CSF1R copy number changes, point mutations, and RNA and protein overexpression in renal cell carcinomas

Maria J Soares[1], Mafalda Pinto[1], Rui Henrique[2,3], Joana Vieira[1], Nuno Cerveira[1], Ana Peixoto[1], Ana T Martins[2], Jorge Oliveira[4], Carmen Jerónimo[1,3,5] and Manuel R Teixeira[1,3]

[1]Department of Genetics, Portuguese Oncology Institute, Porto, Portugal; [2]Department of Pathology, Portuguese Oncology Institute, Porto, Portugal; [3]Institute of Biomedical Sciences (ICBAS), University of Porto, Porto, Portugal; [4]Department of Urology, Portuguese Oncology Institute, Porto, Portugal and [5]Fernando Pessoa University School of Health Sciences, Porto, Portugal

Renal cell carcinomas comprise a heterogeneous group of tumors. Of these, 80% are clear cell renal cell carcinomas, which are characterized by loss of 3p, often with concomitant gain of 5q22qter. Although *VHL* is considered the main target gene of the 3p deletions, none has been identified as the relevant target gene for the 5q gain. We have studied 75 consecutive kidney tumors and paired normal kidney samples to evaluate at the genomic and expression levels the tyrosine kinase genes *CSF1R* and *PDGFRB* as potential targets in this region. Our findings show that RNA expression of *CSF1R*, but not of *PDGFRB*, was significantly higher in clear cell renal cell carcinomas than in normal tissue samples, something that was corroborated at the protein level by immunohistochemistry. The CSF1R staining pattern in clear cell renal cell carcinomas was clearly different from that observed in other renal cell carcinomas, suggesting its potential usefulness in differential diagnosis. FISH analysis demonstrated whole chromosomal gain and relative *CSF1R/PDGFRB* copy number gain in clear cell renal cell carcinomas, which might contribute to *CSF1R* overexpression. Finally, one polymorphism and two novel mutations were identified in *CSF1R* in clear cell renal cell carcinoma patients. Our data allow us to conclude that *CSF1R* plays a relevant role in clear cell renal cell carcinoma carcinogenesis and raise the possibility that CSF1R may represent a future valuable therapeutic target in these patients.
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Renal cell carcinomas comprise a heterogeneous group of tumors that represent about 3% of all malignancies in adults in the Western countries.[1] Clear cell renal cell carcinomas, which represent about 80% of all kidney tumors, are characterized by the loss of the short arm of chromosome 3 (3p), often with a concomitant gain of 5q22qter as the result of an unbalanced 3p;5q translocation.[2] Several genes have been presented as the targets of the 3p deletions seen in this tumor type, with emphasis for *VHL* (3p25~p26). In fact, germline mutations of *VHL* underlie the von Hippel-Lindau syndrome (which includes clear cell renal cell carcinoma as one of its features), whereas somatic inactivation of this gene has been demonstrated in the great majority of sporadic clear cell renal cell carcinomas.[3,4] On the other hand, no target genes have been identified for the 5q22qter gain, the second most common copy number change in clear cell renal cell carcinomas.[2,5]

The *colony-stimulating factor-1 receptor* (*CSF1R*) gene located in 5q33 is a potential target for this chromosomal gain in clear cell renal cell carcinomas. CSF1R is a transmembrane tyrosine kinase receptor and the CSF1R/CSF1 receptor/ligand complex has essential physiological functions in monocyte and macrophage differentiation, embryonic implantation, placental development, and lactogenic differentiation of the human breast.[6] Codons 301 and 969 located in *CSF1R* exons 7 and 22, respectively, have been shown to be mutated in hematological malignancies, with mutations in codon 301 being associated with neoplastic transformation through constitutive activation of the tyrosine kinase receptor[7] and deletions or mutations in codon 969 disrupting its negative regulatory activity.[8,9] Furthermore, a novel fusion gene between

RBM6 and *CSF1R* generated by a t(3;5)(p21;q33) has been identified in acute megakaryoblastic leukemia, coding for a chimeric RBM6-CSF1R protein that confers interleukin-3 independent growth.[10] Molecular cytogenetic studies of *in vitro* models of clear cell renal cell carcinoma have shown frequent *CSF1R* copy number gain,[5,11] but its role in renal carcinogenesis is still unexplored.

In this work, we aimed to study a relatively large series of kidney tumors for *CSF1R* expression both at the mRNA and protein levels, to determine the relationship between *CSF1R* expression and genomic copy number changes, as well as to look for *CSF1R* point mutations in exons 7 and 22. As the *platelet-derived growth factor receptor beta* (*PDGFRB*) gene belongs to the same gene family as *CSF1R* and is located nearby in 5q31 ∼ 32, we also aimed to evaluate its expression in renal cell tumors for comparison.

