

ARTICLE



Diagnostic utility of one-stop fusion gene panel to detect *TFE3/TFEB* gene rearrangement and amplification in renal cell carcinomas

Shuko Harada¹✉, Anna Calì², Karen M. Janowski¹, Diana Morlote¹, Maria D. Rodriguez Pena¹, Sofia Canete-Portillo¹, Djamel Harbi¹, Gina DeFrank¹, Cristina Magi-Galluzzi¹, George J. Netto¹, Guido Martignoni^{1,2,3} and Alexander C. Mackinnon¹

© The Author(s), under exclusive licence to United States & Canadian Academy of Pathology 2021

MiT family translocation renal cell carcinoma (MiT-RCC) harbors translocations involving the *TFE3* or *TFEB* genes. RCC with *TFEB* amplification is also identified and is associated with a more aggressive clinical course. Accurate diagnosis of MiT-RCC is crucial for patient management. In this study, we evaluated the performance of the Archer FusionPlex assay for detection of MiT-RCC with *TFE3* or *TFEB* translocations and *TFEB* amplifications. RNA was extracted from 49 RCC FFPE tissue samples with known *TFE3/TFEB* status (26 *TFE3* FISH positive, 12 *TFEB* FISH positive, 4 *TFEB* amplified (1 case both split and amplified), and 8 FISH negative) using the Covaris extraction kit. Target enriched cDNA libraries were prepared using the Archer FusionPlex kit and sequenced on the Illumina NextSeq 550. We demonstrate that the age of the specimen, quality of RNA, and sequencing metrics are important for fusion detection. Fusions were identified in 20 of 21 cases less than 2 years old, and *TFE3/TFEB* rearrangements were detected in all cases with Fusion QC ≥ 100 . The assay identified intrachromosomal inversions in two cases (*TFE3-RBM10* and *NONO-TFE3*), usually difficult to identify by FISH assays. *TFEB* mRNA expression and the *TFEB/TFE3* mRNA expression ratio were significantly higher in RCCs with *TFEB* fusion and *TFEB* gene amplification compared to tumors without *TFEB* fusion or amplification. A cutoff *TFEB/TFE3* ratio of 0.5 resulted in 97.3% concordance to FISH results with no false negatives. Our study demonstrates that the FusionPlex assay successfully identifies *TFE3* and *TFEB* fusions including intrachromosomal inversions. Age of the specimen and certain sequencing metrics are important for successful fusion detection. Furthermore, mRNA expression levels may be used for predicting cases harboring *TFEB* amplification, thereby streamlining testing. This assay enables accurate molecular detection of multiple subtypes of MiT-RCCs in a convenient workflow.

Modern Pathology (2021) 34:2055–2063; <https://doi.org/10.1038/s41379-021-00858-y>

INTRODUCTION

MiT family translocation renal cell carcinoma (MiT-RCC), incorporated into the World Health Organization classification in 2016 [1], harbors translocations involving the *TFE3* or *TFEB* gene. The *TFE3* gene is located on Xp11, and tumors harboring a *TFE3* gene rearrangement were originally referred to as Xp11 translocation RCC. The three most common translocation partners are *ASPL* (*ASPSR1*), *PRCC*, and *SFPQ* (*PSF*), which are located on chromosomes 17q25, 1q21, and 1p34, respectively [2, 3]. Since then, additional partner genes have been identified including *CLTC*, *PARP14*, *RBM10*, *NONO*, *MED15* [4], *DVL2* [5], and *RBMX* [6]. The *TFEB* gene is located on chromosome 6. *TFEB* is overexpressed in most MiT-RCC involving *TFEB* due to a t(6;11) translocation leading to the fusion of *TFEB* with *MALAT1*. In addition, *TFEB* has several other fusion partners including *KHDRBS2*, *COL21A1*, *CADM2*, *CLTC*, *EWSR1*, and *ACTB* [7]. More recently, RCC with *TFEB* amplification has been described as a unique subtype of RCC associated with poor outcome [8].

MiT-RCC was initially thought to be a childhood tumor characterized by papillary architecture composed of epithelioid

clear cells with clear to eosinophilic cytoplasm [3]. There is now increasing evidence indicating a wide spectrum of morphologies occurs in MiT-RCC [4], making the differential diagnosis challenging. Immunohistochemical stains for markers such as cathepsin K are helpful to a certain degree but are not always reliable [9]. MiT-RCC and RCC with *TFEB* amplification are distinct entities, and recognizing these as a specific category of RCC is important [10–13]. Therefore, accurate diagnosis is crucial because variable clinical behavior and therapy are observed with MiT-RCC [2, 4, 14].

Fluorescent in-situ hybridization (FISH) using break-apart probes for *TFE3* and *TFEB* is the gold standard for the diagnosis of MiT-RCC tumors [10]. However, FISH cannot identify fusion partners and more importantly, FISH is unreliable for rare MiT-RCC cases that have small intrachromosomal gene inversions [6, 15, 16]. Therefore, alternative assays are needed.

The Archer FusionPlex assay utilizes anchored multiplex PCR (AMP) technology [17]. AMP technology combined with next-generation sequencing (NGS) can detect gene fusion events in a partner-agnostic manner and is compatible with low nucleic acid

¹Department of Pathology, The University of Alabama at Birmingham, Birmingham, AL, USA. ²Department of Diagnostic and Public Health, Section of Pathology, University of Verona, Verona, Italy. ³Department of Pathology, Pederzoli Hospital, Peschiera del Garda, Italy. ✉email: sharada@uabmc.edu

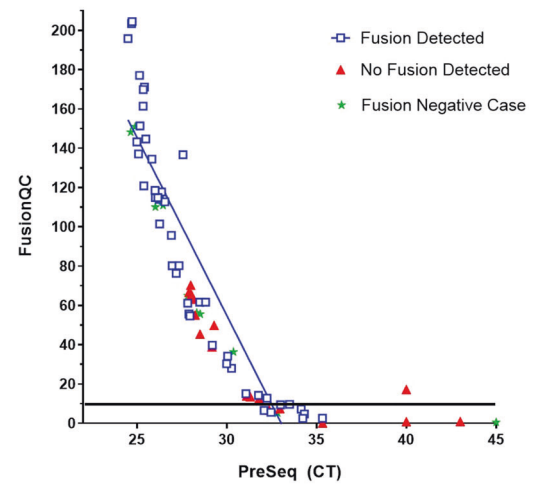
Table 1. Fusion panel results of all 49 renal cell carcinoma cases with known FISH status.

