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

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ABSTRACT (250 words)

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

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

1. INTRODUCTION

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

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

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

2. MATERIAL AND METHODS

2.1. PATIENTS AND HISTOLOGICAL ANALYSIS

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

2.2. IMMUNOHISTOCHEMISTRY (IHC)

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

2.3. FLUORESCENCE IN SITU HYBRIDIZATION (FISH)

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

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

The FISH hybridization patterns were classified as positive or negative as indicated in Table I. The FISH analysis was considering “contributive” when the results led to RCC subtype classification or “non-contributive” when the hybridization patterns did not provide any information for RCC subtyping. Cases with hybridization failure were also mentioned.

2.4. STATISTICAL ANALYSIS

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 considered statistically significant.

3. RESULTS

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 ± 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 solid-sheet 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).

3.2.FISH ANALYSIS

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

The FISH results allowed the differential diagnosis between a CCRCC and a *TFE3* TRCC in 156/173 of cases (90%; Figure 3A). A total of 133 CCRCC were diagnosed. A chromosome 3 loss, encompassing the *VHL* gene was observed in 94/133 tumors. This chromosomal loss was always heterozygous and corresponded most frequently to a deletion on the short arm of the chromosome 3 (1G, 2O signals in 70/94 CCRCC (74%)). For 24/94 cases a larger 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 were classified as CCRCC according to their histological features and a negative *TFE3* FISH pattern. A *TFE3* gene rearrangement was detected in 23 cases, corresponding to *TFE3* TRCC. Among these tumors, TFE3 immunostaining was negative in one case. The hybridization 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%).

A differential diagnosis between oncocytoma and an eosinophilic variant of ChRCC was only achieved after FISH analysis in 9/31 cases (29%, Figure 4A). A rearrangement of the *CCND1* gene was detected in 5/9 tumors classified as oncocytomas. Multiple chromosomal losses were observed 4/9 cases, corresponding to eosinophilic variant of ChRCC. Conversely, cytogenetics investigations did not contribute to the determination of the RCC subtype in 15/31 cases (48%) exhibited none of the previous chromosomal abnormalities and a hybridization failure was observed in 7/31 tumors (23%).

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 *VHL* gene), 24 PRCC (chromosome 7/17 gains), 5 *TFE3* TRCC, 1 *TFEB* TRCC (rearrangement of the corresponding genes) and 1 RCC with a *TFEB* 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 observed in the remaining cases (4/127; 3%).

4. DISCUSSION

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

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

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

Unclassified RCC represented 35% of this cohort. Interestingly, FISH analysis led to the classification of 55% of these RCC showing more frequently CCRCC or PRCC cytogenetics patterns. Other analyses such as array-CGH and next generation sequencing could be useful for the diagnosis of the cases remaining unclassified.

This study shed the light on the interest of FISH analysis for the histological classification of uncommon renal epithelial neoplasms. FISH is a powerful tool to identify chromosomal aberrations associated to CCRCC, PRCC, and TRCC subtypes. Moreover, it allows the distinction between a rearrangement and an amplification of the *TFEB* gene. When FISH results are non-contributive, additional molecular assays could be considered to improve the diagnosis, especially for oncocytic-type and unclassified RCC.

5. REFERENCES

[1] Moch H, Humphrey P, Ulbright T, Reuter V. WHO Classification of Tumours of the Urinary System and Male Genital Organs. Lyon: International Agency for Research on Cancer; 2016.

