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1 Diagnosis of uncommon renal epithelial neoplasms: performances of fluorescence *in situ*

2 hybridization

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30 Author contributions:

- 31 MB and MABR designed the study and wrote the manuscript.
- 32 FD, FC, SFKJ and NRL performed the experiments.
- 33 RM, GV and KB provided clinical data.

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40 ABSTRACT (250 words)

41 Renal cell carcinomas (RCC) are divided in several subtypes, characterized by morphological and histological features, protein expression patterns and genetics criteria. The 42 43 main subtypes include Clear cell renal cell carcinoma (CCRCC), Papillary RCC (PRCC), Chromophobe RCC (ChRCC), oncocytoma, TFE3 and TFEB Translocation renal cell 44 45 carcinoma (TRCC). In most cases, RCC can be easily classified according to histological 46 criteria and immunohistochemistry. Nevertheless, the subtyping process can be more complex 47 in some cases: differential diagnosis (CCRCC or TFE3 TRCC, PRCC or TFE3 TRCC, 48 oncocytic tumors corresponding to ChRCC or oncocytoma), molecular confirmation (TFEB 49 TRCC) and unclassified RCC. Complementary analyses are required such as fluorescence in 50 situ hybridization (FISH) for the detection of chromosomal abnormalities associated to each 51 subtype. In this aim, this study assessed the performance of FISH analysis in the histological 52 classification of 359 RCC exhibiting unusual histological characteristics and/or occurring in 53 young people. FISH probes were selected according to the histological features of each tumor. 54 FISH analysis contributed to the histological classification in 73% of the RCC (261/359). 55 Conversely, FISH did not contribute to the diagnosis in 19% of the cases (69/359) and a 56 hybridization failure was observed for the remaining tumors (8%; 29/359). Considering the 57 different RCC subtypes, FISH analysis was highly efficient to confirm the histological 58 diagnosis of CCRCC, PRCC, and TFE3 TRCC and to identify abnormalities of the TFEB gene. However, this strategy showed some limitations for the diagnosis of oncocytic tumors 59 60 and unclassified RCC, suggesting that additional molecular assays should be evaluated in 61 these cases.

Key words: Renal epithelial neoplasms, Fluorescence *in situ* hybridization, Chromosomal
abnormalities, Histological classification

65 1. INTRODUCTION

66 Renal cell carcinomas (RCC) represent more than 90% of adult renal malignancies [1]. These tumors preferentially affect men, with a mean age at diagnosis of 64 years [2]. 67 68 According to the WHO classification, RCC are divided in several subtypes, characterized by morphological and histological features, protein expression patterns and genetics criteria [1]. 69 70 The conventional or clear cell renal cell carcinoma (CCRCC) is the most frequent subtype [3]. 71 The cytoplasm of tumor cells appears optically empty after hematoxylin eosin safran (HES) 72 staining and immunohistochemistry (IHC) assay frequently reveals positivity for CAIX [4]. 73 Moreover, an inactivation of the VHL gene (3p25.3) is found in at least 70% of the CCRCC 74 [5]. Papillary RCC (PRCC) encompassed two morphological types (type 1 and 2). Cylindrical 75 eosinophilic cytoplasms with nuclei pseudostratifications are observed in type 2 while these 76 features are absent in type 1 [1]. Both types commonly express P504S (AMACR) and CK7 77 [4]. Trisomies of chromosomes 7 and 17 are associated to PRCC [6,7]. Chromophobe RCC 78 (ChRCC) is characterized by large polygonal cells with transparent cytoplasm [1]. The main 79 differential diagnosis is oncocytoma, which is a benign tumor presenting round or polygonal 80 cells with an eosinophilic cytoplasm and a round and regular nuclei [1,8,9]. However, the 81 eosinophilic variant of ChRCC can also exhibit eosinophilic cells [8]. CK7 immunostaining 82 shows diffuse positivity in the majority of ChRCC cases whereas oncocytomas exhibit 83 negative or focally positive patterns [4,8]. Nevertheless, a negative pattern can be observed in 84 about 20% of the ChRCC [10]. Moreover, ChRCC present multiple chromosomal losses 85 while rearrangement of the CCND1 gene is associated to oncocytomas [2,6,11]. Translocation 86 renal cell carcinoma (TRCC) affects preferentially children and young patients [12]. TRCC 87 encompasses TFE3 TRCC and TFEB TRCC, implying respectively rearrangements of TFE3 88 (Xp11.23) and *TFEB* (6p21.1) genes and overexpression of the corresponding fusion proteins 89 [13]. The TFE3 TRCC can show clear cells or papillary architecture, challenging the

