Deregulation of PAX2 expression in renal cell tumours: mechanisms and potential use in differential diagnosis

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Abstract

Expression of PAX2 (Paired-box 2) is suppressed through promoter methylation at the later stages of embryonic development, but eventually reactivated during carcinogenesis. Pax-2 is commonly expressed in the most prevalent renal cell tumour (RCT) subtypes—clear cell RCC (ccRCC), papillary RCC (pRCC) and oncocytoma—but not in chromophobe RCC (chrRCC), which frequently displays chromosome 10 loss (to which PAX2 is mapped). Herein, we assessed the epigenetic and/or genetic alterations affecting PAX2 expression in RCTs and evaluated its potential as biomarker. We tested 120 RCTs (30 of each main subtype) and four normal kidney tissues. Pax-2 expression was assessed by immunohistochemistry and PAX2 mRNA expression levels were determined by quantitative RT-PCR. PAX2 promoter methylation status was assessed by methylation-specific PCR and bisulfite sequencing. Chromosome 10 and PAX2 copy number alterations were determined by FISH. Pax-2 immunoeexpression was significantly lower in chrRCC compared to other RCT subtypes. Using a 10% immunoexpression cut-off, Pax-2 immunoreactivity discriminated chrRCC from oncocytoma with 67% sensitivity and 90% specificity. PAX2 mRNA expression was significantly lower in chrRCC, compared to ccRCC, pRCC and oncocytoma, and transcript levels correlated with immunoexpression. Whereas no promoter methylation was found in RCTs or normal kidney, 69% of chrRCC displayed chromosome 10 monosomy, correlating with Pax-2 immunoexpression. We concluded that Pax-2 expression might be used as an ancillary tool to discriminate chrRCC from oncocytomas with overlapping morphological features. The biological rationale lies on the causal relation between Pax-2 expression and chromosome 10 monosomy, but not PAX2 promoter methylation, in chrRCC.

Keywords: Renal cell tumours ● PAX2 ● differential diagnosis ● chromosome 10 monosomy ● promoter methylation

Introduction

Renal cell tumours (RCTs) are the most common kidney neoplasms in adults, accounting for 2–3% of all non-cutaneous malignant neoplasms, ranking 14th in incidence for both genders and presenting a mortality rate of 1.6/100,000 worldwide [1]. Globally, in 2008, ~167,947 new cases of kidney cancer were diagnosed and 72,030 patients died because of this malignancy [1]. Renal cell tumours are a heterogeneous group of neoplasms which arise from the epithelium of renal tubules [2] and represent more than 90% of all renal tumours occurring in adults in both genders [3]. The most prevalent benign RCT is oncocytoma, whereas among the malignant RCTs, clear cell renal cell carcinoma (ccRCC, 70–75% of cases), papillary renal cell carcinoma (pRCC, 10–15% of cases) and chromophobe renal cell carcinoma (chrRCC, 5–10% of cases) are the most common subtypes [3, 4]. Most RCTs display characteristic histological features, enabling
their distinction in haematoxylin and eosin stained slides, but in a proportion of cases there are overlapping characteristics, which impair a clear diagnostic discrimination. For instance, the eosinophilic variant of chrRCC may be difficult to discriminate from oncocyto ma, yet this distinction is critical because the former is a malignant tumour and the latter is a benign one, thus entailing different clinical approaches [5].

