological and methodological approaches used in each study.

We showed significant correlations between molecules associated with the pathway of angiogenesis such as Flk-1, VEGF, CAIX; molecules related to the pathway of invasiveness/proliferation or inflammation such as EGFR, c-Kit and TGF-β; molecules belonging to the differentiation pathway such as Notch1, Notch3, DLL3 and DLL4; molecules implicated in the transport of sugars such as Glut5, and molecules involved in the antiapoptotic response such as Bcl-2.

VEGF-A is the strongest proangiogenic factor and exerts its effects via interaction with VEGFR-1 and VEGFR-2 (Flk-1). The fact that Hif1-α showed a positive correlation with VEGF in our study corroborates the idea that Hif1-α accumulation leads to enhanced transcription of the respective DNA segments [23]. Carbonic anhydrases are transmembrane enzymes that play a crucial role in the regulation of pH by catalysing the reversible reaction of carbonic acid to carbon dioxide and water [27]. Both hypoxia and VHL inactivation lead to increased cellular levels of Hif1-α, and subsequently, to an increase in carbonic anhydrases, especially CAIX [28].

Hif1-α is involved in apoptosis and cell cycle regulation by controlling p53. Hypoxia induces apoptosis by increasing the stability of p53 [29]. In our study we found no statistical correlation between p53 and Hif1-α, but a negative correlation was found between the key regulator of apoptosis Bcl-2 and Hif1-α.

The placement of Hif-1 both upstream and downstream of cancer metabolism results in a feed-forward mechanism that may play a major role in the development of the invasive, metastatic, and lethal cancer phenotype. Hif1-α activates the transcription of SLC2A1 and SLC2A3, which encode the glucose transporters Glut1 and Glut3, respectively. Our results indicate that expression of Hif1-α is characteristic of clear cell histological type and, in turn, the expression of Hif1-α is positively correlated with the expression of glucose transporter Glut5. Glut5 is the sole transporter specific for fructose with no ability to transport glucose or galactose. The extensive expression of the glucose transporters, and the fact that in most of the clear cell RCC overexpressing Glut5 the rate of fructose uptake is exacerbated indicate that fructose may be a preferred substrate providing energy required for the growth and proliferation of renal cell carcinoma of the clear cell type. This increase of Glut5 could indicate preferential utilisation of fructose by renal cancer cells [30].

Notch signalling is connected to a wide variety of cellular processes, including cell fate specification, cell proliferation, differentiation, apoptosis and the maintenance of stem cells. Culture of lung cancer cells [31] or ovarian cancer cells [32] under hypoxia increases Notch pathway activation. Low oxygen content also potentiates Notch signalling in melanocytes through stabilisation of Hif1-α [33]. Our cohort of RCC samples showed positive statistical correlation between Hif1-α-Notch3, DLL3 and DLL4 ligands. In the case of Notch1, the correlation with Hif1-α was negative. Understanding the implications of Notch signalling in various tumour types will enable the effects of specific Notch signalling effects on tumour angiogenesis and growth to be evaluated as a potential for a novel antiangiogenic therapy in the clinic.

The present study demonstrates the activation of EGFR kinase with increasing tissue concentration of Hif1-α in RCC. As in other tumour types [34], we found that Hif1-α and EGFR are key molecular effectors in the development and progression of RCC, which act in the regulation of tumour angiogenesis as well, show a strong relationship with biological behaviour and prognosis of renal tumours, especially clear cell RCC, of which Hif1-α overexpression is characteristic.

Our results indicate that, in low-Hif1-α tissue, increasing the concentration of Hif1-α increases the concentration of c-Kit, and vice versa. Signalling by the receptor for stem cell factor (SCF), c-Kit, is of major importance for haematopoiesis, melanogenesis and reproduction, and the biological responses include proliferation and cell survival. We hypothesised that, because Hif1-α is expressed in the clear cell histological subtype and c-Kit is expressed in the chromophobe subtype [35], clear cell carcinomas and chromophobe carcinomas may have different signalling mechanisms at the angiogenesis and tumour progression level.

Finally, we emphasise that in our cohort of renal tumours the accumulation of Hif1-α corresponded to an increase in TGF-β. TGF-β is one of several cytokines that regulate angiogenesis. TGF-β can function either as a proangiogenic or antiangiogenic factor in vitro; however, the preponderance of evidence supports a proangiogenic role for TGF-β in vivo. Several lines of evidence support a prominent role for this cytokine signalling pathway in stimulating angiogenesis [36, 37, 38]. TGF-β signalling in endothelial cells is unique in that it can activate two distinct pathways: the classical Smad-dependent pathway through TβRII and TβRI to activate Smads 2 and 3, and the pathway through TβRII and ALK-1 to activate Smads 1, 5, and 8, which are usually activated by the TGF-β superfamily members, the bone morphogenetic proteins. The balance of signalling between these pathways regulates endothelial cell biology through the activation (increased endothelial cell proliferation and migration) and maturation (decreased endothelial cell proliferation and migration) phases of angiogenesis [39].

As protein–protein interactions are central to most biological processes, the systematic identification of all protein interactions is considered a key strategy for uncovering the inner workings of a cell. Understanding the large-scale organising principles of protein interaction networks is one of the prominent goals of post-genomic biology. Computational tools have become critical for the integration, representation and visualisation of heterogeneous biomedical data. Furthermore, several bioinformatics methods have been developed to formulate predictions about the functional role of genes and proteins, including their role in diseases [40]. Actually, the number of protein interaction databases is increasingly extensive, and allows us to use bioinformatics tools as IntAct, a database based on scientific publications that did not give us any binary interaction for Hif1-α search in *Homo sapiens*, but that led us to other databases that showed our query results. So we focused on the results obtained from MINT as it is another database belongs to IMEX consortium. The computational study of Hif1-α interacting proteins we have carried out using bioinformatics tools demonstrates the complexity that exists at the cellular level, taking into account the complex interaction networks that can surround a single protein. A major goal of such computational studies should be to decipher biological functions at the level of protein interactions, which may explain cellular behaviours and allow us to further characterise new tumour classes within the same histological subtype.

Individualised medicine seeks to identify the molecular basis of an individual patient's response to different therapeutic treatments. Individualised medicine is relevant for diseases such as cancer, and it has become a challenge to accomplish more efficient and specific therapeutics. An individualised response to treatment could make the difference between therapeutic success

and failure and could provide a basis for a more individualised prognosis. The use of up-to-date "omic" approaches is changing the way we understand modern medicine in terms of drug efficacy, toxicity and diagnosis. Results from genetic polymorphism studies, gene and protein expression profiling and epigenetic analysis illustrate how pharmacogenomic testing will contribute to the goal of individualised medicine.

To achieve the promise of individualised molecular medicine, the application of bioinformatics tools to the entire hierarchy of biological system interactions and dynamics will be required. Such tools will help to promote the effective discovery, validation and application of new diagnostic and treatment strategies in a real-time environment.

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Conflict of interest
The authors declare that they have no potential conflicts of interest.

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