Gene	Case	Gene fusion	Breakpoint genomic location	FISH (% nuclei)	PreSeq CT	Fusion QC	TFEB expression	TFEB/TFE3 ratio
TFE3	1	PRCC→TFE3	chr1:156756966,chrX:48895967	30% (split)	26.91	95.67	0.14	0.24
	2	ASPSR1→TFE3	chr17:79954722,chrX:48891766	25% (split)	25	140.25	0.04	0.12
	3	RBM10→TFE3	chrX:47041725,chrX:48895639	27% (split)	25.17	151.42	0.04	0.05
	4	MED15→TFE3	chr22:20918975,chrX:48895967	77% (split)	25.41	171.17	0.03	0.13
	5	NONO→TFE3	chrX:70517788,chrX:48891766	55% (1G1F)	24.5	195.83	0.03	0.11
	6	EWSR1→TFE3	chr22:29678546,chrX:48898095	40% (split)	25.5	144.67	0.11	0.15
	7	ASPSR1→TFE3	chr17:79957813,chrX:48895681	85% (split)	32.08	6.58		
	8	PRCC→TFE3	chr1:156764600,chrX:48895967	65% (split)	26.96	80.17	0.09	0.20
	9	PRCC→TFE3	chr1:156738031,chrX:48895967	45% (split)	32.46	5.58		
	10	SFPQ→TFE3	chr1:35652602,chrX:48891766	90% (split)	24.99	143.25	0.03	0.05
	11	ASPSR1→TFE3	chr17:79954722,chrX:48891766	55% (split)	26.37	117.75	0.04	0.29
	12	ASPSR1→TFE3	chr17:79954722,chrX:48891766	50% (split)	31.07	15.08	0.64	0.52
	13	ASPSR1→TFE3	chr17:79954722,chrX:48895639	75% (split)	30.26	28.17	0.11	0.23
	14	SFPQ→TFE3	chr1:35652602,chrX:48895639	80% (split)	28.49	61.67	0.04	0.11
	15	UZAF2→TFE3	chr19:56180158,chrX:48891766	50% (split)	26.26	101.50	0.07	0.17
	16	ASPSR1→TFE3	chr17:79954722,chrX:48891766	75% (split)	26.24	110.17	0.08	0.19
	17	ASPSR1→TFE3	chr17:79954722,chrX:48891766	58% (split)	29.99	30.33	0.32	0.28
18	None	NA	NA	45% (split)	31.31	13.58	0.15	0.14
19	None	NA	NA	80% (split)	28.15	63.17	0.05	0.13
20	None	NA (QC failure)	NA (QC failure)	55% (split)	40	0.83		
21	None	NA	NA	75% (split)	28.51	45.42	0.26	0.27
22	None	NA	NA	50% (split)	27.83	61.17	0.30	0.29
23	None	NA (QC failure)	NA (QC failure)	75% (split)	40	0.92		
24	None	NA (QC failure)	NA (QC failure)	60% (split)	35.34	0.17		
25	None	NA	NA	70% (split)	40	14.75	0.72	0.19
26	None	NA (no tumor in deeper section)	NA (no tumor in deeper section)	88% (split)	27.18	72.42	0.22	0.24
27	None	NA (no tumor in deeper section)	NA (no tumor in deeper section)	90% (split)	31.8	12.67	0.41	0.44
28	None	NA	NA	55% (split)	28.24	55.17	0.33	0.58
TFEB	29	MALAT1→TFEB	chr11:65266581,chr6:41658973	78% (split)	27.89	55.67	4.20	20.96
	30	MALAT1→TFEB	chr11:65266581,chr6:41658973	74% (split)	34.14	7.25		
	31	MALAT1→TFEB	chr11:65266581,chr6:41658973	80% (split)	31.76	14.25	3.80	10.01
	32	MALAT1→TFEB	chr11:65266579,chr6:41673388	58% (split)	34.32	4.75		
	33	ACTB→TFEB	chr7:5568859,chr6:41703094	91% (split)	26.02	114.92	0.63	1.09
	34	MALAT1→TFEB	chr11:65266579,chr6:41673388	75% (split)	27.34	80.25	3.04	11.88
	35	MALAT1→TFEB	chr11:65266581,chr6:41658973	94% (split)	35.33	2.58		
	36	MALAT1→TFEB	chr11:65266581,chr6:41658973	61% (split)	29.19	39.75	4.76	7.43
	37	MALAT1→TFEB	chr11:65266579,chr6:41673388	80% (split)	32.24	12.83	4.48	13.47
	38	MALAT1→TFEB	chr11:65266581,chr6:41658973	85% (split)	61.67	28.82	4.21	10.96

Table 1 continued

Gene	Case	Gene fusion	Breakpoint genomic location	FISH (% nuclei)	PreSeq CT	Fusion QC	TFEB expression	TFEB/TFE3 ratio
	39	NEAT1 → TFE3	chr11:65190461,chr6:41658953	65% (split)	34.23	2.50		
	40	None	NA	80% (split) 80% (amp > 10 signals)	27.99	70.33	0.52	1.19
	41	None	NA	80% (amp > 10 signals)	28.52	55.58	1.03	1.44
	42	None	NA	99% (amp 3–15 signals)	24.65	148.25	0.36	0.51
	43	None	NA	80% (amp > 10 signals)	28.33	56.25	0.32	0.75
No variant	44	None	NA	Negative	32.83	5.25		
	45	None	NA	Negative	26.45	110.92	0.06	0.11
	46	None	NA	Negative	24.9	151.00	0.07	0.13
	47	None	NA	Negative	26.02	110.08	0.11	0.15
	48	None	NA	Negative	45	0.50		
	49	None	NA	Negative	45	0.50		

A



B

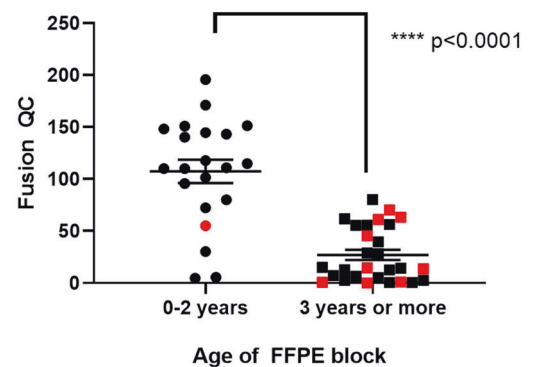


Fig. 1 Effect of age of FFPE block and PreSeq QC on Fusion QC and assay performance. **A** Correlation between PreSeq CT value and Fusion QC value in RCC cases (some cases were run in duplicate). Blue open square: FISH-positive case in which fusion gene transcript was detected. Red triangle: FISH-positive case in which fusion gene transcript was not detected. Green star: FISH-negative case. Linear regression equation $Y = -18.10X + 598$ ($R^2 = 0.838$; $p < 0.0001$). **B** Fusion QC distribution in samples with newer (0–2 years) and older (3 years or older) FFPE tissue blocks. Each black dot (circle and square) represents a case with concordant FISH results and red dots represent the case with discordant results. Lines indicate mean \pm SEM.

input from formalin-fixed paraffin-embedded (FFPE) tissues [17]. The objective of this study is to evaluate the performance of the Archer FusionPlex assay for detection of MiT-RCC with translocations or intrachromosomal inversions. In addition, we evaluated whether elevated *TFEB* expression accurately predicts *TFEB* amplification.