The PAX2 (Paired-box 2) gene encodes a transcription factor from the evolutionarily conserved paired-box family, characterized by the presence of a paired-box DNA-binding domain [6]. This gene is located at 10q24.3-q25.1 and contains 12 exons [7], several of which are alternatively spliced, originating five well characterized alternative splice variants, sharing a common promoter region [8]. The PAX2 gene product is required during development of the central nervous system and genitourinary tract [9], being expressed in the developing kidney, as well as in the optic stalk, midbrain–hindbrain junction, and spinal cord [10]. During kidney development, Pax-2 expression is primarily present during early stages of embryogenesis, where it is thought to suppress apoptosis and promote epithelial aggregation, then it is switched off in most part of the kidney structures in the later phases of differentiation [11]. Indeed, in adult kidney, PAX2 mRNA expression is restricted to cells of the collecting ducts (where it seems to protect medullary cells against the stress induced by high levels of NaCl and urea) and, to a lesser extent, to cells of the distal tubules [12]. Although the precise mechanism of Pax-2 expression downregulation is not fully understood, epigenetic silencing mediated by PAX2 promoter methylation has been reported in the normal adult kidney cells of rats [13]. Conversely, PAX2 promoter methylation has been found in normal endometrium lacking Pax-2 expression and its loss has been associated with Pax-2 re-expression in endometrial carcinoma [14].

This temporal and spatial expression of PAX genes seems to be essential for Pax proteins function as nuclear transcription factors, critically involved in cell proliferation, differentiation and migration, and constitute, therefore, putative targets of disruption during tumorigenesis [15]. Although there is evidence for re-expression of the PAX2 gene in RCTs, the mechanism of oncogenic activation has not been reported [16]. Indeed, significant Pax-2 protein expression has been found in three of the most prevalent RCT subtypes—clear cell RCC, papillary RCC and oncocyto ma—but not in chromophobe RCC [17]. Interestingly, chromophobe RCC frequently display chromosome 10 loss [18, 19], which might justify that finding.

To clarify the mechanism(s) underlying PAX2 deregulation in RCTs, we assessed PAX2 mRNA and protein expression, as well genomic copy number changes and gene promoter methylation status in a large series of RCTs, representing the four major subtypes. Furthermore, the potential usefulness of Pax-2 immunoeexpression for discriminating among RCTs subtypes was determined.

Materials and methods

Patients and sample collection

One hundred and twenty RCT samples, comprising 30 cases of each of the four major subtypes (ccRCC, pRCC, chrRCC, and Oncocytoma) were consecutively selected from a larger series of patients diagnosed and treated at the Portuguese Oncology Institute, Porto, Portugal, whom underwent partial or radical nephrectomy, after informed consent. In each case, a tumour fragment was immediately snap-frozen, stored at −80°C, and subsequently cut in a cryostat for nucleic acid (DNA and RNA) extraction. The bulk material was routinely fixed in buffered formalin and paraffin-embedded. The corresponding hematoxylin-eosin stained sections were examined by a pathologist to determine the tumour type [3], grade classification [20] and pathological stage according to the TNM staging system [21]. A representative paraffin block of each tumour was further selected for immunohistochemical and FISH analyses. Relevant clinical and pathological data were collected and are provided in Table 1.

These studies were approved by the ethics committee of Portuguese Oncology Institute – Porto (Comissão de Ética do IPO Porto).

Nucleic acids extraction and cDNA synthesis

Genomic DNA was extracted from tumour and normal tissues by the phenol–chlorophorm method at pH 8, as previously described [22]. Samples were first submitted to overnight digestion, in a bath at 55°C, using buffer solution SE (75 mM NaCl, 25 mM EDTA), SDS 10% and proteinase K, 20 mg/ml (Sigma-Aldrich®, Schnelldorf, Germany). After digestion, extraction was performed with phenol/chloroform (Sigma-Aldrich®, Schnelldorf, Germany, Merck, Darmstadt, Germany) followed by precipitation with 100% ethanol.

Total RNA from RCT samples was extracted by the PureLink™ RNA Mini Kit (Invitrogen, Carlsbad, CA, USA) and reverse transcription was accomplished using the 120

| Clinicopathological features | RCT |
|-----------------------------|-----|
| Patients, n                 | 120 |
| Gender, n (%)               |     |
| Male                        | 70 (58) |
| Female                      | 50 (42) |
| Median age, years (range)   | 59 (29–83) |
| Pathological stage*, n (%)  |     |
| I                           | 50 (56) |
| II                          | 22 (24) |
| III                         | 16 (18) |
| IV                          | 2 (2) |
| Furhman grade*, n (%)       |     |
| 1                           | 2 (2) |
| 2                           | 29 (32) |
| 3                           | 39 (44) |
| 4                           | 20 (22) |

*Includes RCC cases only.
plied by using the RevertAid™ H Minus First Strand cDNA synthesis Kit (Fermentas, Ontario, Canada), following the manufacturer’s instructions.