[2] Hsieh JJ, Purdue MP, Signoretti S, et al. Renal cell carcinoma. Nat Rev Dis Primer 2017;3:17009. https://doi:10.1038/nrdp.2017.9

[3] Sibony M, Vieillefond A. Les tumeurs du rein qui ne sont pas des carcinomes à cellules claires. État des lieux en 2008. Ann Pathol 2008;28(5):381-401. https://doi:10.1016/j.annpat.2008.07.009

[4] Tan PH, Cheng L, Rioux-Leclercq N, et al. Renal Tumors: Diagnostic and Prognostic Biomarkers. Am J Surg Pathol 2013;37(10):1518-1531. https://doi:10.1097/PAS.0b013e318299f12e

[5] Dagher J, Kammerer-Jacquet S-F, Brunot A, et al. Wild-type VHL Clear Cell Renal Cell Carcinomas Are a Distinct Clinical and Histologic Entity: A 10-Year Follow-up. Eur Urol Focus 2016;1(3):284-290. https://doi:10.1016/j.euf.2015.06.001

[6] Kovacs G. Molecular cytogenetics of renal cell tumors. Adv Cancer Res 1993;62:89-124.

[7] Pignot G, Elie C, Conquy S, et al. Survival analysis of 130 patients with papillary renal cell carcinoma: prognostic utility of type 1 and type 2 subclassification. Urology 2007;69(2):230-235. https://doi:10.1016/j.urology.2006.09.052

[8] Wobker SE, Williamson SR. Modern Pathologic Diagnosis of Renal Oncocytoma. J Kidney Cancer VHL 2017;4(4):1. https://doi:10.15586/jkcvhl.2017.96

[9] Amin MB, Amin MB, Tamboli P, et al. Prognostic impact of histologic subtyping of adult renal epithelial neoplasms: an experience of 405 cases. Am J Surg Pathol 2002;26(3):281-291.

[10] Wu SL, Kothari P, Wheeler TM, Reese T, Connelly JH. Cytokeratins 7 and 20 immunoreactivity in chromophobe renal cell carcinomas and renal oncocytomas. Mod Pathol Off J U S Can Acad Pathol Inc 2002;15(7):712-717. https://doi:10.1097/01.MP.0000017566.29755.8A

[11] Brunelli M, Delahunt B, Gobbo S, et al. Diagnostic Usefulness of Fluorescent Cytogenetics in Differentiating Chromophobe Renal Cell Carcinoma From Renal Oncocytoma: A Validation Study Combining Metaphase and Interphase Analyses. Am J Clin Pathol 2010;133(1):116-126. https://doi:10.1309/AJCPSATJTKBI6J4N

[12] Argani P. MiT family translocation renal cell carcinoma. Semin Diagn Pathol 2015;32(2):103-113. https://doi:10.1053/j.semdp.2015.02.003

[13] Camparo P, Vasiliu V, Molinie V, et al. Renal translocation carcinomas: clinicopathologic, immunohistochemical, and gene expression profiling analysis of 31 cases with a review of the literature. Am J Surg Pathol 2008;32(5):656-670. https://doi:10.1097/PAS.0b013e3181609914

[14] Argani P, Antonescu CR, Couturier J, et al. PRCC-TFE3 renal carcinomas: morphologic, immunohistochemical, ultrastructural, and molecular analysis of an entity associated with the t(X;1)(p11.2;q21). Am J Surg Pathol 2002;26(12):1553-1566.

[15] Argani P, Yonescu R, Morsberger L, et al. Molecular Confirmation of t(6;11)(p21;q12) Renal Cell Carcinoma in Archival Paraffin-embedded Material Using a Break-apart TFEB FISH Assay Expands its Clinicopathologic Spectrum: Am J Surg Pathol 2012;36(10):1516-1526. https://doi:10.1097/PAS.0b013e3182613d8f

[16] Argani P, Reuter VE, Zhang L, et al. TFEB-amplified Renal Cell Carcinomas: An Aggressive Molecular Subset Demonstrating Variable Melanocytic Marker Expression and Morphologic Heterogeneity. Am J Surg Pathol 2016;40(11):1484-1495. https://doi:10.1097/PAS.0000000000000720

[17] Roh MH, Dal Cin P, Silverman SG, Cibas ES. The application of cytogenetics and fluorescence in situ hybridization to fine-needle aspiration in the diagnosis and subclassification of renal neoplasms. Cancer Cytopathol 2010;118(3):137-145. https://doi:10.1002/cncy.20077

[18] Jehan Z, Uddin S, Al-Kuraya KS. In-situ hybridization as a molecular tool in cancer diagnosis and treatment. Curr Med Chem 2012;19(22):3730-3738.