MATERIALS AND METHODS

Tissue samples

A total of 68 FFPE cases including 49 cases of RCCs with known *TFE3* and *TFEB* FISH status (Table 1) and 19 cases of sarcomas and carcinomas harboring non-*TFE3/TFEB* gene fusion events (see Supplementary Table 1) were retrieved from the archives of Departments of Pathology at the University of Verona, Pederzoli Hospital, Peschiera del Garda, Verona, Italy, and The University of Alabama at Birmingham, Birmingham, AL, USA. One hematoxylin and eosin (H&E) stained slide and five 10 μ m thick unstained slides were cut from each selected FFPE block. H&E slides were reviewed for adequacy and tumor cellularity by a board certified pathologist. Seraseq Fusion RNA Mix v4 and Seraseq FFPE tumor fusion RNA v4 reference materials (SeraCare, Milford, MA) with 18 known gene fusion events were used as controls. This study was approved by the Institutional

Table 2. Concordance with FISH results in different Fusion QC values.

FISH results	Fusion QC			Total
	<10	10–100	≥100	
TFE3 positive	2/5 (40%)	6/12 (50%)	9/9 (100%)	17/26 (65%)
TFEB positive	4/4 (100%)	6/7 (86%)	1/1 (100%)	11/12 (92%)
Negative FISH or no tumor	3/3 (100%)	4/4 (100%)	4/4 (100%)	11/11 (100%)
Total	9/12 (75%)	16/23 (70%)	14/14 (100%)	39/49 (80%)

FISH positivity means positive for gene rearrangement. Numbers in each column indicate concordant case/total case (percentage).

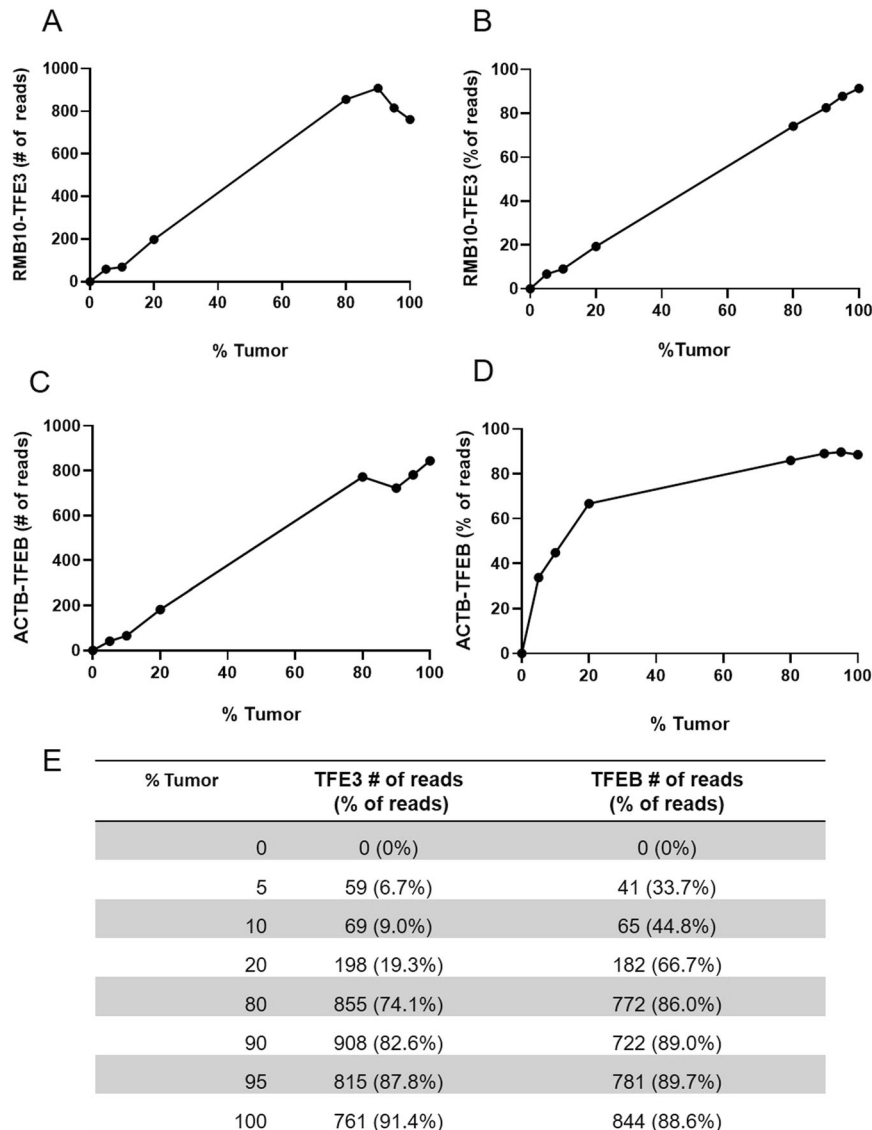


Fig. 2 The mixing experiments using *TFE3* and *TFEB* rearrange to determine the limit of detection (LOD). RNA from a case with *RMB10-TFE3* (A, B) and a case with *ACTB-TFEB* (C, D) were mixed at different ratio and the number (A, C) and the percentage (B, D) of fusion transcript reads were plotted against the percentage of tumor. The number and the percentage of fusion transcript reads at each data point is shown in (E).

Review Board at the both institutions in accordance with the Helsinki Declaration.

Nucleic acid extraction

FFPE tissue samples were scraped off the slides into microfuge tubes and tissue lysis buffer with proteinase K (440 μ L) was added. Tissue was

deparaffinized using Acoustic Paraffin Emulsification on the Covaris ME220 Adaptive Focused-ultrasonicator (Covaris, Inc. Woburn, MA). Total nucleic acid (DNA and RNA) was extracted from the lysate using the Covaris truXTRAC FFPE total Nucleic Acid Plus kit - Column (Cat. No. 520252; Covaris, Inc.), following the manufacturer's instruction [18]. The extracted DNA was not used for this study. RNA quantity was assessed using the Qubit fluorometer (Life Technologies, Carlsbad, CA).