90 differential diagnosis with CCRCC and PRCC, particularly in young patients [12,14]. The 91 TFEB TRCC typically demonstrates biphasic morphology with epithelioid cells and small 92 cells clustered around membrane basement material [15]. This subtype consistently expressed 93 melanoma markers such as Melan A and HMB45 [12]. Recently, amplifications of the TFEB 94 gene have been described [16]. Additionally tumors that do not resemble those of any well 95 characterized RCC subtypes, or low/high grade unclassified oncocytic neoplasms, or RCC 96 with pure sarcomatoid/rhabdoid morphology with no recognizable epithelial component 97 corresponded to Unclassified RCC [1].

98 Prognoses and treatments differ between RCC subtypes [2,9], shedding the light on 99 the importance of an accuracy classification of these tumors. In most cases, RCC can be 100 easily classified according to histological criteria. Nevertheless, some samples can present a 101 combination of features occurring in different subtypes or uncommon characteristics [2]. 102 Furthermore, each tumor occurring in young patient (≤ 40 years) should be screened for a 103 TFE3 TRCC [4]. Thus, uncommon RCC may require complementary analyses to support the 104 diagnosis. Several immunohistochemical markers can lead to equivocal or contradictory 105 results and distinct RCC subtypes can show overlapping histologic patterns [17]. As each 106 RCC subtypes exhibit distinct chromosomal abnormalities, cytogenetics analysis could also 107 be helpful for tumor classification. Fluorescence in situ hybridization (FISH) is a powerful 108 tool to detect balanced and unbalanced-chromosomal rearrangements in cancers [18]. FISH is 109 the principal molecular assay used in clinical diagnosis of RCC [4]. Several authors have 110 assessed the suitability of FISH analysis in the RCC subtyping [19-21], but these studies were 111 carried out on small cohorts. Moreover, the impact of such assays for the classification of 112 RCC with unusual histological characteristics and/or occurring in young people remains to be 113 determined. In this aim, we evaluated the interest of FISH analysis in the histological 114 classification of 359 uncommon renal epithelial neoplasms.

115 2. MATERIAL AND METHODS

116 2.1. PATIENTS AND HISTOLOGICAL ANALYSIS

117 This study focused on 359 RCC addressed for FISH analysis in the Cytogenetics 118 department of Rennes University Hospital from January 2014 to June 2017. Patients 119 originated from the Rennes University Hospital or from others French medical centers. Most 120 of them were included in the French CARARE network (Rare Renal Cancer in Adults) of the 121 INCa (National Institute of Cancer, France) focused on RCC occurring in young people (≤ 40 122 years) and/or every subtypes of RCC, excepted CCRCC and non-metastatic PRCC. 123 Histopathological and immunohistochemical assays were performed on each tumor by 2 124 independent pathologists (NRL and SFKJ) on formalin-fixed paraffin-embedded (FFPE) 125 tissue sections stained by hematoxylin eosin safran (HES). According to these analyses each 126 case was classified into one of the following subgroups: 1) CCRCC or TFE3 TRCC, 2) PRCC 127 or TFE3 TRCC, 3) Oncocytic renal tumors, corresponding to oncocytoma or eosinophilic 128 variant of ChRCC, 4) TFEB TRCC and 5) Unclassified RCC.

