

Association of AXL and PD-L1 Expression with Clinical Outcomes in Patients with Advanced Renal Cell Carcinoma Treated with PD-1 Blockade

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Stéphane Terry, Cécile Dalban, Nathalie Rioux-Leclercq, Julien Adam, Maxime Meylan, et al.. Association of AXL and PD-L1 Expression with Clinical Outcomes in Patients with Advanced Renal Cell Carcinoma Treated with PD-1 Blockade. Clinical Cancer Research, 2021, 27 (24), pp.6749-60. 10.1158/1078-0432.CCR-21-0972 . hal-03477307

HAL Id: hal-03477307 https://hal.science/hal-03477307

Submitted on 7 Jan2022

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Association of AXL and PD-L1 expression with 1 clinical outcomes in Patients with 2 advanced Renal Cell Carcinomas treated 3 4 with PD-1 blockade 5 Stéphane Terry^{1*}, Cécile Dalban², Nathalie Rioux-Leclercq³, Julien Adam¹, Maxime 6 Meylan⁴, Stéphanie Buart¹, Antoine Bougouin⁴, Alexandra Lespagnol⁵, Frédéric 7 Dugay⁶, Irelka Colina Moreno⁴, Guillaume Lacroix⁴, James B. Lorens⁷, Gro Gausdal⁸, 8 Wolf H. Fridman⁴, Fathia Mami-Chouaib¹, Nathalie Chaput^{9,10,11}, Benoit Beuselinck¹², 9 Sylvie Chabaud², Janice Barros Monteiro¹³, Yann Vano^{4,14}, Bernard Escudier^{1,15}, 10 Catherine Sautès-Fridman⁴, Laurence Albiges^{1,15}, and Salem Chouaib^{1,16*} 11 12 13 ¹INSERM UMR 1186, Integrative Tumour Immunology and Immunotherapy, Gustave Roussy, Fac. de 14 Médecine - Université Paris-Sud, Université Paris-Saclay, 94805 Villejuif, France 15 16 ²Clinical research department - Biostatistics unit, Centre de lutte contre le cancer - Centre Léon 17 Bérard, 69008 Lyon, France 18 19 ³Service Anatomie et Cytologie Pathologiques, CHU Rennes, Univ Rennes, Inserm, EHESP, Irset 20 21 22 (Institut de recherche en santé, environnement et travail), UMR_S 1085, 35000 Rennes, France ⁴Centre de Recherche des Cordeliers, INSERM, Sorbonne Université, Université de Paris, Equipe 23 inflammation, complément et cancer, 75006 Paris, France. 24 25 ⁵Service de Génétique Moléculaire, CHU Rennes, 35000 Rennes, France 26 27 ⁶Service de Cytogénétique, CHU Rennes, Univ Rennes, Inserm, EHESP, Irset (Institut de recherche 28 en santé, environnement et travail) - UMR S 1085, 35000 Rennes, France 29 30 31 32 33 ⁷Department of Biomedicine, Centre for Cancer Biomarkers, Norwegian Centre of Excellence, University of Bergen, Bergen, Norway.

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61 Running Title: AXL and PD-L1 in resistance to Nivolumab in ccRCC

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63 Keywords: clear-cell renal cell carcinoma; immunotherapy; AXL; PD-L1; anti-PD64 1;VHL

66 **Word count** (Introduction, results, discussion): 3876 (Method Section): 1706

68 Total number of figures: 5

- 69 Total number of Tables:1
- 70

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- 71 Contains supplementary material
- 72

7374 References: 50

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77 **Disclosure statement:**

