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Brief Communication

PBRM1 and *KDM5C* cooperate to define high-angiogenesis tumors and increased antiangiogenic response in renal cancer

María Santos[1], Javier Lanillos[1], Eduardo Caleiras[2], Carlos Valdivia[1], Juan M Roldan-Romero[1], Nuria Laínez[3], Javier Puente[4], Benoit Beuselinck[5], Stéphane Oudard[6], Jessica Zucman-Rossi[7], Paloma Navarro[8], Mercedes Robledo[1,9], Daniel Castellano[10], Guillermo de Velasco[10], Jesús García-Donas[8], Cristina Rodriguez-Antona[1,9]

[1]Hereditary Endocrine Cancer Group, Human Cancer Genetics Programme, Spanish National Cancer Research Centre (CNIO), Madrid 28029, Spain; [2]Histopathology Core Unit, Spanish National Cancer Research Centre (CNIO), Madrid 28029, Spain; [3]Department of Medical Oncology, Complejo Hospitalario de Navarra, Pamplona, Spain; [4]Medical Oncology Department, Hospital Clínico San Carlos, Instituto de Investigación Sanitaria del Hospital Clínico San Carlos (IdISSC), CIBERONC, Madrid 28029, Spain; [5]Department of General Medical Oncology, Leuven Cancer Institute, Leuven, Belgium; [6]Department of Medical Oncology, Hôpital Européen Georges Pompidou, AP-HP, University of Paris, Paris, France; [7]INSERM UMR1138 Centre de recherche des Cordeliers, University of Paris, Paris, France; [8]Genitourinary and Gynecological Cancer Unit, HM Hospitales-Centro Integral Oncológico HM Clara Campal, Madrid 28029, Spain; [9]Centro de Investigación Biomédica en Red de Enfermedades Raras, Valencia, Spain; [10]Department of Medical Oncology, University Hospital 12 de Octubre, Madrid 28029, Spain

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Abstract: Vascular endothelial growth factor receptor tyrosine kinase inhibitors (VEGFR-TKIs) are key antiangiogenic drugs for renal cancer treatment. While Von Hippel-Lindau dysfunction constitutes the base for VEGFR-TKIs sensitivity, the role for individual and concurrent mutations in the genes encoding for the chromatin remodelers Polybromo-1 (*PBRM1*) and Lysine Demethylase 5C (*KDM5C*) is poorly understood. Here, we analyzed the tumor mutational and expression profiles of 155 unselected clear cell RCC (ccRCC) cases treated with first-line VEGFR-TKIs and the ccRCC cases of IMmotion151 trial were used for validation. We found that concurrent *PBRM1* and *KDM5C* (*PBRM1*&*KDM5C*) mutations occurred in 4-9% of cases and were enriched in Memorial Sloan Kettering Cancer Center favorable-risk patients. In our cohort, tumors only mutated in *PBRM1* or concurrently mutated in *PBRM1* and *KDM5C* had increased angiogenesis (P=0.0068 and 0.039; respectively), and tumors only mutated in *KDM5C* showed a similar trend. Best response to VEGFR-TKIs corresponded to *PBRM1*&*KDM5C* mutated cases, followed by those mutated only in *KDM5C* or only in *PBRM1* (P=0.050, 0.040 and 0.027 versus non-mutated cases, respectively), with a trend for longer progression free survival (PFS) in the group with only *PBRM1* mutated (HR=0.64; P=0.059). Validation in the IMmotion151 trial revealed a similar correlation with increased angiogenesis and the PFS of patients in the VEGFR-TKI-arm was the longest in *PBRM1*&*KDM5C* mutated cases, intermediate for only *PBRM1* or only *KDM5C* mutated patients and the shortest in non-mutated cases (P=0.009 and 0.025, for *PBRM1*&*KDM5C* and *PBRM1* versus non-mutated cases). In conclusion, somatic *PBRM1* and *KDM5C* mutations are common in patients with metastatic ccRCC and likely cooperate increasing tumor angiogenesis and VEGFR-TKI-based antiangiogenic therapy benefit.