DNA and RNA quality and concentration were analysed in a NanoDrop ND-1000 (NanoDrop Technologies, DE, USA) spectrophotometer.

**PAX2 mRNA quantification**

Quantification of PAX2 mRNA was performed by real-time RT-PCR using Taqman probes on an Applied Biosystems 7500 Real-time PCR (Applied Biosystems, Foster City, CA, USA). Primers and probes for PAX2 (Hs01057413_m1) and HPRT1 (Hs 99999909_m1) (endogenous control) were purchased as pre-designed optimized real-time PCR assay reagents from Applied Biosystems. In each reaction, 9 μl of cDNA, 1 μl of Taqman™ Gene Expression Assay and 11 μl of Taqman™ Gene Expression Assay Master Mix (Applied Biosystems) were used. All samples were run in triplicate. Two negative controls, consisting on the replacement of cDNA with DEPC-treated water (MP Biomedicals, Solon, OH, USA), were included in each plate. PCR parameters were as follows: 50°C for 2 min., 95°C for 10 min. and 45 cycles at 95°C for 15 sec. and 60°C for 1 min. For quantitation, a standard curve was built with a series of cDNA dilutions prepared from normal Human Reference total RNA (Agilent Technologies, Stratagene, La Jolla, CA, USA) or from a PAX2 expressing cell line (encephalytoma), for HPRT1 and PAX2 analysis, respectively. PAX2 expression levels were calculated by dividing the values of PAX2 mean quantity by those of hypoxanthine phosphoribosyltransferase 1 (HPRT1), as endogenous control. This ratio was then multiplied by 1000 for easier tabulation.

**Immunohistochemistry**

Immunohistochemistry was performed according to the avidin-biotin method using the VECTASTAIN® Universal Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA). Four micron-thick sections from the representative paraffin block of each RCT and of five normal endometrial samples (collected from women submitted to hysterectomy for uterine leiomyomas, and intended as controls) were deparaffinized with xylene and rehydrated with consecutive ethanol solutions (100%, 90% and 70%). Subsequently slides were steamed in a 1 x sodium citrate buffer solution (Vector Laboratories) for 20 min. in a 700 W microwave oven for antigen retrieval. Endogenous peroxidase was blocked by incubating the slides in a 0.6% H2O2 solution for 20 min. After washing the slides in distilled water and PBS/tween 20 solution, they were incubated with Normal Horse Serum 1:100 in PBS-BSA for 30 min., in a humid chamber, at room temperature. Then, slides were incubated with primary antibody [rabbit monoclonal antibody, Pax-2 (clone ab79389; Abcam, Cambridge, UK)] at a dilution of 1:3000 for 18 hrs, at 4°C, in a humid chamber. The slides were next incubated with a biotinylated secondary antibody 1:100 in PBS/BSA for 30 min. in a humid chamber, at room temperature and localization was performed by incubating the sections with the ABC complex 1:100 in PBS/BSA for 45 min., in a humid chamber, at room temperature. The slides were then immersed in a 3,3’-diaminobenzidine solution for 7 min. Slides were counterstained with hematoxylin for 10 sec., washed in tap water, dehydrated, and mounted with Entellan® (Merck, Germany). An unrelated ccRCC

The results for Pax-2 expression in each tumour were expressed in a semi-quantitative approach according to the estimated percentage of positive tumour cells. Immunostaining of more than 10% of the tumour cell nuclei was required for scoring a case as positive. The positive cases were further divided into two categories according to the proportion of positive cells: >10–50% of the nuclei stained and >50% of the nuclei stained [17, 19, 23].