78 L. Albiges reports grants from BMS during the conduct of the study; other support 79 from Pfizer, Novartis, BMS, IPSEN, Astellas, MSD, Merck & Co, Janssen, Astra 80 Zeneca, EISAI outside the submitted work. Y. Vano reports personal fees from Bristol Myers Squibb, MSD, Novartis, Ipsen, Janssen, Roche, Sanofi, Astellas, Merck 81 82 outside the submitted work. G. Gausdal reports to be an employee at BerGenBio 83 ASA. J. Lorens reports personal fees from BerGenBio ASA, outside the submitted 84 work; in addition, J. Lorens has a patent or patent application pending to BerGenBio ASA. J. Adam reports personal fees from MSD and AstraZeneca outside the 85 submitted work. N. Chaput reports support from AstraZeneca, BMS, GSK, Roche, 86 87 Sanofi, Cytune Pharma, Gilead, outside the submitted work. N. Rioux-Leclercq reports personal fees from Ipsen and BMS outside the submitted work. B. Beuselinck 88 reports grants from BMS and UNICANCER during the conduct of the study; other 89 90 support from BMS, MSD, Astra Zeneca, Ipsen, and Merck outside the submitted 91 work. No potential conflicts of interest were disclosed by the other authors.

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95 Translational relance:

96 This is the first study to demonstrate in a sizable cohort of cases that high tumor AXL 97 expression may affect outcomes of patients with advanced clear-cell renal cell 98 carcinoma treated with anti-PD1 therapy. This study also reveals AXL expression is 99 associated with tumor PD-L1 expression and that co-occurrence of PD-L1 positivity 100 and high AXL expression coincides with reduced overall survival in patients treated 101 with PD-1 blockade. This finding may aid in the selection of treatment for advanced 102 RCC patients.

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112 Abstract

113 A minority of patients currently respond to single agent immune checkpoint blockade 114 (ICB) and strategies to increase response rates are urgently needed. AXL is receptor 115 tyrosine kinase commonly associated with drug-resistance and poor prognosis in 116 many cancer types including in clear-cell renal cell carcinoma (ccRCC). Recent 117 experimental cues in breast, pancreatic and lung cancer models have linked AXL 118 with immune suppression and resistance to antitumor immunity. However, its role in 119 intrinsic and acquired resistance to ICB remains largely unexplored. In this study, 120 tumoral expression of AXL was examined in ccRCC specimens from 316 metastatic 121 patients receiving PD-1 inhibitor, nivolumab, in the GETUG AFU 26 NIVOREN trial 122 after failure of anti-angiogenic therapy. We assessed associations between AXL and 123 patient outcomes following PD-1 blockade, as well as the relationship with various 124 markers including PD-L1, VEGFA, the immune markers CD3, CD8, CD163, CD20, 125 and the mutational status of the tumor suppressor gene VHL. Our results show that 126 high AXL expression levels in tumor cells is associated with lower response rates and a trend to shorter progression-free survival following anti-PD-1 treatment. AXL 127 128 expression was strongly associated with tumor PD-L1 expression, especially in 129 tumors with VHL inactivation. Moreover, patients with tumors displaying concomitant 130 PD-L1 expression and high AXL expression had the worst overall survival. Our 131 findings propose AXL as candidate factor of resistance to PD-1 blockade, and 132 provide compelling support for screening both AXL and PD-L1 expression in the 133 management of advanced ccRCC.

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INTRODUCTION

137 Kidney cancer is responsible for about 175000 deaths each year [1]. The most 138 common type of kidney cancer is clear-cell renal cell carcinoma (ccRCC) accounting 139 for 70%-80% of cases. Treatment with immune checkpoint blockade (ICB) has 140 resulted in striking clinical benefits and changes in the management of patients with 141 advanced ccRCC since anti-PD1 (nivolumab) therapy has been approved as second-142 line treatment for recurrent metastatic diseases, as well as the anti-PD-1 (nivolumab) 143 plus anti-CTLA-4 (ipilimumab) combination approved as first-line treatment for 144 patients with intermediate and high risk metastatic RCC followed by the combination 145 of PD-1 inhibitor with VEGFR TKIs in any risk group in front line [2-5]. Cell surface 146 receptor PD-1 is especially expressed by T lymphocytes, which can prevent T cells 147 from killing tumor target cells upon binding to its ligands PD-L1 and PD-L2, 148 dampening the immune response. Therefore, PD-1 blockade using specific 149 neutralizing antibodies is able to inhibit interaction of PD-1 with its ligands, which may 150 at least partially restore their activity and boost antitumor T-cell immunity. 151 Nevertheless, only a small proportion of patients benefits from these treatments as 152 shown by the low response rate (RR) on one hand, and high rates of disease 153 progression on the other hand, even in initial responders. This illustrates primary and 154 acquired resistances to PD-1 blockade. In an era of progress where anti-PD-1-based 155 combination approaches are expected to increase RR and prolong treatment efficacy 156 [6,7], it is urgent to better define the underlying biology of resistance and response to 157 PD-1 blockade. Thus, new biomarkers and targets that can be easily and routinely 158 inspected in pathological sections might facilitate treatment decisions and give new 159 therapeutic options for metastatic ccRCC (m-ccRCC).