Keywords: Clear cell renal cell carcinoma, *PBRM1, KDM5C*, concurrent mutations, tumor angiogenesis, antiangiogenic response

Introduction

Renal cell carcinoma is among the 10 most common cancers worldwide and its incidence has been steadily increasing [1]. While the long-term survival of patients with metastatic disease remains poor, one-third of cases are diagnosed at advanced stage and about one-third of patients with localized disease relapse. Antiangiogenic treatment through vascular endothelial growth factor receptor tyrosine kinase inhibitors (VEGFR-TKIs), such as sunitinib, con-

stitute a standard therapy for metastatic renal cancer, in combination with immune checkpoint inhibitors (ICIs) or in monotherapy. Current guidelines recommend frontline treatment with a combination of a VEGFR-TKI plus a ICI or two ICIs, as they have demonstrated to extend survival compared to sunitinib alone. Even more, several novel combination schemes that include VEGFR-TKI drugs are being tested for first-line treatment [2-5]. VEGFR-TKIs are also used in monotherapy in second- or subsequent treatment lines, and the adjuvant treatment for patients at high risk of recurrence consists on VEGFR-TKIs or ICIs. Despite these diverse treatment options, renal cancer therapy response varies among individuals and expanding our knowledge on the molecular alterations that lead to increased VEGFR-TKI sensitivity will aid in designing novel treatment strategies effective for the different renal cancer molecular subsets [6].

Clear cell renal cell carcinoma (ccRCC), the most frequent renal cancer histologic subtype, is characterized by Von Hippel-Lindau (VHL) inactivation, which leads to an abnormal accumulation of hypoxia-inducible factors that promote tumor angiogenesis. This enhanced feature is variable among ccRCC tumors and it is associated with VEGFR-TKIs response [7, 8]. Genomic studies have revealed that secondary mutations in chromatin remodeler genes are frequent and critical events for ccRCC progression [9], and suggest an impact of somatic mutations in treatment response. In agreement with this notion, Polybromo-1 (*PBRM1*) mutations have been shown to be associated with increased tumor angiogenesis and are enriched in patients with favorable prognosis [10] and with improved response to antiangiogenic therapy [7, 11, 12]. Although less evidence is available for Lysine Demethylase 5C (*KDM5C*), mutations in this gene have recently been associated with high tumor angiogenesis [6] and Hsieh *et al*. found that mutations in this gene were associated with longer progression free survival (PFS) in patients with first-line sunitinib [13]. However, these preliminary data have not been validated. Furthermore, concurrent mutations in *PBRM1* and *KDM5C* are frequent [6, 12] and *in vitro* studies support convergent transcriptional effects of these different types of chromatin remodelers (*PBRM1* encodes a subunit of the SWI/SNF chromatin remodeling complex while *KDM5C* encodes for a histone lysine demethylase) [14]. While this data may suggest a synergistic effect on anti-angiogenic therapy response, the simultaneous presence of these mutations in tumors and their molecular and clinical consequences have not been studied. Here, we determine the impact of independent and concurrent *PBRM1* and *KDM5C* mutations on tumor angiogenesis, patients' prognosis, and response to VEGFR-TKI-based antiangiogenic treatment.

Materials and methods

Patients

Discovery series: Through an observational study, 155 ccRCC tumor samples (140 from 17 Spanish hospitals; 15 from CIT-rein tumor bank (Paris, France)) were collected. Most samples were primary tumors (n=148; 95%) and a minority were metastases. All the tumor samples had been collected before systemic treatment (i.e. they were treatment naïve). For inclusion in the study, patients had to be 18 years or older, had a histologically confirmed renal cell carcinoma with clear cell histology, had developed metastasis and received antiangiogenic VEGFR-TKI therapy as first-line treatment in the metastatic setting. Patients were excluded if they had received prior systemic treatment of any kind for renal cancer. Written informed consent was obtained from all patients and the study was approved by the corresponding ethical review boards and complies with the declaration of Helsinki.

Validation series: To validate the results, we analyzed data of the IMmotion151 trial (NCT02420821; n=836). We selected only patients with clear cell histology, with or without sarcomatoid component, and with available mutational data (n=693; 343 treated with sunitinib and 350 with atezolizumab/bevacizumab). Data from IMmotion150 (NCT01984242) were also analyzed (n=155 with molecular data: 51 treated with sunitinib, 51 with atezolizumab, 53 with atezolizumab/bevacizumab).