**Bisulfite modification and methylation-specific PCR**

DNA samples from RCTs, normal renal tissue (NK, four cases obtained from patients without renal tumour, used as negative control for methylation status) and normal endometrium (two cases, serving as putative positive control for PAX2 promoter methylation [14]), were subjected to sodium bisulfite modification, using the EZ DNA Methylation-Gold™ Kit (Zymo Research, Orange, CA, USA) according to manufacturer’s instructions.

Methylation analysis of PAX2 promoter was performed by methylation-specific PCR (MSP). Bisulfite-modified genomic DNA was tested using two sets of primers, each recognizing either the methylated or the unmethylated sequences located at the promoter region of PAX2 gene. Two adjacent regions were screened to assess the promoter methylation status [13, 14]. Primer sequences are summarized in Table 2, along with amplicon lengths and positions, and also optimized annealing temperatures.

PCR reactions were performed in an Applied Biosystems Veriti® Thermal Cycler (Applied Biosystems), Each PCR tube contained 14.36 μl of sterile distilled water (B. Braun, Melsungen, Germany), 2 μl of 10 x DyNAzyme™ Hot Start Reaction Buffer (Finnzymes, Espoo, Finland), 0.2 mM of dNTPs (Fermentas, Ontario, Canada), 250 mM of primer forward and 250 nM of reverse primer, 0.24 μl of DyNAzyme™ II Hot Start DNA polymerase 2 U/μl (Finnzymes, Finland), and 2 μl of sample DNA, to a final volume of 20 μl. PCR amplifications were performed as follows: 10 min. at 94°C, followed by 38 cycles of 30 sec. at 94°C, 30 sec. at annealing temperature (see Table 2) and 30 sec. at 72°C. A 10 min. elongation step at 72°C completed the PCR amplification and PCR products were then submitted to electrophoresis on a 2% agarose gel.

Primers’ specificity was assessed using CpGenome™ Universal Methylated DNA (Millipore, Darmstadt, Germany) and CpGenome™ Universal Unmethylated DNA (Millipore) as positive and negative controls, respectively.

**Bisulfite sequencing**

The promoter region of the PAX2 gene was subjected to bisulfite sequencing in the aforementioned normal kidney and endometrial tissue samples. Primer sequences, amplicons, and annealing temperatures are listed in Table S1. PCR reactions included a 94°C denaturation 10 min. step followed by 40 cycles at 94°C for 30 sec., annealing temperature for 30 sec., and 72°C for 30 sec. PCR products were loaded in a 2% agarose gel, stained with ethidium bromide and visualized under an ultraviolet transilluminator. Excess primer and nucleotides were removed by Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, USB Corporation, Cleveland, OH, USA) following manufacturer’s protocol. The purified products were sequenced using the dGTP BigDye Terminator Cycle Sequencing Ready
Reaction kit (Applied Biosystems) in an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems). The approximate amount of methylcytosine of each Cpg site was calculated by comparing the peak height of the cytosine signal with the sum of the cytosine and thymine peak height signals [24]. Cpg sites with ratios 0–0.20, 0.21–0.80, and 0.81–1.0 were considered unmethylated, partially methylated, and fully methylated, respectively.

**Fluorescence in situ hybridization analysis**

Bacterial Artificial Chromosome (BAC) clones targeting the PAX2 gene (RP11_1061B5) were selected using the UCSC Human Genome Browser platform and obtained from the BACPAC Resources Center (Oakland, USA). The Escherichia coli bacteria were first grown in LB agarose medium supplemented with cloramphenicol 12.5 mg/ml, at 37°C overnight. Then, an individual colony was inoculated in 10 ml of liquid LB medium and incubated at 37°C for 16 hrs with continuous agitation. The culture was centrifuged and pellet used for plasmid DNA extraction using the NucleoSpin® Plasmid kit (Macherey-Nagel, Düren, Germany), according to manufacturer’s instructions. DNA concentration was determined in a NanoDrop ND-1000 spectrophotometer.