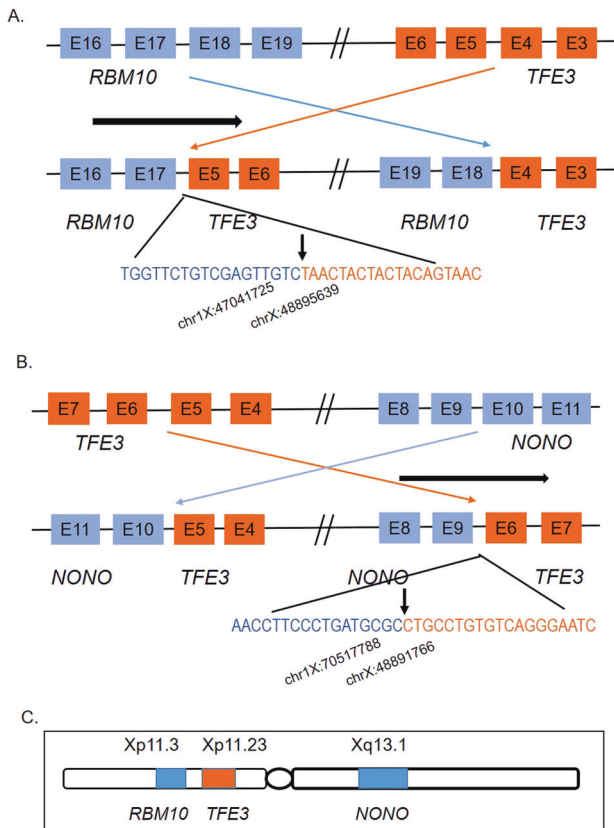


Fig. 3 Schematic diagrams of fusion transcripts by intrachromosomal inversion. Exx represents exon xx of each gene. Thick black arrows indicate oncogenic fusion transcripts. **A** *RBM10* → *TFE3* (breakpoint: chrX:47041725,chrX:48895639). **B** *NONO* → *TFE3* (breakpoint: chrX:70517788,chrX:48891766). **C** Schematic representation of chromosome X with *TFE3*, *RBM10*, and *NONO*.

AMP-based targeted NGS

Target enriched cDNA libraries were prepared with a clinically validated, custom-designed Archer FusionPlex panel targeting 94 target genes, as per the manufacturer's instructions (ArcherDX, Inc. Boulder, CO) [17, 19, 20]. The quality of amplifiable RNA was determined by the presequencing quality control cycle threshold (PreSeq QC CT), which was identified using real-time reverse transcriptase qPCR in duplicate for each sample. The assay utilizes AMP technology, which employs unidirectional gene-specific primers (GSPs) that enrich both known and unknown fusion gene partners [17]. All libraries were purified and quantified using KAPA Biosystems qPCR kit for Illumina (KAPA Biosystems, Wilmington, MA) and then sequenced on the Illumina NextSeq instrument (Illumina, San Diego, CA). Paired-end sequence data were generated by concatenation of lane level FASTQ files with Illumina's Local Run Manager and uploaded to the Archer analysis unlimited software (version 6.2.7). Quality of sequencing was evaluated using Fusion QC score, which reflects the average number of unique reads per GSP2 and the average number of unique start sites per GSP2. Gene fusion events were analyzed using Archer analysis unlimited software (version 6.2.7) and JBrowse genome browser [20].

Analysis of *TFEB* mRNA expression

RNA expression values for all GSP2 primers of *TFE3* and *TFEB* were captured as "rna_expression_visualization.tsv" files using Archer analysis software. Cases with Fusion QC score less than 10 were excluded. Average of all mRNA values of GSP2 primers for *TFEB* and *TFE3* was calculated and compared between different tumor types.

FISH analysis

FISH on FFPE sections was performed with dual-color break-apart probes for *TFE3* on chromosome Xp11.2 and *TFEB* on 6p21 (Cytotest Inc., Rockville, MD) as previously described [21, 22]. Centromeric chromosome 6 probe

(CEP6) was used as the control probe (Vysis-Abbott, Rome, Italy). Scoring was performed by an experienced pathologist. At least 100 nonoverlapping, neoplastic nuclei were counted. Rearrangement, designated as FISH positive, was defined as red and green signals separated more than 2 signal diameters. The FISH-positive cutoff was 25%. Amplification was defined by the ratio of the mean copy number of *TFEB* signals to the mean copy number of CEP6 signals ≥ 10 .

Statistical analysis

When applicable, the results were evaluated using Student's *t* test. A *p* value equal to or less than 0.05 was considered statistically significant.

RESULTS

RNA quality and sequencing quality

We evaluated the performance of the Archer FusionPlex Panel using Seraseq Fusion RNA Mix v4 and Seraseq FFPE tumor fusion RNA v4 control materials. The Seraseq Fusion RNA Mix is not derived from FFPE and is therefore of higher quality compared to the Seraseq FFPE tumor fusion RNA v4, which is similar to a routine FFPE sample. The Seraseq Fusion RNA control was analyzed on five different runs. PreSeq CT values were between 20 and 22 cycles, and Fusion QC values, defined as the average unique start sites per GSP2 control, were over 200, indicating excellent RNA and sequencing quality (Supplementary Fig. 1A). All expected fusion transcripts were detected across all runs. The Seraseq FFPE tumor fusion control was analyzed across 14 different runs. PreSeq CT and Fusion QC values of this control sample varied widely. Fusion QC inversely correlated with PreSeq QC CT values ($y = -52.64X + 1508$; $r^2 = 0.748$) (Supplementary Fig. 1A). All 18 fusions were detected when Fusion QC was ≥ 100 (11/14 samples). However, the number of expected fusions that were detected dropped as the Fusion QC values decreased, with *ROS1* and *MET* dropping out most frequently (Supplementary Fig. 1B). Even though Fusion QC ≥ 10 is considered "PASS" per the manufacturer's recommendation, our data indicated that false negative results are observed when Fusion QC < 100 [23].

Concordance between FISH and fusion panel results

Concordance between the Archer Fusion panel and *TFE3/TFEB* FISH was 80% (39/49) for the 49 RCC cases (Table 1). Figure 1A shows Fusion QC values plotted against PreSeq CT values for these cases. Fusion QC inversely correlated with PreSeq QC CT values ($Y = -18.10X + 598$; $R^2 = 0.838$; $p < 0.0001$). Sequencing quality metrics were important for *TFE3/TFEB* fusion detection. When Fusion QC value was ≥ 100 , concordance of *TFE3* and *TFEB* to FISH was 100% (Table 2). Concordance was 43% for *TFE3* (8/17) and 91% (10/11) for *TFEB* when Fusion QC value was < 100 . Interestingly, even when QC < 10 , concordance was 100% for *TFEB* ($n = 4$) indicating that the detection of gene fusions events is not consistent across all genes in the panel.