129 2.2. IMMUNOHISTOCHEMISTRY (IHC)

130 The protein expression patterns were assessed by IHC using the following antibodies: 131 anti-CAIX (Abcam, Cambridge, UK), anti-CK7 (Dako, Agilent Technologies, USA), anti-132 P504S (Dako, Les Ulis, France), anti-TFE3 (Cell Marque Corporation, Rocklin, California, 133 USA), anti-TFEB (Abcam). Briefly, the reactivity of antibodies was revealed with HRP-134 labeled polymer conjugated secondary antibodies using di-aminobenzidine (DAB) as 135 chromogen (Sigma-Aldrich, Saint-Quentin-Fallavier, France). Antibody staining was 136 observed using an Olympus BX51 microscope and images recorded with an Olympus DP70 137 camera. The tumor expression for each antibody were independently evaluated (NRL, SFKJ). 138 Negative control was performed by omitting the primary antibody.

139 2.3. FLUORESCENCE IN SITU HYBRIDIZATION (FISH)

140 FISH analysis was performed as previously described [22,23]. Briefly, the 5 µm thick, 141 paraffin-embedded sections fixed on slides were deparaffinized and pre-treated using pre-142 treatment solution (Dako). Pepsin solution was added on preparations for 6 minutes (Sigma, 143 100 mg/l) and then dehydrated in ethanol 70°, 85°, and 100° (2 minutes each). Specimens and 144 probes were codenatured (10 minutes at $75^{\circ}C + /-2^{\circ}C$) and hybridized overnight at $37^{\circ}C$. 145 FISH probes were selected to detect the main chromosomal abnormalities associated to each 146 RCC subtypes. Different probes were assayed according to the previous subgroups of tumors. 147 The VHL and TFE3 genes status were assessed for the CCRCC or TFE3 TRCC cases using 148 the Zyto-Light SPEC VHL/CEN3 Dual Color Probe (Zytovision, Clinisciences, Nanterre, 149 France) and the Zyto-Light SPEC TFE3 Xp11 Dual Color Break Apart (Zytovision). 150 Similarly, PRCC or TFE3 TRCC were distinguished using centromeric probes for 151 chromosomes 7 and 17 (CEP 7 and CEP 17 probes; Abbott, Rungis, France) and the Zyto-152 Light SPEC TFE3 Xp11 Dual Color Break Apart (Zytovision). For tumors with TFEB TRCC 153 histological features, the TFEB gene status was assessed using the TFEB Break apart Probe 154 6p21.1 (Empire Genomics, Buffalo, United States). Concerning ChRCC or oncocytomas 155 cases, the ZytoLight SPEC CCND1 Break Apart/2q11/CEN 6 Quadruple Color Probes 156 (Zytovision) and the ZytoLight SPEC VHL/1p12/CEN 7/17 Quadruple Color Probes 157 (Zytovision) were assayed to highlight multiple chromosomal losses or a CCND1 gene 158 rearrangement. Finally, Unclassified RCC were studied for VHL, TFEB, TFE3 and the 159 number of chromosomes 7 and 17, according to the histological or clinical features. After 160 hybridization, slides were washed according to the manufacturers' instructions, and the nuclei 161 were counterstained by DAPI (Dako). Cells were viewed using a fluorescent Axioplan II 162 microscope (Zeiss, Le Pecq, France) or the automatized microscope Bioview Encore 163 (Bioview, Rehovot, Israel) with appropriate filters and 100 non-overlapped nuclei were

164	analysed for each tumor by 2 independents observers (FD and FC). The positive thresholds
165	for the detection of a chromosomal loss/gain or a gene rearrangement were respectively 30%
166	and 15% as previously reported [24,25].

167 The FISH hybridization patterns were classified as positive or negative as indicated in
168 Table I. The FISH analysis was considering "contributive" when the results led to RCC
169 subtype classification or "non-contributive" when the hybridization patterns did not provide
170 any information for RCC subtyping. Cases with hybridization failure were also mentioned.
171 2.4. STATISTICAL ANALYSIS
172 Results are expressed as mean +/- standard error of the mean. Data were compared

using the Chi2 test and the Fisher's Exact test. The Pvalue of <0.05 was consideredstatistically significant.

176

177 **3. RESULTS**

178 3.1.PATIENTS AND TUMORS

Patients and tumors characteristics are summarized in Table II and Figure 1. In this study, male patients were mostly represented (225/359; 63%). The mean age at diagnosis was $47,4 \pm 0,9$ years and 38% of patients were less than 40 years (137/359). Tumors were mainly originated from french medical centers of the CARARE network (324/359; 89%) and sent for a second opinion by an uropathologist expert in the Rennes University Hospital.