160 Notably, ccRCC has specific features: 1) In contrast to most tumor types, ccRCC can

161 be highly infiltrated by CD8+ T cells, but this infiltration is often associated with poor 162 prognosis [8]: 2) RCC has relatively low mutational load and this parameter does not 163 appear to be a good predictor of response to PD-1 blockade [5]; 3) controversies also 164 exist regarding the utility of PD-L1 expression to predict response. Indeed, PD-L1 165 expression is associated with poor prognosis in RCC, but its value to predict 166 response to PD-1 blockade is still a matter of debate [5,9,10]. Furthermore, genetic 167 alterations in PBRM1 and BAP1 genes as well as focal loss in chromosome 168 10q23.31 have demonstrated interesting properties to estimate patients who are 169 likely to benefit from ICB [11,12]. The von Hippel-Lindau (VHL) tumor suppressor 170 gene is also altered in a majority of RCC cases. Defects in VHL gene include 171 germline or somatic mutations, loss of heterozygosity through chromosomal loss of 172 3p and promoter hypermethylation [13]. It is often considered that two "hits" must 173 occur to inactivate VHL. Thus, a combination of these alterations can cause bi-allelic 174 inactivation of VHL in 60-90% of RCC cases leading to a complete loss-of-function 175 (LOF) of VHL gene in some cases, whereas in other cases, such function likely 176 remains intact. VHL LOF in tumor cells results in chronic accumulation of hypoxia-177 inducible factors (HIFs) in the cells entering a pseudohypoxic state likely contributing 178 to tumorigenesis, angiogenesis and changes in the tumor microenvironment (TME), 179 in particular via induction of the HIF-target gene, VEGFA. Recent studies reported 180 clinical benefits for combination of VEGFR TKIs with ICB [5]. However, the impact of 181 the VHL/HIF/VEGF pathway, beyond anti-angiogenic therapy, in regulating refractory 182 responses to ICB remains unclear.

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AXL is member of the TAM (TYRO3-AXL-MER) family of receptor tyrosine kinases, which can regulate tumor cell survival, proliferation, migration, angiogenesis, and interactions with TME [14]. AXL has been associated with drug-resistance and poor prognosis in many cancer types including in RCC [14]. Notably, AXL expression In human RCC has been associated with high risk of relapse after surgery [15], worse clinical outcome in metastatic patients treated with anti-angiogenic agents, as well as epithelial-mesenchymal transition (EMT) features and fibrosis [16]. Recent evidence in breast, pancreatic and lung cancer models have also linked AXL with immune suppression and resistance to antitumor immunity [17–20]. Yet, the role of AXL in resistance to anti-PD1 therapy remains unknown.

In this study, we aimed to elucidate the potential impact of AXL expression on clinical
outcomes of patients with advanced RCC following anti-PD1 treatment.

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Materials and Methods

200 **Patients and samples**

201 The renal cancer samples have been collected as part of the NIVOREN GETUG-AFU 202 26 phase II trial assessing the activity and safety of nivolumab (anti-PD1) in 729 203 patients with m-ccRCC who failed vascular endothelial growth factor-directed 204 therapies (ClinicalTrials.gov identifier: NCT03013335). This trial was conducted by 205 UNICANCER, approved by the human research ethics committee of UNICANCER 206 and conducted in accordance with guidelines from the International Conference on 207 Harmonization and the Declaration of Helsinki. Patients were enrolled across 27 208 institutions between February 12, 2016 and July 27, 2017. Safety activity data was 209 reported previously [21]. The follow-up was censored on January 2019.