After adjusting the plasmid DNA concentration to 10 ng/µl, amplification was carried out using the Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare), according to manufacturer’s instructions. Probes were labelled using a nick translation DNA labelling system (Enzo Life Sciences, Exeter, UK). DNA was eluted in 10 µl of Vysis LSI/WCP Hybridization Buffer (Abbott Molecular, Des Plaines, IL, USA).

FISH analysis for PAX2 was performed in 4 µm thick tissue sections obtained from representative paraffin blocks of each sample and placed in SuperFrost Plus Adhesion slides (Menzel-Glaser, Braunschweig, Germany). Sample processing, hybridization, and analysis were performed as previously described [25]. The PAX2 probe, labelled with SpectrumGreen, was combined with the Vysis centromeric probe for chromosome 10 (CEP10), labelled with SpectrumOrange (Abbott Molecular) and applied to each sample. For scoring purposes, only intact, non-overlapping nuclei were considered. An abnormal population was considered representative when at least 10% of neoplastic cell nuclei presented a copy number change.

**Table 2 Sequences of the primers used in the methylation-specific PCR assays**

| Primer | Primer sequence (5′-3′) | AS (bp) | AL | AnT (°C) | Reference |
|--------|------------------------|--------|----|----------|-----------|
| PAX2_M (F1) | GGGTTTTTTTCGCTGAAGTTTC | 170 | −676 to −846 | 60 | [14] |
| PAX2_M (R1) | ACTAAACCTCGACTCCCGAT | 172 | −673 to −845 | 62 | [14] |
| PAX2_U (F1) | GGGTTTTTTGTTGAGTTTGG | 139 | −489 to −628 | 62 | [13] |
| PAX2_U (R1) | AAAAACTCAAATCCTCCCAAT | 141 | −489 to −630 | 59 | – |
| PAX2_M (F2) | AGTTGTTAGCTCGTCGTTTT | 139 | −676 to −846 | 62 | [14] |
| PAX2_M (R2) | ACAATCCCGAAAATATCCGAATAA | 139 | −676 to −846 | 62 | [14] |
| PAX2_U (F2) | AGAGTTGTTAGCTGTTGTTT | 141 | −489 to −630 | 59 | – |
| PAX2_U (R2) | ACAATCCCGAAAATATCCGAATAA | 139 | −676 to −846 | 62 | [14] |

AS: amplicons size; AL: Amplicons location in relation to the transcriptional start site; Ant: Optimized annealing temperatures; M: Methylated; U: Unmethylated; F: Forward; R: Reverse; bp: base pairs.

**Statistical analysis**

The chi-square test or Fisher’s exact test were used to uncover differences in the frequency of immunoreactivity for Pax-2 protein according to immunohistochemical scoring, among the four RCT types. To assess the value of PAX2 protein immunohistochemical expression for discrimination between chromophobe RCC and oncocytomas, sensitivity, specificity, positive and negative predictive values (PPV and NPV) were determined using the 10% or the 50% cut-off values. Differences among groups were assessed by the Kruskal–Wallis non-parametric test, followed by pairwise comparisons using the Mann–Whitney U test, when appropriate. Association between PAX2 mRNA expression levels and protein expression (immunohistochemistry score) was carried out using the Mann–Whitney U test, when appropriate. Association between PAX2 mRNA expression levels and protein expression (immunohistochemistry score) was carried out using the Mann–Whitney U test, when appropriate. Association between FISH and immunoreactivity for Pax-2 protein was assessed using the Mann–Whitney U test, when appropriate. Association between FISH and immunoreactivity for Pax-2 protein was assessed using the Mann–Whitney U test, when appropriate. Association between FISH and immunoreactivity for Pax-2 protein was assessed using the Mann–Whitney U test, when appropriate.
from those of grade 2 and grade 3 tumours (P < 0.01, for both). However, no correlation was found between PAX2 mRNA expression levels and patients’ age (r = 0.112, P = 0.295) or pathological tumour stage (P = 0.542).