RCC were classified in the following subgroups according to their histological and immunohistochemical features. The most important one was the CCRCC or *TFE3* TRCC subgroup, which represented 173/359 cases (48%). In this group, all the tumors had solid, nested or papillary patterns and consisted of a majority of cells with clear cytoplasm. In some areas, tumor cells displayed granular eosinophilic cytoplasm with more prominent nucleoli, irregular nuclei and clumped chromatin. Two neoplasms contained psammoma bodies (Figure 1A).

Unclassified RCC were observed in 127/359 tumors (35%). These cases represent a
heterogeneous group of carcinomas that do not fit into any of the well-known histologic
subtypes. They include also tumors with pure sarcomatoid histology with no recognized
epithelial subtypes (Figure 1B and 1C).

The distinction between oncocytoma and eosinophilic variant of ChRCC was required in 31/359 RCC (9%). In this group, all the tumors consisted of tubular or focal/diffuse solidsheet pattern. Tumor cells were cuboidal with oncocytic cytoplasm. The cell borders were usually indistinct to focally slightly distinct. Nuclei were round to irregular wrinkled appearance. Perinuclear haloes were rare but focally present (Figure 1D). Differential diagnosis between PRCC and *TFE3* TRCC concerned 20/359 tumors
(6%). These neoplasms demonstrated a papillary architecture with fibrovascular cores. Some
tumors had more compact and solid patterns. Papillae were lined by columnar eosinophilic
cells with more often prominent nucleoli, and a nuclear pseudostratification. In some areas
but in all tumors, a clear cell component was present (Figure 1E).

Finally, histological features of *TFEB* TRCC were observed in only 8/359 cases (2%). All these tumors had a solid pattern of growth with two types of tumor cells. The predominant cells were epithelioid with abundant clear or finely eosinophilic cytoplasm. The second population of tumor cells consisted of smaller cells with dense chromatin around nodules of hyaline basement membrane (Figure 1F).

210 3.2.FISH ANALYSIS

211 FISH analyses were performed for each subgroup of tumors as described in Patients 212 and Methods. The results are indicated in Figure 2. The TFE3 gene rearrangement was 213 assessed in 279/359 cases (78%), following by the status of the VHL gene (246/359; 69%), 214 gain(s) of the chromosomes 7 and/or 17 (103/359; 29%), the TFEB gene rearrangement 215 (81/359; 23%), multiple losses or the CCND1 gene rearrangement (31/359; 9%). The more 216 frequent abnormalities were a loss of the VHL gene (144/246; 59%), following by gains of the 217 chromosomes 7 and/or 17 (52/103; 50%), multiple chromosomal losses or CCND1 218 rearrangement (9/31; 29%), and rearrangements of the TFEB genes (10/81; 12%) and TFE3 219 (29/279; 10%).

A majority of RCC (261/359; 73%) was successfully classified after FISH analysis. Conversely, these cytogenetics investigations did not contribute to the tumor classification in 69/359 of the cases (19%) and a hybridization failure was observed for 29/359 of the tumors (8%). The impact of the FISH was evaluated for each subgroup of tumors (Figures 3 and 4).

224 The FISH results allowed the differential diagnosis between a CCRCC and a TFE3 TRCC in 225 156/173 of cases (90%; Figure 3A). A total of 133 CCRCC were diagnosed. A chromosome 3 226 loss, encompassing the VHL gene was observed in 94/133 tumors. This chromosomal loss 227 was always heterozygous and corresponded most frequently to a deletion on the short arm of 228 the chromosome 3 (1G, 2O signals in 70/94 CCRCC (74%)). For 24/94 cases a larger 229 chromosomal imbalance, including the centromere of the chromosome 3 (1G, 1O) was observed. FISH analysis did not showed a VHL loss in 39/133 cases. However, these tumors 230 231 were classified as CCRCC according to their histological features and a negative TFE3 FISH 232 pattern. A TFE3 gene rearrangement was detected in 23 cases, corresponding to TFE3 TRCC. 233 Among these tumors, TFE3 immunostaining was negative in one case. The hybridization 234 failed in the 17/173 cases (10%).