As part of a biomarker cohort, 324 archived formalin-fixed paraffin-embedded (FFPE) tumor tissue specimens were retrospectively analyzed by IHC for immune cell profiling (CD3, CD8, PD-L1, CD20, CD163, VEGFA, and AXL). Specimens were mostly obtained prior anti-angiogenic therapy as 1st or 2nd line regimens (comprising

Sunitinib (83.3%), Axitinib (27.5%), Pazopanib (23.8%), Sorafenib (6.5%),
Bevacizumab (3.4%), Cabozantinib (0.3%)), or mTOR inhibition (Everolimus 11%).
Cases with paraffin block showing less than 40% of tumor or more than 40% of
necrosis, or corresponding to another histological subtype (TFE3 translocation RCC,
chromophobe RCC or papillary RCC) were excluded.

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220 Alterations in VHL gene

221 VHL inactivation status was determined by PCR, FISH and promoter methylation 222 analysis. Evaluation of VHL point mutations was evaluated by gene sequencing [22]. 223 The three exons and exon-intron junctions of the VHL gene were sequenced on 224 DNA extracted from tumor samples, as described previously [23]. Search for deletion 225 of the VHL genomic region was performed by FISH using ZytoLight ® SPEC 226 VHL/CEN3 Dual Color Probe (Zytovision, Germany) reviewed by FD. Promoter 227 hypermethylation of VHL was assessed by methylation-specific multiplex ligation-228 dependent probe amplification as described [23]. Presumably two hits are needed for 229 VHL inactivation to occur. Therefore, tumors were classified in two groups. When 230 biallelic alteration was evidenced, the case was classified in a group with VHL 231 inactivation, also referred to as VHL-/-. Another group consisted of non-inactivated 232 cases, with no alteration or one allelic alteration, referred to as VHL +/+; +/-. A few 233 cases were excluded from this analysis when biallelic or monoallelic alterations could 234 not be ascertained because of non-interpretable results, or when the mutation 235 detected was simply defined as possibly damaging with no clear loss of VHL function. 236

237 mRNA extraction and analysis of RNA-Seq datasets from RCC patients

238 RNA-seq data were available for 83 FFPE cases of the Nivoren cohort [24]. Briefly, 239 archived FFPE samples were retrieved before anti-PD-1 treatment, and upon review 240 of H&E slides, the tissue blocks with the highest tumoral content were selected for 241 further processing. Blank slides were produced with 5 µm thickness and 242 macrodissected to only include tumoral tissue, using a total surface area of 50-1800 243 mm². RNA was extracted using the Maxwell RSC RNA FFPE kit (Promega) 244 according to the manufacturer's instructions. Subsequently cDNA libraries were 245 prepared using the Forward QuantSeg 3' mRNA-Seg Library Prep Kit for Illumina 246 (Lexogen) according to the manufacturer's instructions with 5 µL of RNA as input and 247 16 PCR cycles. cDNA concentrations and fragment length were measured with the 248 QubitTM dsDNA HS assay (Thermofisher) and Bioanalyzer HS DNA electrophoresis 249 (Agilent). Illumina cBOT was used for clonal cluster generation and RNAseq was 250 performed using the HiSeq 4000 kit (Illumina) according to the manufacturer's 251 instructions. For subsequent survival analysis, samples were classified as AXL high 252 (defined by AXL mRNA expression \geq highest 1/3) or AXL Low (defined by AXL) 253 mRNA expression \leq lowest 2/3) in the dataset. Progression Free Survival (PFS) was 254 estimated using the Kaplan-Meier method to explore the impact of AXL the different 255 subsets. For association studies, VHL status was determined as above. AXL 256 immunostaining also served to classify tumors as AXL^{high} or AXL^{neg/low}, and in this 257 case, RNA-seq data was interrogated to evaluate changes in the expression of 258 known gene expression signatures [25-27].