[19] Barocas DA, Mathew S, DelPizzo JJ, et al. Renal cell carcinoma sub-typing by histopathology and fluorescence in-situ hybridization on a needle-biopsy specimen. BJU Int 2007;99(2):290-295. https://doi:10.1111/j.1464-410X.2006.06607.x

[20] Receveur AO, Couturier J, Molinié V, et al. Characterization of quantitative chromosomal abnormalities in renal cell carcinomas by interphase four-color fluorescence in situ hybridization. Cancer Genet Cytogenet 2005;158(2):110-118. https://doi:10.1016/j.cancergencyto.2004.08.019

[21] Sanjmyatav J, Rubtsov N, Starke H, Schubert J, Hindermann W, Junker K. Identification of tumor entities of renal cell carcinoma using interphase fluorescence in situ hybridization. J Urol 2005;174(2):731-735. https://doi:10.1097/01.ju.0000166749.38455.bc

[22] Dagher J, Dugay F, Verhoest G, et al. Histologic prognostic factors associated with chromosomal imbalances in a contemporary series of 89 clear cell renal cell carcinomas. Hum Pathol 2013;44(10):2106-2115. https://doi:10.1016/j.humpath.2013.03.018

[23] Belaud-Rotureau M-A, Parrens M, Carrere N, et al. Interphase fluorescence in situ hybridization is more sensitive than BIOMED-2 polymerase chain reaction protocol in

detecting IGH-BCL2 rearrangement in both fixed and frozen lymph node with follicular lymphoma. Hum Pathol 2007;38(2):365-372. https://doi:10.1016/j.humpath.2006.08.022

[24] Jiang H, Ren X, Cui X, et al. 1p/19q codeletion and IDH1/2 mutation identified a subtype of anaplastic oligoastrocytomas with prognosis as favorable as anaplastic oligodendrogliomas. Neuro-Oncol 2013;15(6):775-782. https://doi:10.1093/neuonc/not027

[25] Sukov WR, Hodge JC, Lohse CM, et al. TFE3 Rearrangements in Adult Renal Cell Carcinoma: Clinical and Pathologic Features With Outcome in a Large Series of Consecutively Treated Patients. Am J Surg Pathol 2012:1. https://doi:10.1097/PAS.0b013e31824dd972

[26] Skinnider BF, Amin MB. An immunohistochemical approach to the differential diagnosis of renal tumors. Semin Diagn Pathol 2005;22(1):51-68.

[27] Parker D, Belaud-Rotureau MA. Micro-cost Analysis of ALK Rearrangement Testing by FISH to Determine Eligibility for Crizotinib Therapy in NSCLC: Implications for Cost Effectiveness of Testing and Treatment. Clin Med Insights Oncol 2014;8:145‑52. https://journals.sagepub.com/doi/10.4137/CMO.S19236

[28] Khoury T, Sait S, Hwang H, et al. Delay to formalin fixation effect on breast biomarkers. Mod Pathol Off J U S Can Acad Pathol Inc 2009;22(11):1457-1467. https://doi:10.1038/modpathol.2009.117

[29] Belaud-Rotureau M-A, Parrens M, Dubus P, Garroste J-C, de Mascarel A, Merlio J-P. A comparative analysis of FISH, RT-PCR, PCR, and immunohistochemistry for the diagnosis of mantle cell lymphomas. Mod Pathol Off J U S Can Acad Pathol Inc 2002;15(5):517-525. https://doi:10.1038/modpathol.3880556

[30] Gossage L, Eisen T, Maher ER. VHL, the story of a tumour suppressor gene. Nat Rev Cancer 2015;15(1):55-64. https://doi:10.1038/nrc3844