Materials and methods

Patient Data

A consecutive series of 75 kidney tumors diagnosed at the Portuguese Oncology Institute (Porto, Portugal) in 2004 and 2005 were studied. The institutional review board approved the project and patients were enrolled after informed consent. All patients underwent partial or radical nephrectomy before any other kind of treatment. Histologically, 41 were classified as clear cell renal cell carcinoma, 13 as chromophobe renal cell carcinoma, 5 as papillary renal cell carcinoma, 8 as oncocytomas, 2 as unclassifiable renal cell carcinoma (consisting of an admixture of areas of clear cell RCC, papillary RCC, and clear cell lined papillae), and 6 as tumors of other than renal cell origin (Table 1). For control purposes, paired nonneoplastic renal tissue distant from the tumor was obtained when possible. Tissue samples intended for genetic analysis were snap frozen immediately after surgery and stored at -80°C. Five-micrometer thick sections were then cut in a cryostat for molecular studies, and at every 15 cuts were microscopically checked for normal tissue and tumor tissue content. According to routine diagnostic procedures, representative tumor and normal tissues were formalin fixed and subsequently processed for paraffin embedding. Sections were cut and H&E stained for histopathological evaluation. These procedures comprised the diagnosis, grading, and pathological staging. Additional $5\,\mu$m thick sections of paraffin-embedded tissue were cut for immunohistochemical and FISH analyses.

Nucleic Acids Extraction and cDNA Synthesis

Genomic DNA was extracted from tumor and normal tissue using a standard technique comprising overnight digestion with proteinase K (20 mg/ml) in the presence of 10% SDS at 55°C, followed by phenol-chloroform extraction and precipitation with 100% ethanol.[12]

Total RNA was extracted from all samples using the FastRNA Green Kit (Qbiogene, Carlsbad, CA, USA) for 45 s, with a speed rating of 6.0 in a FastPrep FP120 Instrument (Qbiogene). Reverse transcription was performed with the SuperScript III First-Strand Synthesis System for RT–PCR (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions.

CSF1R and *PDGFRB* mRNA Quantification

Quantification of mRNA of the *CSF1R* and *PDGFRB* genes was performed by real-time RT-PCR using TaqMan probes on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Primers and probes for both genes, as well as for the endogenous control gene *HPRT*, were purchased as a predeveloped assay reagent from Applied Biosystems.

PCR reactions were performed in a $20\,\mu$l volume reaction containing $9\,\mu$l of synthesized cDNA, $10\,\mu$l of TaqMan universal master mix (Applied Biosystems), and $1\,\mu$l of primer and probe set. PCR was performed in separate wells for each primer/probe set and each sample was run in triplicate. PCR parameters were as follows: 50°C for 2 min, 95°C for 10 min, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min. Each plate included multiple negative controls consisting of bidistilled water, as well as serial dilutions of a positive control to make the standard curve.

To determine the relative mRNA expression levels of both *CSF1R* and *PDGFRB* for each sample, the expression value of the target gene was divided by the *HPRT* expression value to obtain a normalized target/internal reference ratio. This value was then divided by the average *CSF1R* or *PDGFRB* expression value of the normal renal tissue samples normalized against the average *HPRT* value, to obtain the relative expression level of each target gene in tumors as compared with normal tissue.[13,14] Overexpression of *CSF1R* was defined as a relative expression level higher than 7.35 (mean + 3s.d. in normal tissue), whereas overexpression of *PDGFRB* was defined as a relative expression level higher than 146.82 (mean + 3s.d. in normal tissue).

Immunohistochemistry

The tissue slides were deparaffinized by rinsing with xylol and rehydrated by passing through graded alcohols (absolute ethanol, 90, 80, 70, 50%). Endogenous peroxidase activity was blocked by incubating the slides for 20 min in 0.3% H_2O_2 in a 5% methanol solution. After washing the slides in water and PBS/0.05% Tween 20 solution, they were incubated with normal serum (Vectastain Universal Elite ABC Kit; Vector Laboratories, Burlingame, CA, USA) 1/100 in PBS-bovine serum albumin (BSA) 10% at room

Table 1 RNA and protein expression and genomic data on the *CSF1R* and *PDGFRB* genes in 75 kidney tumors