Pax2 protein expression by immunohistochemistry

As expected, immunoreactivity for Pax-2 was only found in cell nuclei. In normal renal tissue, Pax-2 immunoperoxidase was restricted to distal tubules and collecting duct cells, which showed intense and homogeneous staining (Fig. 2A). Among RCTs, intense staining was shown by ccRCC (Fig. 2B), oncocytomas (Fig. 2C), and pRCC (Fig. 2D), whereas most chrRCC were negative (Fig. 2E). The results of immunostaining scoring for Pax-2 in all RCT subtypes are summarized in Table 3.

In normal endometrium samples, intense immunostaining for Pax-2 was apparent in all nuclei of endometrial glands (Fig. 2F) in all cases tested (n = 5).

Statistically significant differences were observed for quantitative Pax-2 expression among the four subtypes of RCTs (chi-square test, P < 0.001). Pairwise comparisons disclosed significant differences in all cases (P < 0.01) except for pRCC versus chrRCC (P = 0.119) and for oncocytoma versus ccRCC (P = 0.612). Concerning the clinical-pathological data, we found significant differences between the immunoperoxidase frequencies according to Furman grade (P = 0.04) and pathological stage (P = 0.03). These results are shown in Table 4. Both grade and stage were grouped in two categories for statistical purposes (Fig. 3).

PAX2 promoter methylation

Using the previously published set of primers by Wu et al. no methylation was found for the PAX2 promoter in any of the tumour or normal renal tissue samples. As these primers showed PAX2 promoter as methylated in normal endometrium [14], we tested the same primer’s set in two normal endometrium tissue samples and both were unmethylated at the PAX2 promoter (Fig. 5).

Because no methylation was found, either in RCTs or in normal tissues using the two primer sets and PAX2 methylation might occur at regions that were not assessed by MSP, we performed bisulfite sequencing of the entire PAX2 promoter region CpG island. Strikingly, the full characterization of the methylation status of PAX2 promoter allowed us to confirm absence of methylation in most of CpG dinucleotides within the CpG island (Fig. 6).

PAX2/Chromosome 10 copy number alterations

All 30 chrRCC cases were tested for PAX2/chromosome 10 copy number alterations using FISH probes targeting the PAX2 gene and chromosome 10 centromeric region, respectively. Chromosome 10 loss was observed in 18 (69%) tumours, whereas gain was detected in two (8%) cases, and no alterations were found in six (23%) out of the 26 chrRCC analysable (four chrRCC samples were not included due to technical reasons). Interestingly, no isolated PAX2 locus

| Table 3 Immunohistochemical expression of Pax-2 in histological sections of renal cell tumours (RCTs) |
| RCT subtype       | Negative, n (%) | Positive, n (%) |
|                  | ≤10%            | >10–50%         | >50%            |
| Clear cell (n = 30) | 1 (3.3)         | 4 (13.4)        | 25 (83.3)       |
| Papillary (n = 30)  | 13 (43.3)       | 4 (13.4)        | 13 (43.3)       |
| Chromophobe (n = 30) | 20 (66.7)    | 6 (20)          | 4 (13.3)        |
| Oncocytoma (n = 30) | 3 (10)          | 5 (16.7)        | 22 (73.3)       |

Pax-2 negative cases, categorized according to the 10% cut-off value, showed significantly lower median mRNA levels compared to Pax-2 positive cases (P = 0.011), as illustrated in Figure 4.

Because the morphological differential diagnosis between chrRCC and oncocytoma might be troublesome, we tested whether Pax-2 immunostaining might be used as an ancillary tool for histopathological assessment. Indeed, the best Pax-2 immunostaining cut-off value for discriminating between chrRCC and oncocytoma was 10%, with a sensitivity and specificity of 67% and 90%, respectively. For comparison purposes, we also calculated the performance of Pax-2 immunostaining for discriminating chrRCC from oncocytoma in previous publications, and the results are summarized in Table 5.
deletion was found in any of the examined samples. Figure 6 shows representative FISH images of chrRCC tested.