The FISH analysis contributed to the differential diagnosis between PRCC and *TFE3* TRCC in 19/20 RCC (95%; Figure 3B). A majority of these tumors (18/19) exhibited gains of chromosomes 7/17 or a normal hybridization pattern for the *TFE3* gene and were classified as PRCC. The gain of the chromosome 17 was observed more frequently but not significantly in type 2 PRCC than in type 1 PRCC (respectively 4/8, 50%, and 8/10, 80%; p=0.32). Only one case (1/19) showed a *TFE3* gene rearrangement but a negative TFE3 immunostaining and was classified as a *TFE3* TRCC. A hybridization failure was observed in one case.

A *TFEB* gene rearrangement (6p21.1) was observed in all but one tumor exhibiting *TFEB* TRCC histological features (7/8; 88%; Figure 3C). Among these tumors, one case showed a complex rearrangement including a distal 6p21.1 deletion (no red signals) associated to a *TFEB* gene amplification (numerous green signals). This result was confirmed by array-CGH (data not shown). A *TFEB* gene rearrangement could not be retained for the last RCC, showing a splitted FISH signals in only 10% of nuclei, lower than the positive threshold (15%).

249	A differential diagnosis between oncocytoma and an eosinophilic variant of ChRCC
250	was only achieved after FISH analysis in 9/31 cases (29%, Figure 4A). A rearrangement of
251	the CCND1 gene was detected in 5/9 tumors classified as oncocytomas. Multiple
252	chromosomal losses were observed 4/9 cases, corresponding to eosinophilic variant of
253	ChRCC. Conversely, cytogenetics investigations did not contribute to the determination of the
254	RCC subtype in 15/31 cases (48%) exhibited none of the previous chromosomal
255	abnormalities and a hybridization failure was observed in 7/31 tumors (23%).
256	Unclassified RCC was a heterogeneous group included tumors with different
256 257	Unclassified RCC was a heterogeneous group included tumors with different cytogenetics profiles. The FISH analysis was contributive in 70/127 cases (55%; Figure 4B)
256 257 258	Unclassified RCC was a heterogeneous group included tumors with different cytogenetics profiles. The FISH analysis was contributive in 70/127 cases (55%; Figure 4B) corresponding to 39 CCRCC (deletion of the <i>VHL</i> gene), 24 PRCC (chromosome 7/17 gains),
256 257 258 259	Unclassified RCC was a heterogeneous group included tumors with different cytogenetics profiles. The FISH analysis was contributive in 70/127 cases (55%; Figure 4B) corresponding to 39 CCRCC (deletion of the <i>VHL</i> gene), 24 PRCC (chromosome 7/17 gains), 5 <i>TFE3</i> TRCC, 1 <i>TFEB</i> TRCC (rearrangement of the corresponding genes) and 1 RCC with a
256 257 258 259 260	Unclassified RCC was a heterogeneous group included tumors with different cytogenetics profiles. The FISH analysis was contributive in 70/127 cases (55%; Figure 4B) corresponding to 39 CCRCC (deletion of the <i>VHL</i> gene), 24 PRCC (chromosome 7/17 gains), 5 <i>TFE3</i> TRCC, 1 <i>TFEB</i> TRCC (rearrangement of the corresponding genes) and 1 RCC with a <i>TFEB</i> amplification without rearrangement of the gene (numerous fusions signals). The FISH
256 257 258 259 260 261	Unclassified RCC was a heterogeneous group included tumors with different cytogenetics profiles. The FISH analysis was contributive in 70/127 cases (55%; Figure 4B) corresponding to 39 CCRCC (deletion of the <i>VHL</i> gene), 24 PRCC (chromosome 7/17 gains), 5 <i>TFE3</i> TRCC, 1 <i>TFEB</i> TRCC (rearrangement of the corresponding genes) and 1 RCC with a <i>TFEB</i> amplification without rearrangement of the gene (numerous fusions signals). The FISH assays were non contributive in 53/127 tumors (42%) and a hybridization failure was