Since Fusion QC and PreSeq QC values likely reflect the quality of the RNA, we correlated the age of the FFPE specimen with both the Fusion QC and the detection of *TFE3* and *TFEB* gene rearrangements. Comparing FFPE samples less than 2 years old with samples older than 2 years, both Fusion QC (107.4 ± 11.2 versus 27.02 ± 4.93 , respectively) and detection of *TFE3/TFEB* fusion events (95% versus 64%, respectively) were superior in samples less than 2 years old (Fig. 1B and Supplementary Table 2). In fact, all samples with Fusion QC ≥ 100 are less than 2 years old (Supplementary Table 3). Given that the majority of FFPE samples submitted for routine clinical testing are less than 2 years old, these data indicate that the Archer Fusion panel is reliable for clinical testing of both *TFE3* and *TFEB* gene rearrangements.

We analyzed input RNA ranging from 250 (manufacturer's recommendation) to 20 ng and obtained sufficient numbers of sequence reads for fusion detection as long as tumor percentage is $\geq 20\%$ (data not shown). These results are consistent with a

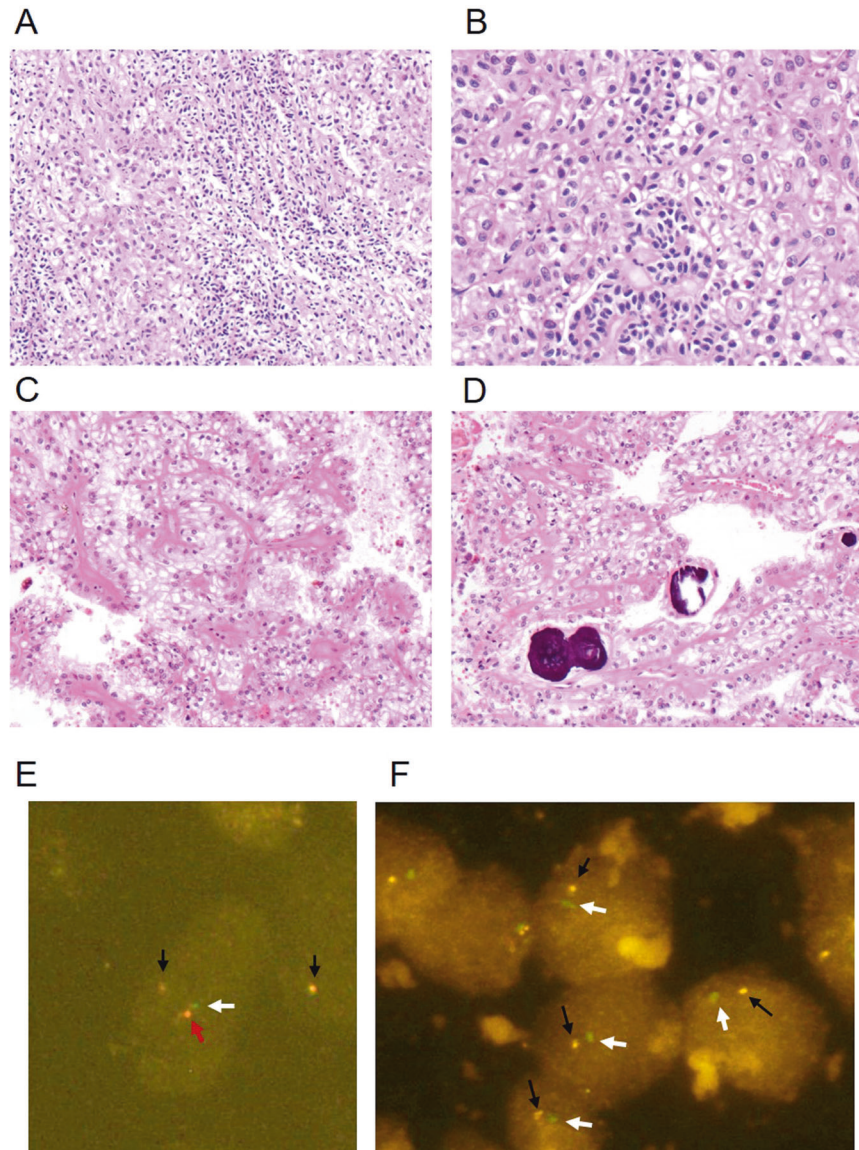


Fig. 4 Morphology (H&E) and fluorescent in-situ (FISH) images of RCCs with intrachromosomal inversions. H&E of RCCs with *RBM10*→*TFE3* (case 3) shows a solid tumor with distinctive biphasic morphology, composed of larger epithelioid cells with clear and eosinophilic cytoplasm and smaller cells occasionally clustering around basement membrane material (**A, B**). *TFE3* break-apart FISH shows 1R1G1F pattern with closely split signals (**E**). H&E of RCCs with *NONO*→*TFE3* (case 5) shows a tumor with predominant papillary architecture lined by cells with clear cytoplasm with relatively uniform and round nuclei. Perivascular hyalinization is also apparent. Focal psammoma bodies are occasionally observed (**C, D**). *TFE3* break-apart FISH shows predominant pattern of one green and one fusion (1G1F) (**F**). White arrows indicate green signals, red arrow indicates red signal, and black arrows indicate fusion signals (yellow).

previous study by Sussman et al. [24]. The limit of detection (LOD) of the Archer Fusion panel was determined by mixing RNA from two fusion positive cases at different ratios (Fig. 2). The mixing experiments were performed using *TFE3* and *TFEB* cases with high Fusion QC scores to help mitigate any potential influence that poor amplification may have on determining the LOD. The mixing experiments were designed based on the premise that mixing FFPE-derived RNAs from MiT-RCCs with different fusion events is equivalent to a dilution study using RNA extracted from normal tissue. In addition, mixing studies are cost effective because you can determine the LOD for both targets in the same experiment. Mixing studies demonstrated *TFE3* and *TFEB* fusion transcripts were detected with 5% variant fraction (Fig. 2).

The Archer Fusion panel identified intrachromosomal inversions in two cases (*TFE3*-*RBM10* and *NONO*-*TFE3*) (Figs. 3 and 4; cases 3 and 5, respectively, in Table 1). The FISH patterns on these cases

were unusual. Case 3 was a female whose tumor exhibited ~30% FISH closely split signals which is close to the 25% threshold for positivity (Fig. 4E). Case 5 was a male whose tumor exhibited a unusual split pattern with one green signal and one fusion signal (1G1F) (Fig. 4F). The distance between green and fusion signals appears to be 2–3 signal diameters.