Tumor DNA sequencing and variant interpretation

DNA was isolated from 140 formalin-fixed paraffine-embedded (FFPE) and 15 fresh frozen tumor samples using Maxwell® RSC DNA FFPE

Kit (Promega) and DNeasy Blood and Tissue Kit (Qiagen), respectively. For cases in which whole exome sequencing (WES) was performed, genomic DNA was also purified from peripheral blood leucocytes using Maxwell® RSC Blood DNA Kit (Promega). WES was performed in the tumor and paired normal tissue of 23 extreme antiangiogenic responders using SureSelect Human All Exon V5 kit (Agilent). For the remaining 132 cases, DNA libraries were prepared using a capture panel (SeqCap EZ Choice Enrichment Kit; Roche) that targeted the coding region of 43 genes found frequently mutated in renal cancer tumors [15]. Sequencing was performed in a HiSeq sequencer (Illumina) configured to generate 100 bp paired-end reads. Experimental protocols and analyses have been described elsewhere [15]. WES mean coverage was 80× in tumors and 79× in matched blood samples. The mean coverage in tumor samples undergoing targeted sequencing was 388× (detailed next-generation sequencing metrics are available upon request).

For read alignment GRCh37/hg19 assembly was used as reference and Mutect2 was used for the calling of somatic variants. Somatic variants with a minor allele frequency >0.01% in gnomAD and those with a fraction of altered reads <0.15 were filtered out. Ensembl Variant Effector Predictor annotation tool was used to predict variant impact and only variants with high impact (nonsense, frameshift and start/stop loss variants) and moderate impact (missense and inframe indels variants) were considered for the analysis.

Tumor gene expression profiling

Angiogenesis-related gene expression was assessed in 93 tumors and in 8 renal normal tissue samples used as controls. Total RNA was isolated using RNeasy FFPE Kit (QIAGEN) and quantified using NanoDrop ND-1000 (Thermo Fisher Scientific). Quantification of the expression of 16 genes related with angiogenesis and hypoxia (*ANG1, ANG2, ENG, FGF2, HGF, HIF1A, HIF2A, NOTCH1, NRP2, PDGFR, PGF, VEGFA, VEGFB, VEGFC, VEGFR1, VEGFR2*) was performed using the nCounter Elements Technology (NanoString Technologies) following the manufacturer's instructions, using three housekeeping genes for normalization (*ACTB, GAPDH, HPRT1*). Oligonucleotide probe pairs were from Integrated DNA technologies (IDT). Hybridization, magnetic beads washing, hybridized complexes capture, barcodes imaging and counting was performed on the nCounter® FLEX Analysis System (NanoString Technologies). The nSolver 4.0 Analysis Software (NanoString Technologies) was used for quality control assessments and to normalize the expression data according to internal positive and negative controls and the housekeeping genes. Cluster analysis was made using Gene Cluster 3.0 software.

Treatment response and statistical analysis

For VEGFR-TKI therapy response analysis, treatment responses were classified as good (PFS>18 months; and complete response (CR), partial response (PR) or stable disease (SD) as best Response Evaluation Criteria in Solid Tumors (RECIST) response), intermediate (PFS between 18 and 6 months; and CR, PR or SD as best RECIST response) and poor (progressive disease (PD) in <6 months). Categorical variables, including mutational status, expression cluster/signature and overall VEGFR-TKI response were compared using Pearson's chi-squared test. PFS was defined as the time between the first day of VEGFR-TKI treatment and the date of radiological PD, clear clinical evidence of PD or death. Patients who had not progressed at database closure were censored at final follow-up. The Kaplan-Meier and Cox regression methods were used to estimate median survival and compare groups. Statistical analyses were conducted in SPSS version 19.0. Differences were considered significant for *P* values <0.05.

Results

Clinical and molecular characteristics of ccRCC patients

Tumors collected from 155 metastatic ccRCC patients (median age of 62 years, all treated with first-line VEGFR-TKIs, 93% sunitinib; **Table 1**) were subjected to tumor mutational and expression profiling analysis.

Tumor mutational screening, revealed that mutations in genes encoding chromatin remodelers are frequent: *PBRM1*, *SETD2*, *BAP1* and *KDM5C* were mutated in 33%, 24%, 12% and

Table 1. Characteristics of the 155 metastatic ccRCC patients included in the discovery series of the study