[31] Rao Q, Williamson SR, Zhang S, et al. TFE3 Break-apart FISH Has a Higher Sensitivity for Xp11.2 Translocation–associated Renal Cell Carcinoma Compared With TFE3 or Cathepsin K Immunohistochemical Staining Alone: Expanding the Morphologic Spectrum. Am J Surg Pathol 2013;37(6):804-815. https://doi:10.1097/PAS.0b013e31827e17cb

[32] Just P-A, Letourneur F, Pouliquen C, et al. Identification by FFPE RNA-Seq of a new recurrent inversion leading to RBM10-TFE3 fusion in renal cell carcinoma with subtle TFE3 break-apart FISH pattern. Genes Chromosomes Cancer 2016;55(6):541-548. https://doi:10.1002/gcc.22356

[33] Klatte T, Pantuck AJ, Said JW, et al. Cytogenetic and Molecular Tumor Profiling for Type 1 and Type 2 Papillary Renal Cell Carcinoma. Clin Cancer Res 2009;15(4):1162-1169. https://doi:10.1158/1078-0432.CCR-08-1229

[34] Williamson SR, Grignon DJ, Cheng L, et al. Renal Cell Carcinoma With Chromosome 6p Amplification Including the TFEB Gene: A Novel Mechanism of Tumor Pathogenesis? Am J Surg Pathol 2017;41(3):287-298. https://doi:10.1097/PAS.0000000000000776

[35] Zheng G, Chaux A, Sharma R, Netto G, Caturegli P. LMP2, a novel immunohistochemical marker to distinguish renal oncocytoma from the eosinophilic variant of chromophobe renal cell carcinoma. Exp Mol Pathol 2013;94(1):29-32. https://doi:10.1016/j.yexmp.2012.05.008

[36] Sukov WR, Ketterling RP, Lager DJ, et al. CCND1 rearrangements and cyclin D1 overexpression in renal oncocytomas: frequency, clinicopathologic features, and utility in differentiation from chromophobe renal cell carcinoma. Hum Pathol 2009;40(9):1296-1303. https://doi:10.1016/j.humpath.2009.01.016

6. FIGURE / TBALE LEGENDS

Figure 1. Histological RCC features

H & S staining. **A. CCRCC or *TFE3* TRCC:** RCC with alveolar arrangement of clear cells and a low nucleolar grade (100x). **B and C. Unclassified RCC:** RCC with leiomyomatous stroma showing prominent smooth muscle bundles (50x, B). RCC showing high-grade epithelioid cells with eosinophilic cytoplasm and necrosis (100x, C). **D. Oncocytic tumors:** Oncocytic cells with eosinophilic cytoplasm and nuceli showing a raisinoid aspect with perinuclear halo (200x). **E. PRCC or *TFE3* TRCC:** RCC with a papillary architecture. Abundant and eosinophilic cytoplasm in tumor cells with a nuclear pseudostratifiation and a high nucleolar grade (100x). **F. *TFEB* TRCC:** RCC showing a solid pattern with eosinophilic/clear cells. Nuclei were rounded and vesicular with no prominent nucleoli (200x).

Figure 2. FISH analysis of 359 RCC

The FISH results were classified as Positive or Negative according to the hybridization patterns described in Table A.

Figure 3. FISH analysis: CCRCC, PRCC, and TRCC

A. Loss of the *VHL* gene in CCRCC (1G, 2O ; star) and *TFE3* gene rearrangement in TRCC (1F, 1G, 1O; female patient; arrow). **B.** Gains of chromosomes 7 and 17 in PRCC (3G, 3O; star) and *TFE3* gene rearrangement in TRCC (1G, 1O; male patient; arrow). **C.** Common *TFEB* gene rearrangement in TRCC (1F, 1G, 1O; star) and tumor showing a complex 6p21,1 rearrangement including a distal deletion (no red signals) associated to a *TFEB* gene amplification (numerous green signals; arrow)