Evaluation of *TFEB* expression

We calculated the mRNA expression levels of *TFEB* and *TFE3* and determined the *TFEB*/*TFE3* mRNA expression ratio for cases with Fusion QC (≥ 10) (Fig. 5 and Table 3; individual results are shown in Table 1). *TFEB* mRNA expression and *TFEB*/*TFE3* ratio were significantly higher in RCCs with *TFEB* fusion compared to RCCs with *TFE3* fusion, RCCs with no fusion, or nonrenal tumors. Similarly, RCCs with *TFEB* amplification had significantly higher *TFEB* expression levels and *TFEB*/*TFE3* ratios compared to cases

with *TFE3* fusion events and cases without fusion or amplification events (Table 3). There was no statistically significant difference in *TFE3* mRNA levels between the groups. Using a cutoff value of 0.5 for the *TFEB/TFE3* ratio, all *TFEB* altered tumors (11/11) were above 0.5, and 96% (25/26) of the tumors without *TFEB* alteration were below 0.5. The overall concordance of the 0.5 expression ratio with *TFEB* alteration is 97% (36/37) with 100% sensitivity, 96% specificity, 91% positive predictive value, and 100% negative predictive value. These data suggest that *TFEB* expression levels

and the *TFEB/TFE3* expression ratio are very accurate for predicting RCC cases harboring *TFEB* amplification that require confirmatory testing.

DISCUSSION

It has been more than two decades since the Xp11 translocation RCC was first reported [25–28]. Clinical and histopathological features of MiT-RCCs are more diverse than originally described [4, 29], and the accurate diagnosis of these tumors is challenging. Currently, there is no standardized work-up for MiT-RCC. *TFE3* immunohistochemistry (IHC) for screening has been proposed [29, 30]. However, IHC can be problematic due to low specificity and inconsistent performance [29]. FISH is currently the gold standard for diagnosis but has several drawbacks. The fusion partner of *TFE3* and *TFEB* is not identified by FISH. The lack of a consensus threshold for interpreting FISH-positive nuclei [29] and the technical challenge of interpretation—particularly for cases with *TFE3* intrachromosomal inversions, are also limitations for FISH analysis [15, 31]. By analyzing a large cohort of MiT-RCC with a variety of *TFE3* or *TFEB* gene alterations, we demonstrated that the Archer FusionPlex assay reliably identifies *TFE3* and *TFEB* gene fusion events—including intrachromosomal *TFE3* inversions and *TFEB* gene amplification.

Our study demonstrates that fusion detection is not uniform across all genes in the panel. Fusion genes involving *TFEB* were detected in 92% of all cases including those with low Fusion QC, whereas detection of *TFE3* fusions was 100% when Fusion QC is >100 but falls to 40–50% when Fusion QC < 100 [23]. In support of nonuniform detection, we observed that identification of *ROS1* fusions and *Met* exon14 skipping, which are both targets of the Archer Fusion panel, correlates strongly with Fusion QC score, whereas detection of *ALK1-EML4* occurs over a range of Fusion QC scores including Fusion QC < 40 (Supplementary Fig. 1 and data not shown). Fusion QC values indicate confidence in the assay's performance and demonstrate the critical importance of pre-analytical assessment for quality control and quality assurance purposes. When no fusion transcript is detected and Fusion QC is less than 100, the results should be interpreted with caution. Clinical labs need to have policies for alternative testing to confirm the negative results in this scenario.

The reason for the differences in target detection is not clear but likely reflects variation in PCR amplification efficiency for the different GSPs in the panel [32]. When input RNA becomes the limiting factor, as reflected by a decrease in Fusion QC score, the differences in GSP efficiency becomes apparent. The importance of RNA quality and sequencing metrics for successful fusion detection has not been well characterized. A few studies have shown correlation between the PreSeq QC CT value and Fusion QC [23, 24], consistent with our study. Garcia et al. demonstrated that average read depth is significantly lower in cases where fusion events were not detected [32]. We observed robust detection and very little dropout of *TFE3* and *TFEB* gene alterations using RNA from cases less than 2 years old. Taken together, certain

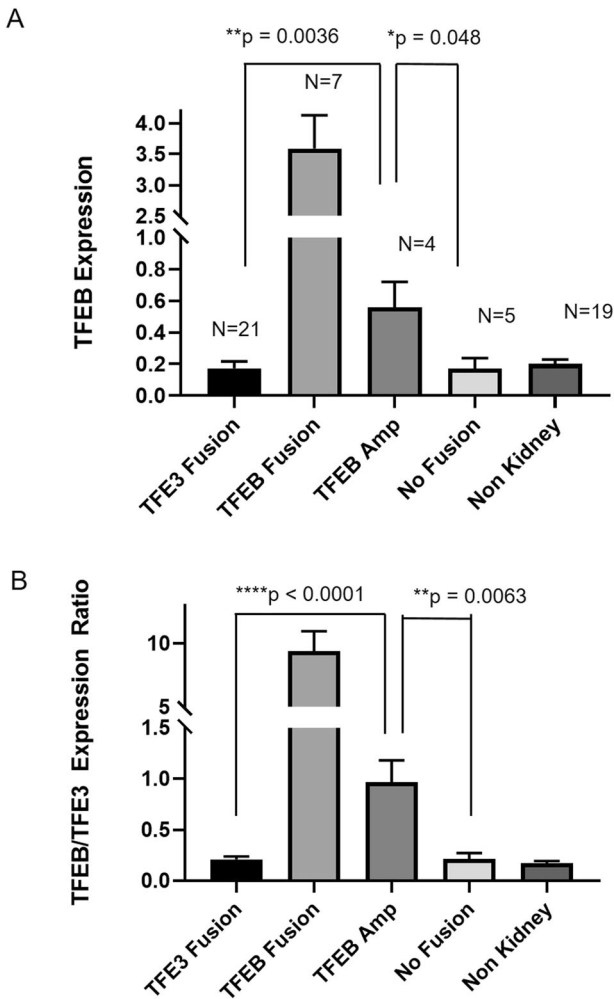


Fig. 5 Evaluation of *TFEB* expression levels in different types of tumors. Expression levels of *TFEB* mRNA (A) and *TFEB/TFE3* mRNA expression ratio (B) in renal tumors with *TFE3* fusion, *TFEB* fusion, *TFEB* amplification (Amp), no fusion, and tumors from other organs (carcinomas and sarcomas). *N* indicates number of cases and *p* value was calculated by Student's *t* test.

Table 3. Summary of RNA expression levels of *TFE3*, *TFEB*, and *TFE3/TFEB* ratio in different tumor types.