Characteristics	n (%)
Age at diagnosis, years	
Median [Min, Max]	62 [34-85]
Gender	
Female	48 (31)
Male	107 (69)
MSKCC prognostic risk group	
Favorable	56 (36)
Intermediate	82 (53)
Poor	8 (5)
Not available	9 (6)
Nr. of metastatic sites at VEGFR-TKI	
1	59 (38)
2	61 (39)
3	22 (14)
≤4	8 (5)
Not available	5 (3)
First-line VEGFR-TKI	
Sunitinib	144 (93)
Pazopanib	11 (7)
RECIST response	
CR	15 (10)
PR	67 (43)
SD	48 (31)
PD	15 (10)
Not available	10 (6.5)
VEGFR-TKI response classification	
Good	68 (44)
Intermediate	48 (31)
Poor	33 (21)
Not available	6 (3.9)
PFS (months)	
Median [95 CI]	21.2 [14.4-28.0]
OS (months)	
Median [95 CI]	56.8 [35.6-78.0]

10% of cases, respectively (**Figure 1A**). Concurrent mutations in *PBRM1* and *KDM5C* (*PBRM1*&*KDM5C*) occurred in 4% of our cases, with 12% of *PBRM1* and 40% of *KDM5C* mutated tumors having mutations in both genes.

Tumor gene expression profiling revealed two clusters, reflecting high and low angiogenesis (52% and 48% of cases and referred as angio-high and angio-low, respectively; **Figure 1B**). The angio-high group had an overrepresentation of *PBRM1* and *KDM5C* mutations (P=0.00084, P=0.039, respectively), while no differences were observed for other frequently mutated chromatin remodeler genes (i.e. *SETD2* and *BAP1*).

Mutations in PBRM1 and KDM5C are associated with increased tumor angiogenesis and better antiangiogenic treatment response

Tumors were classified according to *PBRM1* and *KDM5C* mutational status in 4 groups (those without mutations (WT/WT); those with only *PBRM1* mutated; those with only *KDM5C* mutated; those with concurrent mutations in *PBRM1* and *KDM5C*) and their characteristics were compared.

As shown in **Figure 2A**, tumors with only *PBRM1* mutation, with only *KDM5C* mutation, or with concurrent *PBRM1* and *KDM5C* mutations had increased angio-high features, with the differences for *PBRM1* and *PBRM1*&*KDM5C* groups compared to WT/WT group being statistically significant (*P* values 0.0068 and 0.039, respectively; **Figure 2A**). Regarding Memorial Sloan Kettering Cancer Center (MSKCC) prognostic risk groups, tumors with *PBRM1* and *KDM5C* mutations had a trend towards increased proportion of favorable-risk patients (34%, 41%, 67%, and 50% of favorable risk cases for WT/WT, *PBRM1, KDM5C* and *PBRM1*&*KDM5C* groups, respectively; **Figure 2A**).

VEGFR-TKI treatment response, evaluated by an endpoint combining RECIST response and PFS, was compared among the four groups of patients, finding the best response in *PBRM1*&*KDM5C* mutated cases, followed by those with *KDM5C* and *PBRM1* mutations (P=0.050, 0.040 and 0.027, respectively, for comparisons with WT/WT cases). Similarly, the median PFS was longer in patients with mutated tumors, although differences were not statistically significant (HR=0.64 and P=0.059 for *PBRM1* mutated cases; **Figure 2B**).

Validation of PBRM1 and KDM5C associations in IMmotion151 trial

Results were validated in the ccRCC cases of the IMmotion151 trial, in which 9% of patients corresponded to the *PBRM1*&*KDM5C* group. In this case, 19% of *PBRM1* and 62% of *KDM5C* mutated tumors had mutations in both genes.

Consistent with the findings in our series, high angiogenesis was more frequent in *PBRM1* and