Figure 4. FISH analysis: oncocytic and unclassified RCC

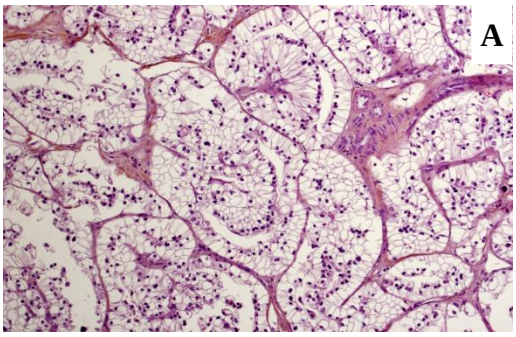
A. Rearrangement of the *CCND1* gene in an oncocytoma (1F, 1O, 1G, 2A, 2Go; star) associated to losses of 2q11 locus and chromosome 6 centromere (2F, 1A, 1Go; arrow). **B.** Unclassified RCC showing a *TFEB* gene amplification (1F, numerous green signals; star)

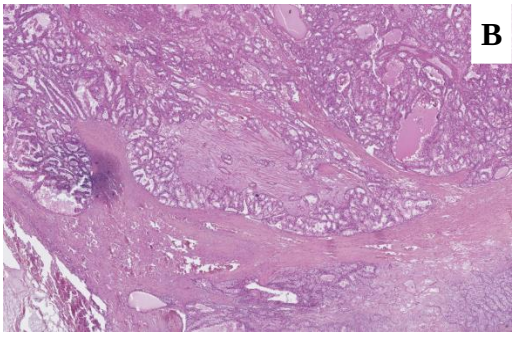
Table I. FISH hybridization patterns associated to the different RCC subtypes

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

Table II. Patients and tumors characteristics

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


A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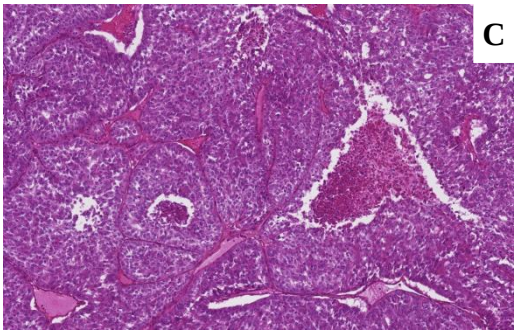
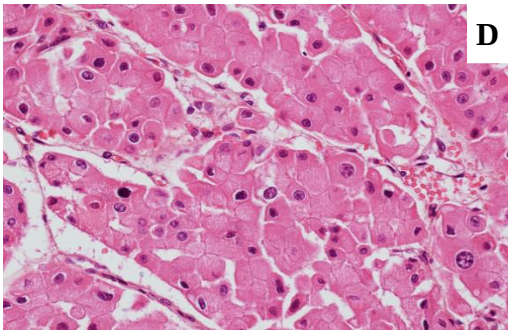