Histology	Sample	qRT-PCR CSF1R	qRT-PCR PDGFRB	IHC CSF1R	FISH[a,b] CSF1R/PDGFRB	Mutations CSF1R
Clear cell RCC (*n* = 41)	RT 128	18.60	2.50^{-3}	Positive	Normal	Normal
	RT 130	27.61	3.54^{-2}	Positive	(3:3)/4:4	Normal
	RT 131	16.15	9.60^{-3}	Positive	Normal	Normal
	RT 132	3.34	2.00^{-4}	Positive	3:2/6:4	Normal
	RT 133	4.52	9.00^{-4}	Positive	Normal	Normal
	RT 137	1.78	8.40^{-3}	Positive	3:2	Normal
	RT 138	9.05	1.12^{-1}	Positive	Normal	Normal
	RT 140	13.24	1.85^{-2}	Positive	Normal	Normal
	RT 142	4.66	3.00^{-4}	Positive	4:2	c.12C > T
	RT 143	1.11	2.00^{-4}	Positive	3:2/3:3/4:4	Normal
	RT 144	18.92	6.20^{-3}	Positive	Normal	Normal
	RT 150	3.19	5.27	Positive	4:4/3:3	c.908T > C
	RT 151	4.53	5.16	Positive	4:4/5:5/3:3	Normal
	RT 152	3.49	1.00^{-4}	Positive	3:2/3:3/4:2/4:4	Normal
	RT 154	31.58	1.72^{4}	Positive	3:2/6:4	c.2799T > C
	RT 155	11.75	4.19	Positive	3:3/4:4/6:6	Normal
	RT 157[c]	9.08	1.83^{4}	Positive	3:3/3:2	Normal
	RT 158	20.07	6.79^{4}	Positive	3:3/3:2	Normal
	RT 159	8.82	2.90	Positive	4:4/3:2	Normal
	RT 160	5.24	NA	Positive	Normal	Normal
	RT 161	5.96	NA	Positive	3:3/4:4	Normal
	RT 163	2.66	2.68^{-1}	Positive	Normal	Normal
	RT 164	13.74	2.35	Positive	3:3	Normal
	RT 165	5.5	NA	Positive	Normal	Normal
	RT 166	6.92	4.56	Positive	4:3/3:3/4:2/3:2	Normal
	RT 168	10.89	7.76^{-1}	Positive	Normal	Normal
	RT 170	5.97	4.25^{-1}	Positive	3:2/3:3	Normal
	RT 172	12.00	1.06	Positive	Normal	Normal
	RT 173	7.36	1.94	Positive	4:4/3:3	Normal
	RT 174	1.54	9.27^{-1}	Positive	3:3	Normal
	RT 177	18.39	1.51	Positive	Normal	Normal
	RT 180	NA	NA	Positive	Normal	Normal
	RT 181	1.93	4.72	Positive	4:4/3:3	Normal
	RT 186	14.02	3.30	Positive	NA	Normal
	RT 188	10.10	1.60	Positive	Normal	Normal
	RT 190	35.03	8.59	Positive	3:3/4:4	Normal
	RT 191	19.64	6.15	Positive	3:3/5:5/4:4/6:6	Normal
	RT 197[c]	NA	NA	Positive	3:2	Normal
	RT 199	4.24	2.10	Positive	Normal	Normal
	RT 200	11.66	NA	Positive	3:3	Normal
	RT 201	5.20	2.98^{-1}	Positive	Normal	Normal
Chromophobe RCC (*n* = 13)	RT 129	0.11	ND	Negative	4:4	ND
	RT 134	0.21	ND	Negative	4:4/8:8	ND
	RT 136	0.15	ND	Negative	1:1	ND
	RT 153	0.08	ND	Negative	4:4/3:3	ND
	RT 156	2.51	ND	Negative	3:3/4:4/6:6	ND
	RT 169	10.50	ND	Negative	Normal	ND
	RT 175	0.32	ND	Negative	Normal	ND
	RT 183	0.57	ND	Negative	NA	ND
	RT 192	0.01	ND	Negative	3:3/4:4/6:6	ND
	RT 193	1.07	ND	Negative	Normal	ND
	RT 194	0.04	ND	Focal	4:4	ND
	RT 195	NA	ND	Focal	NA	ND
	RT 196	NA	ND	Negative	Normal	ND
Papillary RCC (*n* = 5)	RT 147	72.03	ND	Positive[d]	Normal	ND
	RT 179	NA	ND	Negative	Normal	ND
	RT 182	1.16	ND	Negative	Normal	ND
	RT 187	7.04	ND	Focal	Normal	ND
	RT 198	2.58	ND	Positive[d]	Normal	ND
Unclassifiable RCC (*n* = 2)	RT 139	2.67	ND	Positive	Normal	ND
	RT 149	NA	ND	Positive[d]	Normal	ND
Oncocytomas (*n* = 8)	RT 135	0.04	ND	ND	ND	ND
	RT 141	1.93	ND	ND	ND	ND
	RT 145	0.03	ND	ND	ND	ND
	RT 146	NA	ND	ND	ND	ND
	RT148	NA	ND	ND	ND	ND

Table 1 Continued

Histology	Sample	qRT-PCR CSF1R	qRT-PCR PDGFRB	IHC CSF1R	FISH[a,b] CSF1R/PDGFRB	Mutations CSF1R
	RT 162	0.23	ND	ND	ND	ND
	RT 171	0.03	ND	ND	ND	ND
	RT 178	0.79	ND	ND	ND	ND
Urothelial carcinoma (n = 3)	RT 167	1.58	ND	ND	ND	ND
	RT 184	0.58	ND	ND	ND	ND
	RT 189	1.29	ND	ND	ND	ND
Leiomyosarcoma	RT 126	0.67	ND	ND	ND	ND
Follicular lymphoma	RT 127	13.76	ND	ND	ND	ND
Angiomyolipoma	RT 185	4.53	ND	ND	ND	ND

RCC, renal cell carcinoma; NA, sample not analyzable; ND, not determined.

[a]Number of copies of CSF1R/PDGFRB: number of copies of 5p15.2 (LSI D5S23. D5S721).

[b]Most representative cell population indicated at first.

[c]Multilocular cystic renal cell carcinoma (variant of clear cell renal cell carcinoma).