264 **4. DISCUSSION**

265 RCC encompass several histological subtypes, differing by their clinical outcomes and 266 treatments [2,9]. The classification of renal tumors is mainly based on morphological and 267 histological data. Nevertheless, RCC are characterized by a huge heterogeneity and several 268 tumors require ancillary assays, as IHC or FISH analyses [4,20,26]. To assess the impact of 269 the latest one, we studied 359 RCC with uncommon characteristics. In routine diagnosis, a 270 majority of RCC exhibits classical histological features and FISH assay is not necessary for 271 their classification. However, numerous uncommon renal epithelial neoplasms are addressed 272 to our department which is the French reference center for the diagnosis of renal tumors 273 (CARARE network). This large and specific recruitment and our high-volume activity (more 274 than 5000 analyses per year of all tumors), allowed the analysis of large series of tumors 275 using rapid and automatized processes (deparaffinisation, sample preatreatment, nuclei 276 pictures acquisition). As previously reported, this strategy is cost effective and suggests that 277 testing cost is an insufficient reason to limit the use of FISH [27]. To our knowledge, this 278 study is the first and larger one showing 1) that FISH assay improves the histological 279 classification of 73% of the RCC with uncommon characteristics and 2) that this strategy is 280 highly efficient on targeted-RCC subgroups. A majority of the RCC studied herein (89%) 281 were originated from various medical centers. Consecutively, variations in the pre-analytic 282 parameters, such as time of fixation, may lead to a limitation of FISH analysis [28]. 283 Interestingly, the hybridization failure rate was low (8%) and similar to previous studies, 284 confirming that this testing is a robust ancillary assay [29]. FISH assay does not require DNA 285 extraction and appears as a quick and easy method to assess chromosomal aberrations on 286 **RCC FFPE samples.**

According to the literature, the mean age for RCC diagnosis is 64 years [2]. Interestingly, it was only 47,4 years in this cohort. This difference could reflect the high rate of patients

289 from the CARARE network, which includes especially all the RCC occurring in young 290 people. As a TFE3 TRCC should be suspected for each tumor with a clear cell contingent 291 occurring in a young patient, the distinction between a CCRCC and a TFE3 TRCC is 292 frequently observed in our study. In this subgroup, FISH analysis was 100% contributive for 293 the histological classification and supported the diagnosis of 133 CCRCC. A biallelic 294 inactivation of the VHL gene is associated to CCRCC. Three different mechanisms are 295 involved: chromosome 3 deletion encompassing 3p25-p26 region, mutations on the coding 296 regions of the gene and/or methylation of its promotor [30]. Two positive FISH patterns were 297 observed herein: loss of the VHL gene and losses of both VHL gene and centromere of the 298 chromosome 3. In the last case, FISH analysis does not allow the distinction between partial 299 and complete chromosome 3 losses. Array-CGH analysis can overlap this limitation by an 300 accurate determination of the extent of the deletion but no prognostic value was associated to 301 these monosomies. In this series, some CCRCC cases (39/133) did not exhibit a loss of the 302 VHL gene by FISH analysis. The classification of these tumors as CCRCC was based on their 303 histological characteristics and the absence of TFE3 rearrangement. As previously reported, 304 these CCRCC may present other VHL inactivating events (mutation and/or promoter 305 methylation) or a wild-type VHL gene leading to a worse clinical outcome [5]. The diagnosis 306 of other clear cell renal tumors such as clear cell papillary RCC was excluded by 307 morphological analysis and immunohistochemistry (CK7, CAIX, P504S) [1] and did not 308 require complementary analyses. FISH testing also allowed the diagnosis of 23 TFE3 TRCC. 309 The TFE3 immunostaining was negative for one of these cases supporting, as previously 310 described, that IHC and FISH analysis should be associated [31,32]. 311 As for CCRCC, FISH analysis always contributed to the differential diagnosis 312 between PRCC and TFE3 TRCC. Centromeric probes of chromosomes 7 and 17 were used to 313 identify gains of the corresponding chromosomes observed in PRCC. PRCC is divided in type 1 and type 2, respectively associated to favorable and pejorative outcomes [2,7]. The gain of chromosome 17 has been described more frequently in the type 1 and is correlated to an improvement of the survival [33]. Conversely, in our study, this aneuploidy was observed more frequently but not significantly in the type 2 (p=0.32). Characteristics of our tumors or recruitment of young patients may explain differences between our study and data of the literature. In this subgroup, one tumor exhibited a rearrangement of the *TFE3* gene. As previously, the TFE3 immunostaining was negative, highlighting the interest of FISH assay.