Tumor type	<i>TFE3</i> (mean ± SEM)	<i>TFEB</i> (mean ± SEM)	<i>TFEB/TFE3</i> ratio (mean ± SEM)
<i>TFEB</i> fusion tumor (n = 7)	0.422 ± 0.052	3.588 ± 0.535 ^a	9.402 ± 1.550 ^a
<i>TFEB</i> amplified tumor (n = 4)	0.573 ± 0.080	0.557 ± 0.162 ^a	0.971 ± 0.210 ^a
<i>TFE3</i> fusion tumor (n = 21)	0.760 ± 0.162	0.174 ± 0.043	0.211 ± 0.029
Kidney with no fusion/amplification (n = 5)	0.724 ± 0.091	0.172 ± 0.066	0.213 ± 0.060
Non-kidney tumor (n = 19)	1.175 ± 0.157	0.200 ± 0.030	0.175 ± 0.020

The results are expressed as mean ± SEM.

^aStatistically significantly higher compared to *TFE3* fusion tumor and kidney with no fusion/amplification (*p* < 0.05).

target genes in the Archer Fusion panel are more susceptible to dropout. Fusion QC scores can predict these false negatives, but it is important to empirically determine which targets are susceptible. Using RNA from cases less than 2 years old largely avoids this pitfall.

Our study also demonstrated that the *TFEB/TFE3* expression ratio was significantly higher in RCCs with *TFEB* amplification as compared to tumors with *TFE3* fusion or no alterations. Consequently, the *TFEB/TFE3* expression ratio calculated using the RNASeq data is highly useful for predicting cases with *TFEB* amplification. A simple *TFEB/TFE3* ratio threshold of 0.5 results in 97% concordance to FISH results with no false negatives. Since *TFEB* amplification is relatively uncommon, the number of the cases with *TFEB* amplification that we were able to analyze was low. In addition, we did not have any cases exhibiting gain of *TFEB*/chromosome 6p (low levels of increased copy number rather than amplification) in our cohort. Those cases may represent chromosome 6 polysomy and seen in a variety of high-grade cancers [8, 12], but may be challenging to distinguish from the case with true amplification. Further prospective validation of this ratio for predicting *TFEB* amplification using various types of RCCs including cases with chromosome 6p gain is warranted. Interestingly, *TFEB* expression levels in *TFEB* amplified RCC are not as high as those in *TFEB* rearranged RCCs. These results are consistent with previous work, where *TFEB* gene expression levels were evaluated by digital droplet PCR [33]. Our work underscores the value of applying this same principle for other tumors with gene amplification, such as liposarcoma with *MDM2* amplification, when running Archer FusionPlex sarcoma panel. However, expression levels for different gene amplifications events will vary, and appropriate validation studies are needed rather than generalizing a cutoff value from this study.

In summary, our study demonstrates that the Archer FusionPlex assay enables accurate molecular diagnosis of all MiT-RCCs in a convenient, single workflow by reliably identifying *TFE3* and *TFEB* fusions including intrachromosomal inversions, as well as *TFEB* amplification. The assay performs well down to 20 ng of input RNA allowing testing of challenging or limited samples, such as core needle biopsies. Age of the specimen, RNA quality, and sequencing metrics are important parameters for successful detection of *TFE3* and *TFEB* gene alterations. Caution is needed when sequencing is suboptimal, and alternative testing strategies should be considered in fusion-negative cases where Fusion QC values are <100 to avoid false negative results.