[d]Cytoplasmatic and membrane immunoreactivity.

temperature for 30 min in a humid chamber. After incubation, the primary antibody (rabbit polyclonal antibody; c-Fms/CSF-1R: sc-692; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added at a dilution of 1:200 in PBS-BSA 10%, and the slides were incubated overnight at 4°C in a humid chamber. The slides were then rinsed in PBS/0.05% Tween 20 solution and bound antibody was detected by applying biotinylated secondary antibody and ABC reagent from the Vectastain Universal Elite ABC kit. The slides were washed in PBS and incubated for 7 min in 3′,3-diaminobenzidine (DAB; Sigma-Aldrich, Steinheim, Germany) in a solution of 50 ml PBS-BSA 1% + 50 μl H_2O_2. Counterstaining was then performed with hematoxylin (Harris Modified Hematoxylin Stain; Fisher Scientific, Fair Lawn, NJ, USA) for 30 s and washed for 10 min in H_2O. Slides were mounted with Entellan (Merck KGaA, Darmstadt, Germany). A placenta sample was used as positive control. Sections were scored as negative, focal (<25%), or diffuse staining.

Fluorescent In Situ Hybridization

Five-micrometer thick sections from a representative paraffin-embedded block of each tumor were cut onto SuperFrost Plus Adhesion slides (Menzel-Glaser, Braunschweig, Germany). Slides were deparaffinized in two series of xylol followed by two series of ethanol (5 min each), rinsed in 2 × SSC, and placed in a solution of NaSCN 1 M at 80°C for 20 min (Merck KGaA). The tissue was then digested with 6 mg/ml pepsin (Sigma-Aldrich) for 20 min at 37°C, after which slides were rinsed in 2 × SSC and dehydrated in a series of ethanol. A dual-color probe targeting 5p15.2 (control) labeled with SpectrumGreen and the CSF1R and PDGFRB genes on 5q labeled with SpectrumOrange (Vysis LSI; Abbott Laboratories, IL, USA) was used for each sample. Slides were then placed in a Hybrite denaturation/hybridization system (Abbott Laboratories) and codenatured at 80°C for 8 min. Hybridization took place for 18 h at 37°C, followed by posthybridization washes in 2 × SSC/0.5% Igepal (Sigma-Aldrich) at 73°C for 5 min and 2 × SSC/0.1% Igepal at room temperature for 3 min. Slides were counterstained with DAPI (Vector Laboratories). The cut-off value for FISH scoring was calculated using the Microsoft Excel inverse function as described by Wolff et al[15] after analyzing 100 cells from each of 10 normal tissue samples (99% confidence level). The cutoff for amplification was a 5q/5p copy number ratio equal or above 2.2.

Mutation Analysis

The mutation hotspots in exons 7 and 22 of the CSF1R gene were screened by direct sequencing in an automatic DNA sequencer ABI PRISM 310™Genetic Analyser (Applied Biosystems), using primers described by Abu-Duhier et al.[16] Data analysis was performed with Sequencing Analysis Software (version 5.2; Applied Biosystems).

Statistical Analysis

Statistical calculations were performed with SPSS for Windows version 15.0 (SPSS, Chicago, IL, USA). The Mann–Whitney U-test was used as nonparametric test for unpaired samples to assess the significance of differences between histological groups with respect to gene expression and to evaluate the relationship between gene copy number and gene expression. All two-tailed P values less than 0.05 were considered significant.

Results

CSF1R and PDGFRB Expressions by Quantitative RT-PCR

All 75 kidney tumors and paired normal kidney samples were analyzed for CSF1R RNA expression by

quantitative RT-PCR (Table 1). Increased *CSF1R* RNA expression was observed in kidney tumors when compared with normal kidney tissue samples ($P = 3.19^{-9}$). When considering the histological subtypes, the level of *CSF1R* RNA expression was significantly higher in clear cell renal cell carcinomas compared with the remaining tumors altogether ($P = 1.00^{-7}$) and with most of the other tumor types individually (all but papillary renal cell carcinomas; Figure 1).

Significant *CSF1R* RNA overexpression (expression level higher than mean + 3s.d. of that in normal tissue) was seen in 21 of the 41 (51%) patients with clear cell renal cell carcinoma, in one of five (20%) patients with papillary renal cell carcinoma, in one of 13 (8%) patients with chromophobe renal cell carcinoma and in a renal follicular lymphoma. No altered *CSF1R* expression was observed in the two unclassifiable renal cell carcinomas, in the eight oncocytomas, or in the remaining five tumors of other than renal cell origin.

For *PDGFRB* RNA expression, all 41 clear cell renal cell carcinomas and corresponding normal kidney samples were analyzed (Table 1). *PBGFRB* RNA overexpression was seen in 3 (7.3%) of the 41 clear cell renal cell carcinomas, but no statistically significant differences were found between this carcinoma subtype and paired nonneoplastic tissue.