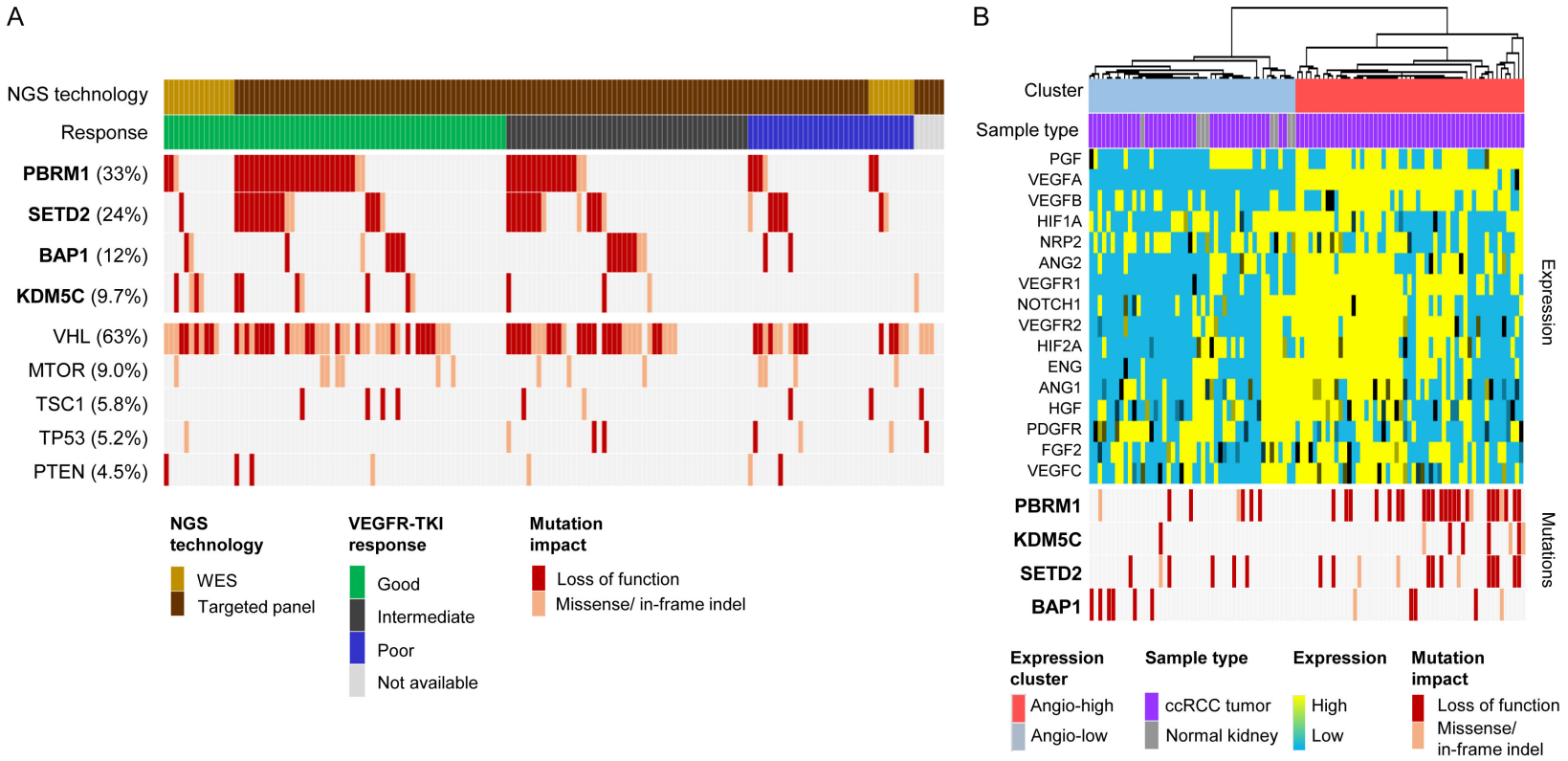

Figure 1. Molecular features of ccRCC tumors. A. Mutational status of chromatin remodelers (*PBRM1*, *SETD2*, *BAP1* and *KDM5C*) and other genes with a mutation frequency ≥4.5% in 155 ccRCC tumors (columns). For each gene (rows), mutations with high impact (loss of function) are shown in dark red and those with moderate impact (missense and inframe indels) are shown in light red. The overall mutation frequency of each gene is shown between brackets. The next generation sequencing (NGS) technology used and the VEGFR-TKI response group are shown in the upper two rows of the graph. B. Heatmap showing the expression of 16 angiogenesis-related genes (rows) in 93 ccRCC tumors (purple) and 8 normal kidney tissue samples (grey). Normalized counts were z-score transformed before visualization. Angio-high and angio-low clusters are shown in red and blue, respectively. The mutational status of *PBRM1*, *KDM5C*, *BAP1* and *SETD2* genes is shown in the last four rows.