Significant differences in Pax-2 immunoexpression were observed among the three categories of PAX2/CEP 10 copy number changes, when the 10% ($P < 0.001$) or the 50% ($P = 0.018$) cut-off values were used (Table 6). Moreover, Somers’d directional measure revealed a

**Table 4** Correlation between PAX2 immunoexpression and Furhman nuclear grade and pathological tumour stage, $n$ (%)

| Furhman grade | Immunoexpression scoring | $P$   |
|---------------|-------------------------|-------|
|               | $\leq 10\%$ | $> 10\%$ |
| 1–2           | 7 (23%) | 24 (77%) | 0.04 |
| 3–4           | 27 (46%) | 32 (54%) |
| Stages        |            |        |
| I–II          | 23 (32%) | 49 (68%) | 0.03 |
| III–IV        | 11 (61%) | 7 (39%) |

**Fig. 2** Pax-2 protein expression by immunohistochemical analysis in normal kidney (A), and endometrial glands (F) and renal cell tumours (C–E). Both normal distal tubules and the collecting duct cells with intense homogeneous nuclear immunoreactivity (A). Higher intensity staining was depicted in ccRCC (B) and oncocytomas (C), as well as in pRCC (D), whereas most of chrRCC were negative (E).

**Fig. 3** Distribution of PAX2 mRNA expression levels (Log 10 transformed) according to Pax-2 immunoexpression scoring using the 10% cut-off value ($n = 120$).
strong positive association between the immunoexpression frequency and PAX2/CEP 10 copy number changes, both using the 10% ($d = 0.8$, $P < 0.001$) or 50% ($d = 0.498$, $P = 0.041$) immunoexpression cut-off values. No statistically significant association was found between PAX2/CEP 10 copy number and PAX2 mRNA expression ($P = 0.919$).

Discussion

PAX2 is an important transcription factor intensely expressed during kidney development [10], but which is downregulated in the latter phases of differentiation in most of the kidney structures [11]. In the mature kidney, Pax-2 expression is restricted to the collecting ducts and distal tubules, where it promotes osmotic tolerance and protects cells from apoptosis due to high sodium chloride and urea exposure [12]. The mechanisms leading to PAX2 silencing at the end of kidney development, however, are still controversial and hypotheses include PAX2 promoter methylation [13] and WT1 mediated repression [26]. Moreover, PAX2 has
been reported to function as an oncogene, conferring proliferative and apoptosis inhibitory characteristics to cells in several tumour models [16], including RCTs [27]. Finally, diffuse protein expression of Pax-2 has been reported to be frequent in three of the most prevalent RCT subtypes - ccRCC, pRCC and oncocytoma - but not in chrRCC, suggesting its use for differential diagnosis in problematic cases [17, 19, 23, 28].

In this study, immunohistochemical analysis of Pax-2 expression disclosed significant differences among the four major subtypes of RCT. Whereas ccRCC and oncocytoma showed the highest frequency of positive cases (immunoexpression >10% of tumour cells), chrRCC showed the opposite trend, in line previous reports [17, 19, 23], although some differences were apparent mainly in the frequency of positive cases among chrRCC. As the same cut-off value was used, it is likely that methodological differences underlie these discrepant results. Indeed, we used a monoclonal antibody (which tends to be more specific), in a 1/3000 dilution, whereas all the aforementioned studies used polyclonal

Table 6 FISH results for PAX2/CEP10 copy number and distribution of the immunopexpression frequencies and mRNA expression levels, in chrRCC cases

| FISH analysis     | Immunopexpression, n (%) | mRNA expression | 10% cut-off | 50% cut-off | Median (interquartile range) |
|-------------------|--------------------------|-----------------|-------------|-------------|-----------------------------|
| Monosomy          |                          |                 |             |             |                             |
|                   | 17 (94)                  | 1 (6)           | 18 (100)    | 0 (0)       | 213.2 (104.9–1000.4)        |
| No ch10 loss      | 1 (17)                   | 5 (83)          | 4 (67)      | 2 (33)      | 524.7 (114.1–1508.3)        |
| Polysomy          | 0 (0)                    | 2 (100)         | 1 (50)      | 1 (50)      | 384.7 and 527.7*            |