C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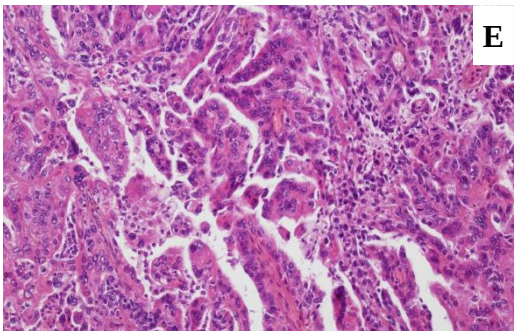
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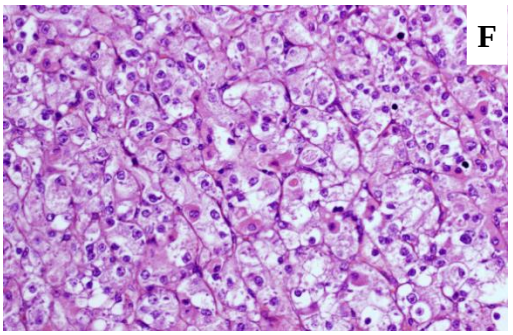
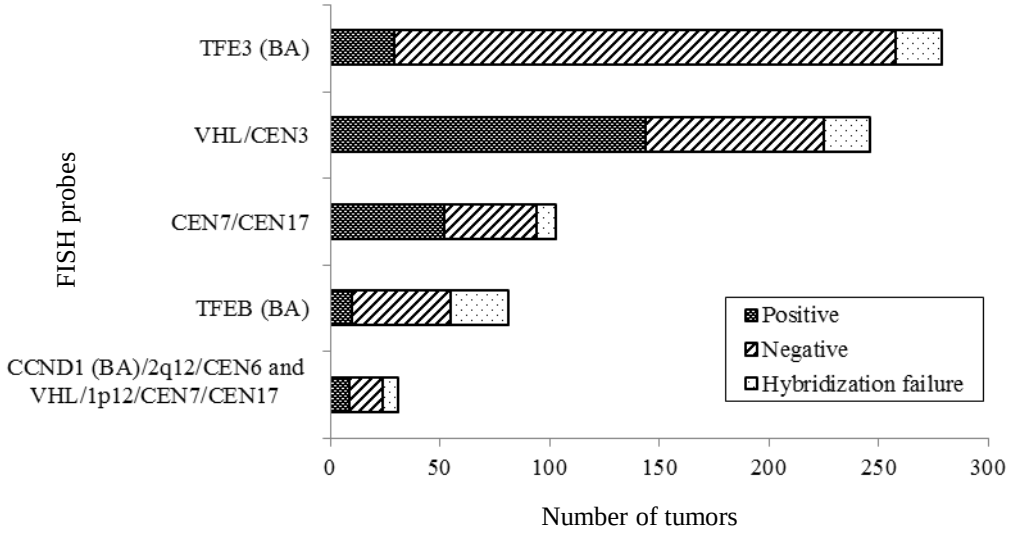
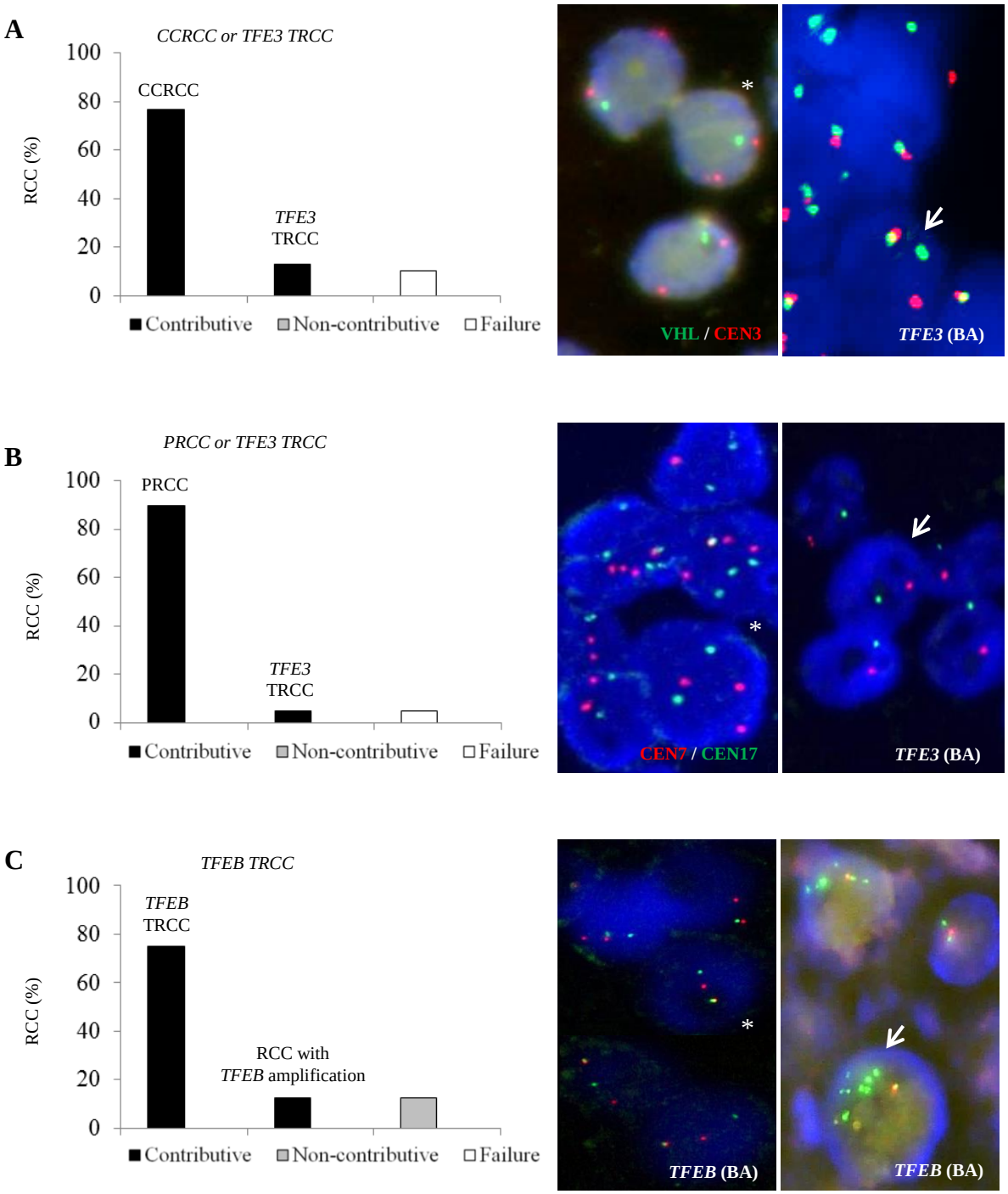
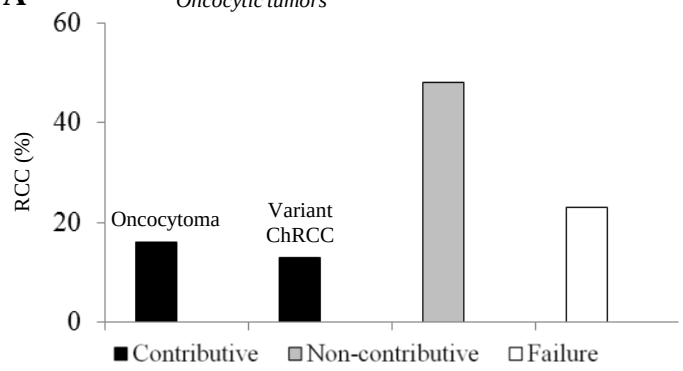
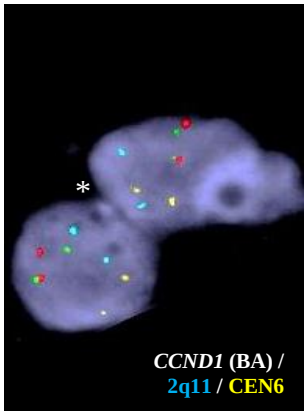