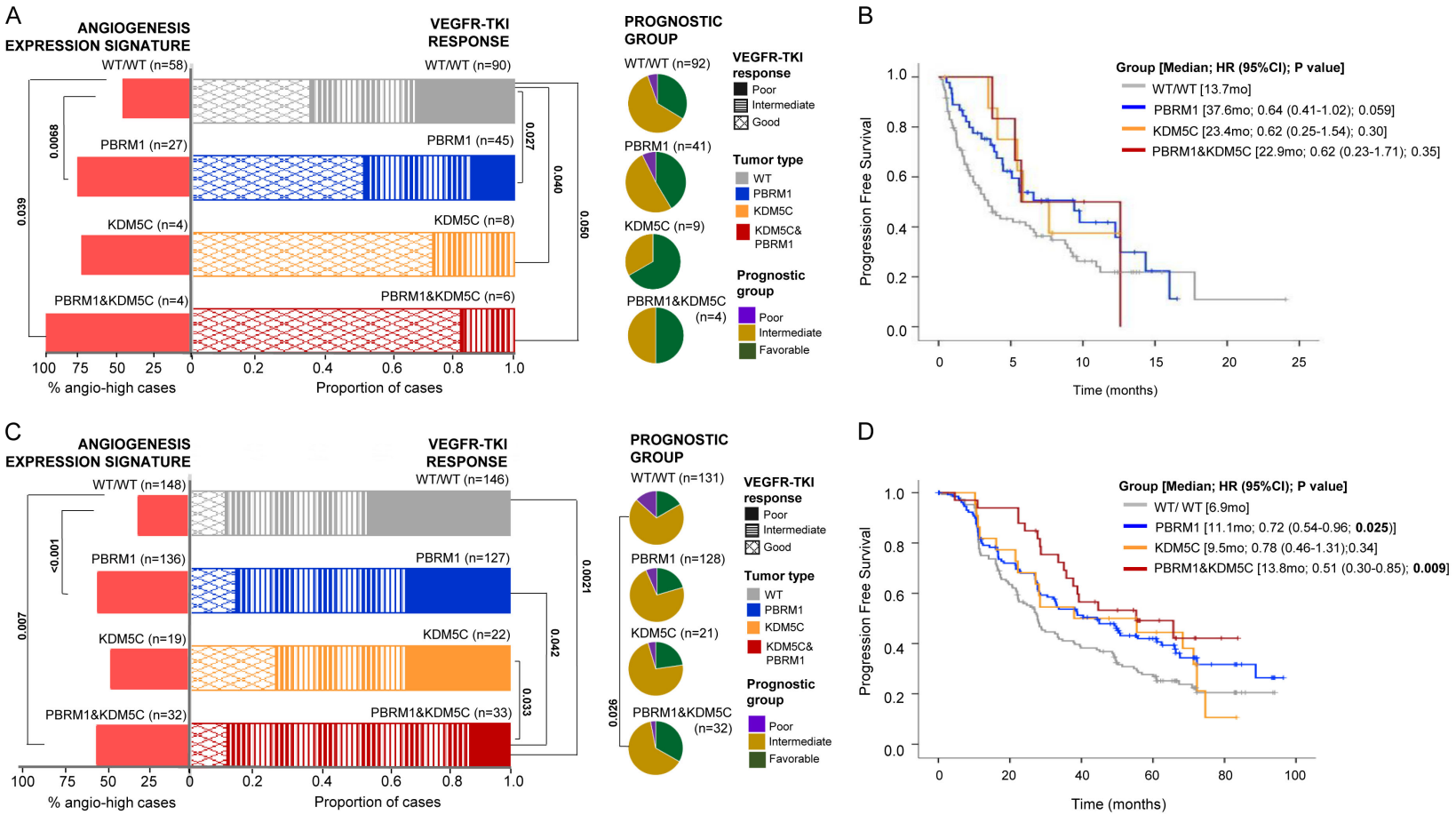

Figure 2. Association between mutations in *PBRM1* and *KDM5C* and tumor features and VEGFR-TKI response. The results corresponding to the discovery series (n=155) are shown as bar and pie charts (A) and Kaplan-Meier curves (B). Results corresponding to the validation series, which consists of IMmotion151 ccRCC patients treated with sunitinib (n=343), are shown as bar and pie charts (C) and Kaplan-Meier curves (D). Red bar charts on the left depict the proportion of cases with a high-angiogenesis (angio-high) expression signature. Colored bar charts on the right show the patients' VEGFR-TKI treatment response stratified by the mutational status of *PBRM1* and *KDM5C* (poor, intermediate and good response to first-line VEGFR-TKI treatment are shown in solid, stripped and rhomboid colors, respectively). The numbers of patients are shown between brackets. *P* values obtained from Pearson's chi-square test comparing poor and good responses in *PBRM1*, *KDM5C* and *PBRM1&KDM5C* mutated groups, are shown. Pie charts show the frequency of the different prognostic groups according to the mutational status of *PBRM1* and *KDM5C* (poor, intermediate and favorable prognostic groups are shown in purple, yellow and green, respectively). Kaplan-Meier plots show the PFS of patients according to *PBRM1* and *KDM5C* mutational status. Median PFS is shown between brackets. Hazard ratios and *P* values obtained from comparing *PBRM1* or/and *KDM5C* mutated groups with double wild type (WT/WT) cases are shown between brackets. HR, hazard ratio; 95% CI, 95% confidence interval.