*Descriptive values.
antigen, associations between PAX2 reported a decrease or lack of Pax-2 immunoexpression with increasing Fuhrman grade [23], and of Luu et al., although a different grading system was used and only ccRCC were analysed [32]. Concerning pathological tumour stage, however, only PAX2 immunoexpression showed a significant inverse correlation, whereas the same trend was not apparent at the mRNA level. The reason for this discrepancy is not immediately apparent and may derive from the higher heterogeneity of the staging system compared to the more homogeneous nuclear grading system.

Another main objective of the present study was to ascertain the mechanism underlying the differential expression of Pax-2 among RCT subtypes. Two possibilities for PAX2 downregulation have been suggested: PAX2 promoter hypermethylation and/or gene deletion. The former possibility was founded upon existence of a CpG island in the promoter region and the putative role of promoter methylation in PAX2 gene silencing at the final stages of kidney development [13]. The latter hypothesis stemmed from the observation that loss of chromosome 10 (to which PAX2 is mapped) is a frequent event in chrRCC [17, 19, 23].

To test the PAX2 promoter methylation hypothesis, methylation-specific PCR was performed not only in chrRCC but also in the remainder RCT subtypes and normal kidney tissue samples, using two different sets of primers that anneal to two adjacent regions in the promoter [13, 14]. These regions have been previously reported to be involved in PAX2 gene transcription regulation. Remarkably, no methylation was detected in those regions either in any of the analysed RCT samples, including chrRCC nor in normal kidney tissues. Furthermore, no methylation was found in normal endometrium (five random samples), which was previously reported to harbour PAX2 promoter methylation using one of the primer sets [13, 14]. In addition, we tested for methylation using the other set of primers and the result was again negative. Importantly, we demonstrated Pax-2 protein expression in endometrial glands [which were negative according to previous studies [13, 14], which is consistent with absence of methylation by MSP analysis. Moreover, our results are in accordance with those of Luu et al., which also did not find PAX2 promoter methylation in ccRCC or in normal kidney tissues [32]. As bisulfite sequencing allows for a more detailed characterization of the methylation status for individual CpGs, we used this approach to further confirm the absence of methylated residues in the promoter region of PAX2. Bisulfite sequencing in normal kidney and endometrium samples validated MSP results, showing that no methylation was present in PAX2 promoter region. Taken together our results indicate that methylation does not explain PAX2 different expression levels both when comparing groups of tumours and stages of kidney development.

To explore the chromosome 10 loss hypothesis, FISH analysis using specific probes for chromosome 10 and for the PAX2 gene was performed in all cases of chrRCC. As expected, the vast majority of chrRCC cases exhibited chromosome 10 monosomy, which is in accordance with a previous report [18], and no case of isolated PAX2 locus deletion was observed. This might explain both the lower PAX2 mRNA levels and protein expression globally observed in this tumour subtype. Remarkably, a significant association between the immunoexpression findings was observed, indicating that chromosome 10 loss is the most likely mechanism underlying
PAX2 loss of expression. This is further supported by the higher frequency of Pax-2 immunooexpression in cases with no copy number change or with polysomy, a trend confirmed by the Somers’d test. Interestingly, the highest correlation value was found for the 10% immunooexpression cut-off, suggesting that this cut-off might be more biologically meaningful. Our results indicate that PAX2 promoter methylation is not implicated in gene silencing in chrRCC or in normal kidney. Instead, the frequent chromosome 10 loss observed in chrRCC samples, and the significant correlation with Pax-2 immunooexpression categories, indicate that this genetic alteration is the major cause for PAX2 underexpression in this tumour subtype. Importantly, this feature might be used for discriminating chrRCC from oncocytoma in challenging cases. Nonetheless, additional studies are required to further determine the genetic and/or epigenetic regulation mechanisms underlying PAX2 differential expression in other RCTs.

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Conflicts of interest

The authors declare that they have no competing interests.

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