F

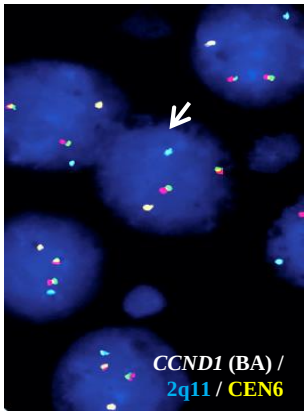

TFE3 (BA)
VHL/CEN3
CEN7/CEN17
TFEB (BA)
CCND1 (BA)/2q12/CEN6 and VHL/1p12/CEN7/CEN17
FISH probes
Positive
Negative
Hybridization failure
0
50
100
150
200
250
300
Number of tumors


A
CCRCC or TFE3 TRCC
RCC (%)
100
80
60
40
20
0
CCRCC
TFE3
TRCC
Contributive
Non-contributive
Failure
VHL / CEN3
TFE3 (BA)
B
PRCC or TFE3 TRCC
RCC (%)
PRCC
TFE3
TRCC
Contributive
Non-contributive
Failure
CEN7 / CEN17
TFE3 (BA)
C
TFEB TRCC
RCC (%)
TFEB
TRCC
RCC with
TFEB amplification
Contributive
Non-contributive
Failure
TFEB (BA)
TFEB (BA)

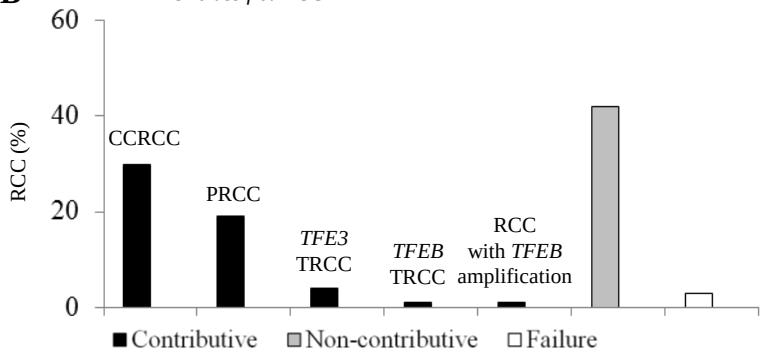
A

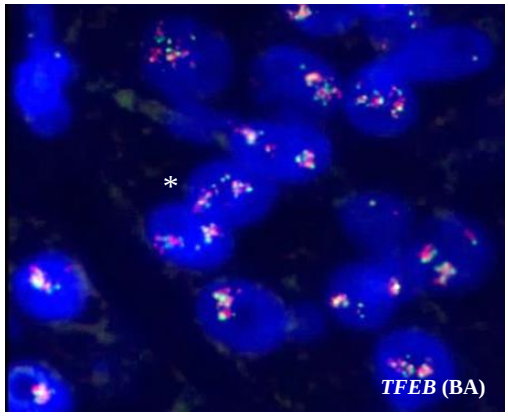

Oncocytic tumors
60
40
20
0
RCC (%)
Oncocytoma
Variant ChRCC
Contributive
Non-contributive
Failure


*
CCND1 (BA) / 2q11 / CEN6


CCND1 (BA) / 2q11 / CEN6

B


Unclassified RCC
60
40
20
0
RCC (%)
CCRCC
PRCC
TFE3 TRCC
TFEB TRCC
RCC with TFEB amplification
Contributive
Non-contributive
Failure


*
TFEB (BA)

RCC subtype	Probes	Hybridization patterns	
		Positive	**Negative**
CCRCC	*VHL*(G)/CEN3(O)	▪ 1G, ≥ 1O	▪ 2G, 2O
PRCC	CEN7(G)/CEN17(O)	▪ ≥ 3G and/or ≥ 3O	▪ 2G, 2O
TFE3 TRCC	*TFE3*(BA)	▪ 1G, 1O (male) ▪ 1F, 1G, 1O (female)	▪ 1F (male) ▪ 2F (female)
TFEB TRCC	*TFEB*(BA)	▪ 1F, 1G, 1O (translocation) ▪ Numerous G signals (amplification)	▪ 2F
ChRCC	*CCND1*(BA)/ 2q11(A)/CEN6(Go) and *VHL*(G)/1p12(R)/ CEN7(Go)/CEN17(A)	▪ Multiple chromosomal losses without *CCND1* rearrangement (2F)	▪ 2F, 2A, 2Go 2G, 2R, 2Go, 2A
Oncocytoma		▪ *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	
≤ 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