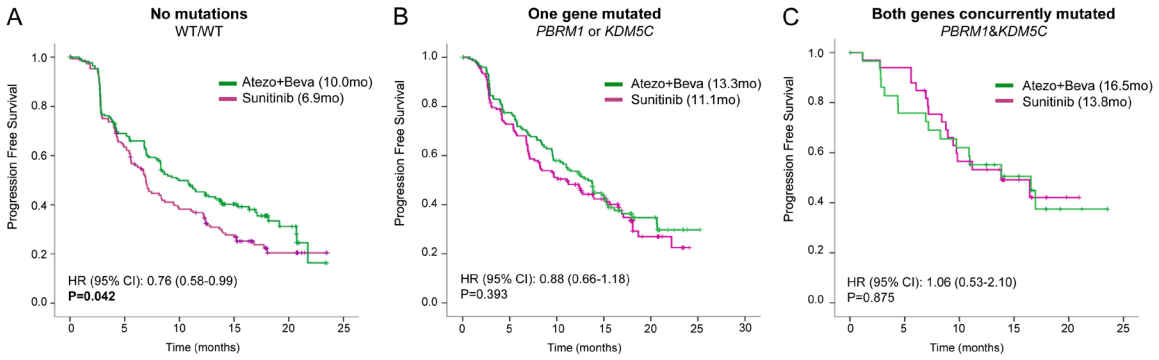

Figure 3. IMmotion151 trial treatment outcome according to *PBRM1* and *KDM5C* mutations. The Kaplan-Meier plots show the PFS of the IMmotion151 trial ccRCC patients (n=343 sunitinib; n=350 atezolizumab plus bevacizumab) grouped by the mutational status of *PBRM1* and *KDM5C*. Patients with no mutations in *PBRM1* or *KDM5C* (WT/WT) (A), patients with only one gene mutated (*PBRM1* or *KDM5C)* (B) and patients with both genes mutated (*PBRM1&KDM5C*) (C) are shown Sunitinib arm is shown in purple; atezolizumab plus bevacizumab arm (Atezo+Beva) is shown in green. In the Kaplan-Meier graphs, the median survival time is shown between brackets.

KDM5C mutated tumors compared to tumors without mutations (P<0.001 and P=0.007, for *PBRM1* and *PBRM1&KDM5C* groups, respectively; **Figure 2C**) and favorable prognostic-risk was more frequent in cases with concurrent mutations than in the WT/WT group (P=0.026). In VEGFR-TKI (sunitinib) treatment arm, those with *PBRM1&KDM5C* mutations again displayed the best response (P=0.0021 compared with WT/WT, **Figure 2C**).

Regarding PFS, the longest corresponded to patients with *PBRM1&KDM5C* mutated tumors, it was intermediate when tumors had only one gene mutated (*PBRM1* or *KDM5C*), and the shortest corresponded to patients with WT/WT tumors (13.8, 11.1, 9.5 and 6.9 months, respectively; **Figure 2D**). The IMmotion150 trial showed similar trends and *PBRM1&KDM5C* mutated patients had a better response than WT/WT patients (P=0.009; data not shown).

Antiangiogenic versus ICI plus antiangiogenic combination in IMmotion151 trial

As shown in **Figure 3**, the PFS of WT/WT patients was longer in the ICI (atezolizumab) plus antiangiogenic (bevacizumab) combination arm, than in the antiangiogenic (sunitinib) arm (10.0 versus 6.9 months; P=0.042; **Figure 3A**). When the same comparison was performed in the group of patients with only one gene mutated (either *PBRM1* or *KDM5C*) and in those with both genes mutated (*PBRM1&KDM5C*), no statistically significant differences were observed in PFS.

Discussion

Several renal cancer clinical trials have shown that the combination of an ICI plus a VEGFR-TKI improves PFS when compared to a VEGFR-TKI alone, even in favorable-risk patients [2-5]. Also, the combination of two ICIs leads to improved overall survival compared to sunitinib in intermediate and poor-risk patients [16]. VEGFR-TKIs are also used in second and posterior lines of treatment and in the adjuvant setting. However, response variability among patients is large and there is a need of molecular knowledge, which could be used to guide future therapeutic strategies.