259 CheckMate RNA-Seq data, clinical, and genomic information were interrogated from 260 the Braun et al. study [11] for 181 advanced ccRCC treated with anti-PD1 in the 261 CheckMate 009, 010 and CheckMate 025. The CheckMate cohorts consist mostly of 262 previously treated anti-angiogenic-refractory patients. Experimental details including

263 whole-exome and RNA sequencing analyses have been reported previously [11]. 264 Among the samples with both RNA and genomic information, 93% exhibited loss of 265 chromosome 3p carrying VHL gene, and 64% were reported with a point mutation in 266 VHL. Cases with at least one WT copy of VHL were considered as non-inactivated 267 VHL cases, whereas cases with at least two alterations were considered as 268 inactivated. Samples were classified as above with AXL^{high} (defined by AXL mRNA 269 expression \geq highest 1/3) and AXL^{low} cases (defined by AXL mRNA expression \leq 270 lowest 2/3). PFS and OS estimates using the Kaplan-Meier method were generated 271 to explore the impact of AXL in all patients (n=181), VHL-inactivated (n=78), or VHL-272 non-inactivated (n=44) subgroups.

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274 Immunohistochemistry

275 All tumor specimens were centrally reviewed by a genitourinary pathologist (NRL). 276 For each case, one representative archived FFPE tissue block was used for IHC. 277 Primary antibodies to stain for PD-L1 (clone 22C3, Dako), CD8 (clone C8/144B, 278 Dako), VEGF (clone SP28, Spring Bioscience Corp), CD163 (clone 10D6 Diagnostic 279 BioSystem), CD20 (clone L26, Agilent), CD3 (polyclonal rabbit antibody, Agilent) 280 were used according to standard protocols. For PD-L1, AXL, CD8, and VEGF, slides 281 were independently evaluated by a genitourinary pathologist (JA, NRL) blinded to the 282 patients' treatment outcomes. For AXL IHC assessment, epitope retrieval was 283 performed in a citrate buffer pH 7.3 (Diapath) during 30 min in a water bath set to 284 98°C. Endogenous peroxidase activity was inactivated with 3% hydrogen peroxide 285 for 10 minutes. Blocking was performed with IHC/ISH Super Blocking (Leica 286 biosystems) for one hour at 37°C. The primary antibody (clone C89E7, Cell Signaling 287 Technology), was incubated overnight. Omission of the primary antibody was used

as negative control. A multiplicative H-score (0-300) was calculated as the product of intensity scores and the percentage of stained tumor cells. Representative stainings are shown in **Supplementary Fig. S1**. To note, AXL staining could also be detected in various non-tumor cells, in particular immune cells with morphology evocative of myeloid cells (i.e. monocytes/macrophages/DC) (**Supplementary Fig. S1**). A score of 0-3 was given based on the abundance of AXL⁺ immune cells.

294 Patients were considered with Negative (H-score 0), Low (>0 <=50), or High (>50) 295 AXL tumor expression. H-score > 50 was used as cutoff to discriminate between 296 AXL^{high} and AXL^{neg/low} expression. For PD-L1, VEGF and CD8, stainings were 297 performed using a Discovery XT research instrument (Roche) using standardized 298 protocols. For PD-L1 (anti-PD-L1, clone 22C3, Dako). The % of positive tumor cells 299 was evaluated. $\% \ge 1$ was used as cutoff to discriminate between PD-L1 TC ^{neg} and 300 PD-L1 TC⁺ expression. For assessment of PDL1 staining in TILs, a manual semi 301 quantitative 3-point scale was employed with score 0 (no PDL1⁺ TILs), score 1 (rare 302 and focal PDL1⁺ TILs), score 2 (several foci of PDL1⁺ TILS), score 3 (diffuse and 303 numerous PDL1⁺ TILs). For subsequent analysis, the cutoff \geq 1 was used to discriminate PD-L1^{neg} and PD-L1⁺ in TILs. CD8 positivity (anti-CD8, clone C8/144B, 304 305 Dako) in TILs was scored according to the proposal of the International 306 ImmunoOncology Biomarkers Working Group [28], using a manual semi quantitative 307 four point scale : score 0 (no CD8⁺ TILs or very rare CD8+ TILS), score 1 (rare 308 diffuse or focal CD8⁺ TILs), score 2 (diffuse numerous CD8⁺ TILs), and score 3 309 (diffuse and numerous CD8⁺ TILs with some aggregates of TILs). Anti-VEGF (clone 310 SP28, Spring Bioscience Corp) was used and the % of positive tumor cells assessed. 311 For CD3 (polyclonal rabbit antibody, Agilent), CD20 (clone L26, Agilent), and CD163 312 (clone 10D6 Diagnostic BioSystem) immunostaining, tissues were deparaffinized and