321 The TFEB gene rearrangement is the molecular hallmark of TFEB TRCC [12]. FISH 322 analysis confirmed a rearrangement of this gene in almost all the cases with characteristic 323 histological features. Interestingly, a complex 6p21.1 rearrangement associated to a TFEB 324 gene amplification was detected by FISH in one RCC and confirmed by array-CGH. Another 325 case (Unclassified-RCC) showed a TFEB amplification without gene rearrangement. The 326 TFEB gene amplifications have been recently reported and individualized as a rare RCC 327 subtype, more aggressive and occurring in older patients than rearrangements [16,34]. Thus, 328 FISH analysis is a particularly useful all-in-one assay for the detection of these different 329 TFEB gene aberrations.

330 Oncocytoma is a benign tumor. However, some malignant ChRCC cases showed 331 histological characteristics of oncocytoma (eosinophilic variant), shedding the light on the 332 importance of the differential diagnosis [35]. FISH analysis contributed to the histological 333 classification in only 29% of the cases. These results are consistent with the literature: 334 rearrangement of the CCDN1 gene is observed in 22% of the oncocytomas and multiple 335 chromosomal imbalances in ChRCC can concern other chromosomes than those targeted by 336 the selected FISH probes [2,6,36]. As Array-CGH is a very sensitive tool allowing a 337 pangenomic analysis of chromosomal imbalances, the combination of these assays could be 338 considered to improve the differential diagnosis between oncocytomas and ChRCC.

339	Unclassified RCC represented 35% of this cohort. Interestingly, FISH analysis led to
340	the classification of 55% of these RCC showing more frequently CCRCC or PRCC
341	cytogenetics patterns. Other analyses such as array-CGH and next generation sequencing
342	could be useful for the diagnosis of the cases remaining unclassified.
343	This study shed the light on the interest of FISH analysis for the histological
344	classification of uncommon renal epithelial neoplasms. FISH is a powerful tool to identify
345	chromosomal aberrations associated to CCRCC, PRCC, and TRCC subtypes. Moreover, it
346	allows the distinction between a rearrangement and an amplification of the TFEB gene. When
347	FISH results are non-contributive, additional molecular assays could be considered to
348	improve the diagnosis, especially for oncocytic-type and unclassified RCC.

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467 6. FIGURE / TBALE LEGENDS

468 Figure 1. Histological RCC features

- 469 H & S staining. A. CCRCC or *TFE3* TRCC: RCC with alveolar arrangement of clear cells
- 470 and a low nucleolar grade (100x). **B and C. Unclassified RCC:** RCC with leiomyomatous
- 471 stroma showing prominent smooth muscle bundles (50x, B). RCC showing high-grade
- 472 epithelioid cells with eosinophilic cytoplasm and necrosis (100x, C). D. Oncocytic tumors:
- 473 Oncocytic cells with eosinophilic cytoplasm and nuceli showing a raisinoid aspect with
- 474 perinuclear halo (200x). **E. PRCC or** *TFE3* **TRCC:** RCC with a papillary architecture.
- 475 Abundant and eosinophilic cytoplasm in tumor cells with a nuclear pseudostratifiation and a
- 476 high nucleolar grade (100x). F. TFEB TRCC: RCC showing a solid pattern with
- 477 eosinophilic/clear cells. Nuclei were rounded and vesicular with no prominent nucleoli478 (200x).
- 479 Figure 2. FISH analysis of 359 RCC
- 480 The FISH results were classified as Positive or Negative according to the hybridization481 patterns described in Table A.
- 482 Figure 3. FISH analysis: CCRCC, PRCC, and TRCC
- 483 A. Loss of the VHL gene in CCRCC (1G, 2O; star) and TFE3 gene rearrangement in TRCC
- 484 (1F, 1G, 1O; female patient; arrow). **B.** Gains of chromosomes 7 and 17 in PRCC (3G, 3O;
- 485 star) and *TFE3* gene rearrangement in TRCC (1G, 1O; male patient; arrow). C. Common
- 486 *TFEB* gene rearrangement in TRCC (1F, 1G, 1O; star) and tumor showing a complex 6p21,1
- 487 rearrangement including a distal deletion (no red signals) associated to a *TFEB* gene
- 488 amplification (numerous green signals; arrow)
- 489 Figure 4. FISH analysis: oncocytic and unclassified RCC