After *VHL* loss, the mutation of genes encoding chromatin remodelers is a key event in the development of renal tumors with clear cell histology. *PBRM1* loss has been shown to amplify hypoxia-inducible factor response in ccRCC models [17] and, thus, modify tumor microenvironment. *PBRM1* mutations have recently been associated with tumors with increased vascularization and a better response to antiangiogenic drugs [6, 11, 12]. These mutations also modulate the recruitment of T-effector cells [18], however, the connection between *PBRM1* loss and ICI response is complex, with some studies finding improved response [19, 20], but not others [8, 21]. Even increased ICI resistance was recently reported in a *Pbrm1* knockout murine model [22]. In addition, mutations in some chromatin remodelers are not independent events and while mutations in *PBRM1* and *BAP1* tend to be mutually exclusive [23], there

is co-occurrence for *PBRM1* and *KDM5C* mutations (IMmotion151 both arms, P=0.002). TRACERx project in ccRCC [9] indicates that *PBRM1* mutations are associated with branched tumors with larger intratumor heterogeneity. Regarding *PBRM1* and *KDM5C* mutations, this study shows that in most tumors both mutations occur together, rather than in different subclones of the primary tumor.

Determining the molecular and clinical features associated with *KDM5C* mutation requires the subgrouping of tumors, to separate those that are only mutated in *KDM5C* from those that have concurrent mutations with *PBRM1* (about half). Thus, while *KDM5C* mutated tumors were described to be enriched in the high angiogenesis cluster [6], this initial analysis failed to differentiate *KDM5C* and *PBRM1* single and concurrently mutated cases. Our analyses investigating the two genes separately, confirm an association of *KDM5C* with higher tumor vasculature. It is important to stress that, *PBRM1* and *KDM5C* encode epigenetic modifiers with different modes of action. *PBRM1* gene encodes BAF180 protein, a component of the PBAF form of the SWI/SNF chromatin remodeling complex, which mediates chromatin accessibility through modification of nucleosome positioning. Regarding *KDM5C* (also known as *JARID1C*), it encodes a histone demethylase that removes di- and tri-methylation of histone H3 lysine 4 (H3K4me2/3). Thus, the concurrent inactivation of *PBRM1* and *KDM5C* in the tumor cells suggests a functional interaction. This is supported by our results, in which *PBRM1*&*KDM5C* mutated cases tend to have stronger effects in terms of tumor vasculature, prognostic risk group enrichment, and antiangiogenic response.

In the IMmotion151 ccRCC cases, we only found statistically significant differences in PFS between VEGFR-TKI alone (sunitinib) versus ICI plus antiangiogenic (atezolizumab plus bevacizumab) treatment arms in WT/WT patients, and not in *PBRM1* or/and *KDM5C* mutated cases. Likewise, in the IMmotion150 trial, only patients with low-angiogenesis tumors showed better response in the ICI plus antiangiogenic combination arm versus a VEGFR-TKI alone, and no differences between treatment arms were observed for patients with angio-high tumors [8]. Moreover, *PBRM1* mutated cases displayed better outcomes on atezolizumab-bevacizumab or sunitinib alone compared to atezolizumab monotherapy. Thus, the results obtained in our study support that patients with *PBRM1* and *KDM5C* mutations, and especially those harboring concurrent mutations, can possibly benefit more from therapies incorporating a VEGFR-TKI than from two ICIs combined. However, these results are based on retrospective analyses and prospective and mechanistic studies investigating the role of single and concurrent *PBRM1* and *KDM5C* mutations as tumor microenvironment modulators are needed.

In summary, *PBRM1* and *KDM5C* concurrent mutations occur in a substantial number of ccRCC tumors, and define a subgroup of patients with increased tumor angiogenesis, associated with favorable prognosis and in which a synergistic effect between these mutations likely increase antiangiogenic therapy benefit. Future investigations are warranted to further characterize and validate these results.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Cristina Rodriguez-Antona, Hereditary Endocrine Cancer Group, Spanish National Cancer Research Centre (CNIO), Melchor Fernández Almagro Street 3, Madrid 28029, Spain. Tel: +34 917328000; E-mail: crodriguez@cnio.es

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