CSF1R Protein Expression by Immunohistochemistry

We examined all 61 renal cell carcinomas (Table 1) and 10 samples of normal tissue by immunohistochemical analysis targeting CSF1R (Figure 2). Strong and diffuse membrane CSF1R immunoreactivity was observed in all 41 clear cell renal cell
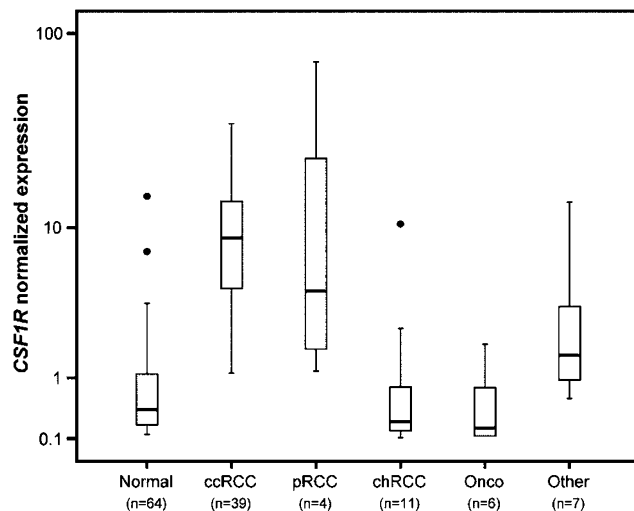
carcinomas. Two of the five papillary renal cell carcinomas (one of them with RNA overexpression) showed diffuse cytoplasmatic and membrane CSF1R immunoreactivity, whereas one presented
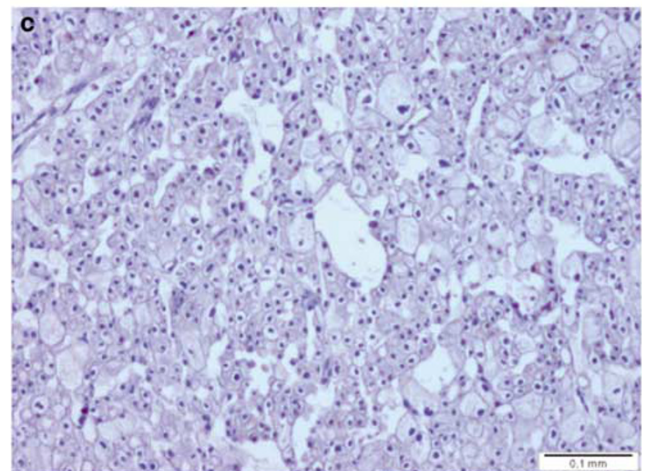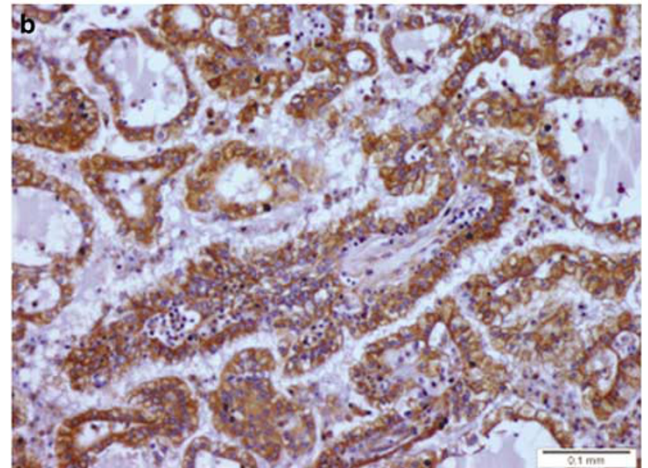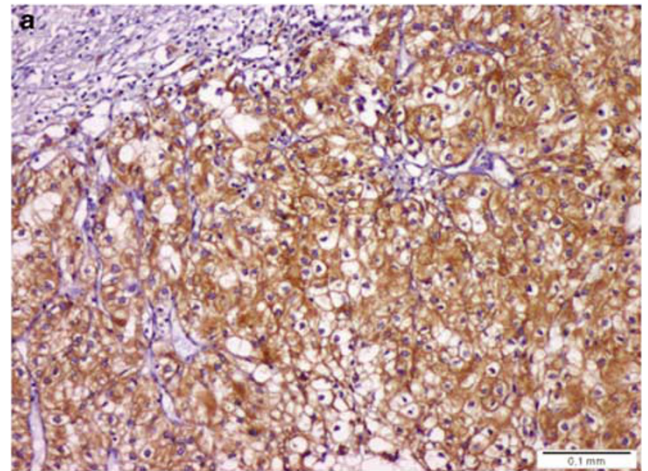

Figure 2 CSF1R protein expression by immunohistochemical analysis. (**a**) Clear cell renal cell carcinoma with diffuse membrane CSF1R immunoreactivity and its absence in stromal cells. (**b**) Papillary renal cell carcinoma with diffuse membrane and cytoplasmatic CSF1R immunoreactivity. (**c**) Chromophobe renal cell carcinoma with negative CSF1R staining.