DATA AVAILABILITY

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

REFERENCES

- Moch H, Cubilla AL, Humphrey PA, Reuter VE, Ulbright TM. The 2016 WHO classification of tumours of the urinary system and male genital organs-part a: renal, penile, and testicular tumours. *Eur Urol*. 2016;70:93–105.
- Wang XT, Xia QY, Ye SB, Wang X, Li R, Fang R, et al. RNA sequencing of Xp11 translocation-associated cancers reveals novel gene fusions and distinctive clinicopathologic correlations. *Mod Pathol*. 2018;31:1346–60.
- Xie L, Zhang Y, Wu CL. Microphthalmia family of transcription factors associated renal cell carcinoma. *Asian J Urol*. 2019;6:312–20.
- Caliò A, Segala D, Munari E, Brunelli M, Martignoni G. MiT family translocation renal cell carcinoma: from the early descriptions to the current knowledge. *Cancers*. 2019;11:1110.
- Argani P, Zhong M, Reuter VE, Fallon JT, Epstein JI, Netto GJ, et al. TFE3-fusion variant analysis defines specific clinicopathologic associations among Xp11 translocation cancers. *Am J Surg Pathol*. 2016;40:723–37.
- Argani P, Zhang L, Sung YS, White MJ, Miller K, Hopkins M, et al. A novel RBMX-TFE3 gene fusion in a highly aggressive pediatric renal perivascular epithelioid cell tumor. *Genes Chromosomes Cancer*. 2019;59:58–63.
- Xia QY, Wang XT, Fang R, Wang Z, Zhao M, Chen H, et al. Clinicopathologic and molecular analysis of the TFEB fusion variant reveals new members of TFEB translocation renal cell carcinomas (RCCs): expanding the genomic spectrum. *Am J Surg Pathol*. 2020;44:477–89.
- Argani P, Reuter VE, Zhang L, Sung YS, Ning Y, Epstein JI, et al. TFEB-amplified renal cell carcinomas: an aggressive molecular subset demonstrating variable melanocytic marker expression and morphologic heterogeneity. *Am J Surg Pathol*. 2016;40:1484–95.
- Caliò A, Brunelli M, Segala D, Pedron S, Remo A, Ammendola S, et al. Comprehensive analysis of 34 MiT family translocation renal cell carcinomas and review of the literature: investigating prognostic markers and therapy targets. *Pathology*. 2020;52:297–309.
- Skala SL, Xiao H, Udager AM, Dhanasekaran SM, Shukla S, Zhang Y, et al. Detection of 6 TFEB-amplified renal cell carcinomas and 25 renal cell carcinomas with MITF translocations: systematic morphologic analysis of 85 cases evaluated by clinical TFE3 and TFEB FISH assays. *Mod Pathol*. 2018;31:179–97.
- Caliò A, Brunelli M, Segala D, Pedron S, Doglioni C, Argani P, et al. VEGFA amplification/increased gene copy number and VEGFA mRNA expression in renal cell carcinoma with TFEB gene alterations. *Mod Pathol*. 2019;32:258–68.
- Williamson SR, Grignon DJ, Cheng L, Favazza L, Gondim DD, Carskadon S, et al. Renal cell carcinoma with chromosome 6p amplification including the TFEB gene: a novel mechanism of tumor pathogenesis? *Am J Surg Pathol*. 2017;41:287–98.
- Gupta S, Johnson SH, Vasmatzis G, Porath B, Rustin JG, Rao P, et al. TFEB-VEGFA (6p21.1) co-amplified renal cell carcinoma: a distinct entity with potential implications for clinical management. *Mod Pathol*. 2017;30:998–1012.
- Peckova K, Vanecek T, Martinek P, Spagnolo D, Kuroda N, Brunelli M, et al. Aggressive and nonaggressive translocation t(6;11) renal cell carcinoma: comparative study of 6 cases and review of the literature. *Ann Diagn Pathol*. 2014;18:351–7.
- Argani P, Zhang L, Reuter VE, Tickoo SK, Antonescu CR. RBM10-TFE3 renal cell carcinoma: a potential diagnostic pitfall due to cryptic intrachromosomal Xp11.2 inversion resulting in false-negative TFE3 FISH. *Am J Surg Pathol*. 2017;41:655–62.
- Xia QY, Wang XT, Zhan XM, Tan X, Chen H, Liu Y, et al. Xp11 translocation renal cell carcinomas (RCCs) with RBM10-TFE3 gene fusion demonstrating melanotic features and overlapping morphology with t(6;11) RCC: interest and diagnostic pitfall in detecting a paracentric inversion of TFE3. *Am J Surg Pathol*. 2017;41:663–76.
- Zheng Z, Liebers M, Zhelyazkova B, Cao Y, Panditi D, Lynch KD, et al. Anchored multiplex PCR for targeted next-generation sequencing. *Nat Med*. 2014;20:1479–84.
- Kresse SH, Namløs HM, Lorenz S, Berner JM, Myklebost O, Bjerkehagen B, et al. Evaluation of commercial DNA and RNA extraction methods for high-throughput sequencing of FFPE samples. *PLoS ONE*. 2018;13:e0197456.
- Lam SW, Cleton-Jansen AM, Cleven AHG, Ruano D, van Wezel T, Szuhai K, et al. Molecular analysis of gene fusions in bone and soft tissue tumors by anchored multiplex PCR-based targeted next-generation sequencing. *J Mol Diagn*. 2018;20:653–63.
- Chang F, Lin F, Cao K, Surrey LF, Aplenc R, Bagatell R, et al. Development and clinical validation of a large fusion gene panel for pediatric cancers. *J Mol Diagn*. 2019;21:873–83.
- Green WM, Yonescu R, Morsberger L, Morris K, Netto GJ, Epstein JI, et al. Utilization of a TFE3 break-apart FISH assay in a renal tumor consultation service. *Am J Surg Pathol*. 2013;37:1150–63.
- Caliò A, Harada S, Brunelli M, Pedron S, Segala D, Portillo SC, et al. TFEB rearranged renal cell carcinoma. A clinicopathologic and molecular study of 13 cases. Tumors harboring MALAT1-TFEB, ACTB-TFEB, and the novel NEAT1-TFEB translocations constantly express PDL1. *Mod Pathol*. 2021;34:842–50.
- Seager M, Aisner DL, Davies KD. Oncogenic gene fusion detection using anchored multiplex polymerase chain reaction followed by next generation sequencing. *J Vis Exp*. 2019;149:10.3791.
- Sussman RT, Oran AR, Paolillo C, Lieberman D, Morrisette JJD, Rosenbaum JN. Validation of a next-generation sequencing assay targeting RNA for the multiplexed detection of fusion transcripts and oncogenic isoforms. *Arch Pathol Lab Med*. 2020;144:90–98.
- Hernandez-Martí MJ, Orellana-Alonso C, Badía-Garrabou L, Verdeguer Miralles A, Paradís-Alós A. Renal adenocarcinoma in an 8-year-old child, with a t(X;17)(p11.2;q25). *Cancer Genet Cytogenet*. 1995;83:82–83.
- Sidhar SK, Clark J, Gill S, Hamoudi R, Crew AJ, Gwilliam R, et al. The t(X;1)(p11.2;q21.2) translocation in papillary renal cell carcinoma fuses a novel gene PRCC to the TFE3 transcription factor gene. *Hum Mol Genet*. 1996;5:1333–8.
- Weterman MA, Wilbrink M, Geurts van Kessel A. Fusion of the transcription factor TFE3 gene to a novel gene, PRCC, in t(X;1)(p11;q21)-positive papillary renal cell carcinomas. *Proc Natl Acad Sci USA*. 1996;93:15294–8.
- Argani P, Antonescu CR, Illei PB, Lui MY, Timmons CF, Newbury R, et al. Primary renal neoplasms with the ASPL-TFE3 gene fusion of alveolar soft part sarcoma: a distinctive tumor entity previously included among renal cell carcinomas of children and adolescents. *Am J Pathol*. 2001;159:179–92.

29. Akgul M, Williamson SR, Ertoy D, Argani P, Gupta S, Caliò A, et al. Diagnostic approach in TFE3-rearranged renal cell carcinoma: a multi-institutional international survey. *J Clin Pathol.* 2021;74:291–9.
30. Akgul M, Cheng L, Idrees M. A simplified diagnostic approach on TFE3 gene fusion-associated renal cell carcinoma. *Arch Pathol Lab Med.* 2020;145:132–4.
31. Kato I, Furuya M, Baba M, Kameda Y, Yasuda M, Nishimoto K, et al. RBM10-TFE3 renal cell carcinoma characterised by paracentric inversion with consistent closely split signals in break-apart fluorescence in-situ hybridisation: study of 10 cases and a literature review. *Histopathology.* 2019;75:254–65.
32. García R, Patel N, Uddin N, Park JY. Development and clinical validation of a multiplex gene fusion assay. *Lab Med.* 2020;51:512–8.
33. Gupta S, Argani P, Jungbluth AA, Chen YB, Tickoo SK, Fine SW, et al. TFEB expression profiling in renal cell carcinomas: clinicopathologic correlations. *Am J Surg Pathol.* 2019;43:1445–61.

AUTHOR CONTRIBUTIONS

SH, ACM, DM, and GN performed study concept and design; KMJ, GD, SH, ACM, DM, and DH performed development of methodology; SH, ACM, DM, and DH provided acquisition, analysis, and interpretation of data and statistical analysis; AC, GM, MDRP, SCP, CM-G, and KMJ provided technical and material support; and SH, ACM, DM, GN, and GM performed writing and reviewing of the paper. All authors read and approved the final paper.

FUNDING

Institutional grant to GN (The University of Alabama at Birmingham).

COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL

This study was approved by the Institutional Review Board at the both institutions in accordance with the Helsinki Declaration.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41379-021-00858-y>.

Correspondence and requests for materials should be addressed to S.H.

Reprints and permission information is available at <http://www.nature.com/reprints>

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.