- 490 A. Rearrangement of the *CCND1* gene in an oncocytoma (1F, 1O, 1G, 2A, 2Go; star)
- 491 associated to losses of 2q11 locus and chromosome 6 centromere (2F, 1A, 1Go; arrow). B.
- 492 Unclassified RCC showing a *TFEB* gene amplification (1F, numerous green signals; star)

493 Table I. FISH hybridization patterns associated to the different RCC subtypes

- 494 A: Aqua ; BA: Break apart ; CEN: Centromeric probe ; F: Fusion; G: Green; Go: Gold;
- 495 O: Orange; R: Red

496 **Table II. Patients and tumors characteristics**

497 -: negative ; +: positive ; -*: negative or focally positive ; NR: Not realized



















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■ Contributive

TRCC amplification

□Failure

■Non-contributive





RCC	Probes	Hybridization patterns		
subtype		Positive	Negative	
CCRCC	VHL(G)/CEN3(O)	■ 1G, ≥ 10	• 2G, 2O	
PRCC	CEN7(G)/CEN17(O)	• \geq 3G and/or \geq 3O	• 2G, 2O	
<i>TFE3</i> TRCC	<i>TFE3</i> (BA)	 1G, 1O (male) 1F, 1G, 1O (female) 	 1F (male) 2F (female) 	
TFEB TRCC	<i>TFEB</i> (BA)	1F, 1G, 1O (translocation)Numerous G signals (amplification)	• 2F	
ChRCC	<i>CCND1</i> (BA)/ 2q11(A)/CEN6(Go)	• Multiple chromosomal losses without <i>CCND1</i> rearrangement (2F)	 2F, 2A, 2Go 2G, 2R, 2Go, 2A 	
Oncocytoma	- and <i>VHL</i> (G)/1p12(R)/ CEN7(Go)/CEN17(A)	 CCND1 rearrangement (1F, 1G, 1O) without multiple chromosomal losses 	• 2F, 2A, 2Go 2G, 2R, 2Go, 2A	

Characteristics		%
• Sex		
Male	225/359	63
Female	134/359	37
 Age 		
Mean (y)	47,4	
Range (y)	2-87	
\leq 40 years	137/359	38
Center		
Rennes University Hospital	39/359	11
Others (CARARE network)	324/359	89
 Categories of RCC 		
CCRCC or TFE3 TRCC	173/359	48
CAIX - / + / NR	11 / 75 / 87	7 / 43 / 50
TFE3 - / + / NR	99 / 47 / 27	57 / 27 / 16
Oncocytic tumors	31/359	9
CK7 -* / + / NR	15 / 12 / 4	48 / 39 / 13
PRCC or TFE3 TRCC	20/359	6
CK7 - / + / NR	6 / 11 / 3	30 / 55 / 15
P504S - / + / NR	0 / 17 / 3	0 / 85 / 15
TFE3 - / + / NR	8 / 6 / 6	40 / 30 / 30
TFEB TRCC	8/359	2
TFEB - / + / NR	1 / 2 / 5	13 / 25 / 62
Unclassified RCC	127/359	35
CAIX - / + / NR	29 / 51 / 47	23 / 40 / 37
TFE3 - / + / NR	57 / 20 / 50	45 / 16 / 39
CK7 -* / + / NR	46 / 30 / 51	36 / 24 / 40
P504S - / + / NR	17 / 59 / 51	13 / 47 / 40