Figure 1 *CSF1R* RNA expression by real-time PCR in kidney tumors and in nonneoplastic tissue (ccRCC: clear cell renal cell carcinoma; pRCC: papillary renal cell carcinoma; chRCC: chromophobe renal cell carcinoma; onco: oncocytoma; other: non renal cell carcinoma tumors; normal: normal kidney tissue). RNA overexpression was detected in most clear cell renal cell carcinomas and occasionally in other renal cell carcinomas (black dots indicate outliers).

focal immunoreactivity and two were negative for CSF1R staining. Of the 13 chromophobe renal cell carcinomas, 2 (15%) showed occasional (focal) staining, with all remaining cases classified as negative (including the single case with RNA over-expression). One of the two unclassifiable renal cell carcinomas showed diffuse cytoplasmatic and membrane CSF1R immunoreactivity, whereas the other presented diffuse membrane CSF1R immunoreactivity. All 10 normal renal tissue samples displayed negative staining in proximal tubules and positive staining in distal and collecting tubules.

CSF1R/PDGFRB Copy Number

All 61 renal cell carcinomas were analyzed for *CSF1R/PDGFRB* copy number using a FISH probe targeting the two genes in 5q31~33 (control probe in 5p15; Table 1). Of the 41 clear cell renal cell carcinomas, 24 (59%) had copy number gain of *CSF1R/PDGFRB*. Cell populations with relative *CSF1R/PDGFRB* gain when compared with the control probe (3:2, 4:2, or 6:4) was observed in 29% of clear cell renal cell carcinomas, but 3:3 (44%) and 4:4 (29%) signal patterns were also seen (Figure 3). Other chromosome 5 alterations were
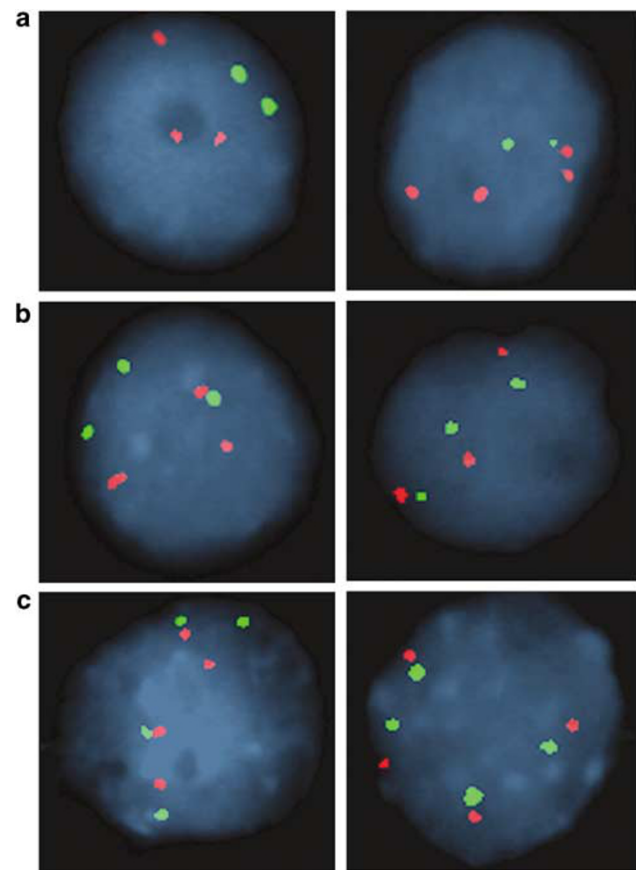
observed more rarely. Of the 13 chromophobe renal cell carcinomas, 7 (54%) had numerical changes of chromosome 5, namely trisomy (3:3; 23%), tetrasomy (4:4; 46%), and monosomy (1:1; 8%). 5q31~33 changes were not observed in papillary renal cell carcinomas. No statistically significant correlation
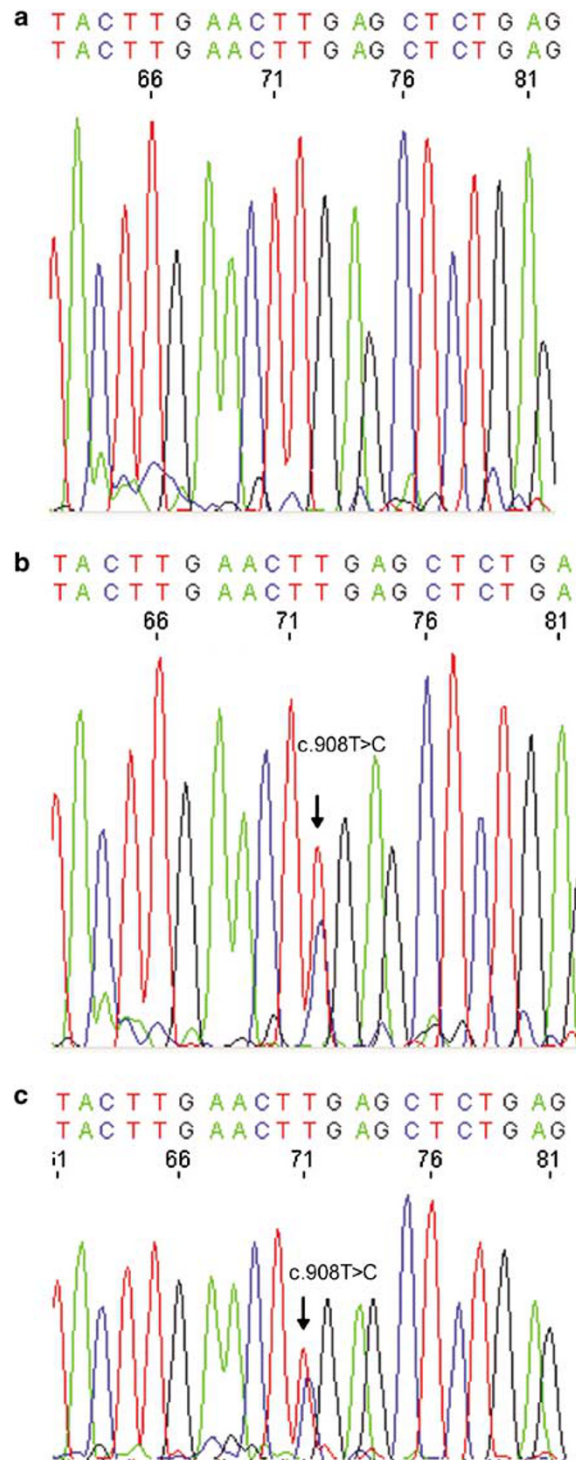

Figure 4 Sequencing analysis of exon 7 of *CSF1R*. (**a**) Wild type. (**b**) Tumor tissue showing the c.908T>C mutation. (**c**) The same mutation was found in the patient's blood sample, demonstrating its germline origin.

Figure 3 *CSF1R/PDGFRB* copy number changes in clear cell renal cell carcinomas evaluated by fluorescence *in situ* hybridization (*CSF1R/PDGFRB*, red; control probe, green). (**a**) Relative *CSF1R/PDGFRB* copy number gain. (**b**) Trisomy. (**c**) Tetrasomy.

was found between gene copy number and RNA expression.

CSF1R Structural Changes

All 41 clear cell renal cell carcinomas and 100 healthy blood donors were investigated for the presence of *CSF1R* mutations in exons 7 and 22. Three genetic alterations were detected (Figure 4). A novel exon 7 mutation (c.908T>C), which results in a leucine to serine substitution at codon 303, was detected in one patient in tumor tissue, in peripheral blood leukocytes, and in normal renal tissue. This novel mutation was absent in the 200 chromosomes from healthy blood donors. A second mutation, c.12C>T, was found in the noncoding region of exon 22 (3′ UTR), was also detected in the patient's normal renal tissue, but not in the 200 chromosomes from healthy blood donors. Finally, the third change, c.2799T>C, was detected in exon 22 in the tumor, in the patient's normal renal tissue, and in 2 of the 100 healthy blood donors, therefore, being classified as a polymorphism.

Discussion

Clear cell renal cell carcinoma is characterized by loss of genetic material from 3p, including the region 3p25∼26 where the *VHL* gene is located.[2] As germline *VHL* mutations underlie the von Hippel-Lindau syndrome and *VHL* somatic mutations are found in most sporadic clear cell renal cell carcinomas, this gene is considered the most relevant target of the recurrent 3p deletion seen in this tumor type.[3,4] On the other hand, no target genes have been identified for the 5q22qter gain, the second most common chromosome aberration in clear cell renal cell carcinomas. We therefore tested whether two potential oncogenes mapping to that chromosomal region, *CSF1R* and *PDGFRB*, could be the relevant target genes of the recurrent 5q gain. We found that RNA overexpression of *CSF1R* was much more common than that of *PDGFRB* in clear cell renal cell carcinomas. On the contrary to what was observed for *PDGFRB*, the RNA expression of *CSF1R* was significantly higher in clear cell renal cell carcinomas than in normal kidney tissue samples. These findings agree with gene expression profile data showing *CSF1R* as one of the genes more differentially expressed in renal cell carcinoma.[17]

The *CSF1R* RNA overexpression demonstrated in clear cell renal cell carcinomas was corroborated by protein overexpression observed by immunohistochemistry. Indeed, strong CSF1R membrane staining was present in most neoplastic cells of all cases of clear cell renal cell carcinomas, in clear contrast with other renal tumor types. These results indicate that CSF1R may be a useful immunohistochemical marker for the differential diagnosis of renal cell carcinoma. Indeed, the eosinophilic variant of chromophobe renal cell carcinoma is particularly difficult to distinguish from oncocytoma, whereas the typical variant of chromophobe renal cell carcinoma may closely resemble classical clear cell renal cell carcinoma.[18] These difficulties are illustrated by the fact that one clear cell renal cell carcinoma of this series was reclassified as a chromophobe renal cell carcinoma by the pathologist after the tumor displayed a genetic pattern typical of the latter (Vieira J *et al*, in preparation). Interestingly, the staining pattern of this case was clearly different from that observed in all other clear cell renal cell carcinomas, indicating its potential usefulness for the differential diagnosis of clear cell renal cell carcinoma.

The immunohistochemical findings in nonneoplastic kidney tissue strengthen our proposal that CSF1R plays a role in clear cell renal cell carcinoma carcinogenesis. Positive staining was present in distal and collecting tubules, but not in the cells of the proximal tubule from which clear cell renal cell carcinoma presumably originate. Thus, the absence of immunoreactivity in cells of the proximal tubule and the presence of *CSF1R* overexpression in the vast majority of clear cell renal cell carcinomas is compatible with the activation of an oncogene pathogenetically relevant for the development of this tumor subtype. On the other hand, the presence of positive staining in the cells of the collecting tubule, from which chromophobe renal cell carcinoma presumably originate, may explain the presence of focal CSF1R staining in some chromophobe renal cell carcinomas. The biological meaning of the diffuse cytoplasmic and membrane CSF1R immunoreactivity observed in two of the five papillary carcinomas is not known, as this receptor tyrosine kinase is a membrane protein. However, it is noteworthy that papillary renal cell carcinoma also originates from proximal tubule and this may explain that a proportion of these tumors share some gene expression changes with clear cell renal cell carcinoma.

Proto-oncogene activation can result from chromosomal translocations, gene amplifications, or subtle intragenic mutations affecting crucial amino-acid residues that regulate the activity of the gene product.[19,20] FISH analysis showed whole chromosomal gains and relative *CSF1R/PDGFRB* copy number gain, but no gene amplification. In particular, we confirmed that relative 5q genomic gain is typical of clear cell renal cell carcinoma. However, no statistically significant association was observed between *CSF1R/PDGFRB* copy number and expression, indicating that copy number changes might contribute to, but do not fully explain, the *CSF1R* overexpression seen in renal cell carcinomas. Another mechanism to activate proto-oncogenes is through point mutations. One *CSF1R*-silent single nucleotide polymorphism (SNP) and two novel mutations of unknown significance were identified in our panel of clear

cell renal cell carcinomas. Silent SNPs have largely been assumed to exert no discernible effect on gene function or phenotype. However, some studies have provided evidence that synonymous SNPs can affect protein function by altering mRNA stability.[21] One of the two mutations we found, results in a leucine to serine substitution at codon 303 (c.908T>C). The same alteration was described by Ridge *et al*[7] in codon 301 and seems to be associated with ligand independence and constitutive activation of the tyrosine kinase receptor. As the mutations we detected were present in the germline, one might conceive that they might confer hereditary predisposition to malignancy, but we have currently no evidence that this is the case in these three families.

Abnormally high *CSF1R* expression has been associated with aggressive behavior and poor outcome in a variety of malignancies, including breast,[22,23] endometrial,[24,25] ovarian,[26,27] and prostate cancers.[28] The follow-up time in this series is still relatively short to allow a meaningful evaluation of the prognostic value of *CSF1R* overexpression in renal cell carcinoma. The mechanisms whereby *CSF1R* overexpression might promote renal carcinogenesis may be similar to those of other neoplasms. Several studies indicate that autocrine and paracrine interaction of CSF1R and its ligand CSF1 may participate in the biology of breast cancer, being associated with tumor progression and metastasis.[22,23,29] In ovarian cancer autocrine intracellular or extracellular interactions between CSF1R and CSF1 produced by the tumor cells are more relevant than paracrine effects of stromal CSF1.[26,27] Concomitant expression of CSF1R and CSF1 was also observed in endometrial cancer cells and it is absent in the adjacent normal tissue.[24,25] Finally, it has been shown in uterine cervical carcinomas that the CSF1/CSF1R-signaling pathway is involved in enhanced survival and invasion of cancer cells via an autocrine mechanism.[30] Our finding of increased *CSF1R* expression at both the mRNA and protein level in most clear cell renal cell carcinomas suggests that tumor cells may be hypersensitive even to normal CSF1 levels, presumably resulting in decreased apoptosis and increased ability to invade. Furthermore, our immunohistochemical data show that tumor cells, but not tumor-associated macrophages or stromal cells, are the main source of this receptor in clear cell renal cell carcinomas. Usually, papillary renal cell carcinomas show more macrophage infiltration than clear cell renal cell carcinomas, and these cells may also collaborate in a paracrine manner in tumor angiogenesis and invasion.[31–33] Thus, the study of cytokine CSF1 expression separately in tumor and stromal cells is warranted to better understand its role together with CSF1R in renal tumorigenesis.

Sunitinib is currently approved as a second-line treatment of metastatic renal cell carcinoma in patients who have either not responded to or are not eligible to receive interleukin-2 therapy.[34,35] Sunitinib targets selectively VEGF, KIT, FLT3, RET, PDGFRA, and PDGFRB[36,37] and the fact that CSF1R shares structural and organizational homology with those receptor tyrosine kinases suggests a relevant role of CSF1R as a target of sunitinib in renal cell carcinoma patients. Furthermore, recent studies using hematopoietic colony assays demonstrated that imatinib targets CSF1R at therapeutic concentrations,[38,39] further indicating that CSF1R may represent a future valuable therapeutic target of receptor tyrosine kinase drug inhibitors in renal cell carcinoma patients.

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Disclosure/conflict of interest

We have no conflict of interest to declare.

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