313 rehydrated by successive baths of Clearene (Leica Biosystems, 3803600E) and 314 ethanol gradient (100%, 90%, 70% and 50%). Antigen retrieval was performed with a 315 PT-link (Dako) at pH 6.1 (EnVision FLEX Target Retrieval Solution, Low pH 50x 316 concentrated Citrate buffer, Dako, K8005) by an incubation of 30 minutes at 97°C. 15 317 minutes of Endogenous Peroxidase block (H2O2 3% Gifrer, 1060351) and 10 318 minutes of Protein Block (Dako, X0909) reagents were used for alkaline phosphatase 319 and FcR blocking. The primary and secondary antibodies were incubated for 30 320 minutes respectively. All these markers were processed on an automated 321 immunostainer (AutostainerPlusLink 48, Dako). Secondary antibodies were revealed 322 by Permanent HRP Green (Zytomed Systems, ZUC070-100) for CD3 (10 minutes), 323 High-Def red IHC chromogen (AP) (Enzo, ADI-950-140-0030) for CD20 (5 minutes) 324 and AEC Substrate Kit, Peroxidase HRP: 3-amino-9-ethylcarbazole (Vector 325 Laboratories, SK-4200) for CD163 (23 minutes). The nuclei were counterstained with 326 hematoxylin (Dako, S3301) during 5-7 minutes and slides were scanned with a 327 Nanozoomer (Hamamatsu) after mounting with Ecomount (Biocare, EM897L). For 328 CD20 and CD3, and glycergel Mounting Medium (Dako, C056330-2) for CD163. The 329 density of positive cells/mm² was quantified in the Tumor Core (TC) by two 330 independent analysts (AB, GL) using Halo10 software (Indica labs).

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332 Statistical analysis

333 Qualitative data are reported by frequency and proportion. The Chi-square and 334 Fisher's exact tests were used to identify associations between biomarkers, status, or 335 groups as categorical variables. Median follow-up is calculated using the reverse 336 Kaplan-Meier method and presented associated with its 95% Cl. PFS was defined as 337 the time between treatment initiation and first progression or death, and Overall

Survival (OS) was defined as the time between treatment initiation and death or date of final contact for patients alive. OS and PFS were estimated using the Kaplan-Meier method and were described in terms of median or specific time point estimation in each subgroup, along with the associated two-sided 95% CI for the estimates and were compared with the log-rank test. A multivariate cox regression model was used to estimate the hazard ratio and 95% CI and adjusted for the following baseline characteristics: International Metastatic Renal Cell Carcinoma Database Consortium (IMDC) risk groups, age, sex and number of previous systemic therapies. Objective Response Rate (ORR) as per central radiology review using Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 and previously described [21]. Quantitative data are reported by means and range. Non-parametric Mann-Whitney U or Kruskal-Wallis test were applied. A value of p<0.05 was considered statistically significant, and all p values were two-sided. Data analyses were carried out using GraphPad (GraphPad Prism), Excel (Microsoft Corp) and SAS 9.4 (SAS Institute Inc, Cary, NC).

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Results

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368 AXL expression is associated with worse PFS and resistance to anti-PD-1 369 therapy

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AXL tumor cell staining was detectable in 54.4% of cases (172/316) and AXL 371 372 expression was considered with high expression (H-score >50) in 23.7% of cases (75/316). Population characteristics of patients with AXL^{high} vs AXL^{neg/low} tumors were 373 374 comparable in terms of gender, age at diagnosis, metastatic features, radiotherapy, 375 IMDC (International Metastatic RCC Database Consortium), or Eastern Cooperative 376 Oncology Group (ECOG) Performance Status (Table 1). Median follow-up was 23.5 377 months (95% CI, 22.6 to 24.0) and median PFS was 4.5 months (95% CI, 2.9 to 5.2). 378 ORR was 25% for the evaluable cases (78/312). In the AXL^{high} group of patients (H-379 score > 50), median PFS was shorter (2.8 months (95% CI, 2.5 to 4.8)) than in the 380 AXL^{neg/low} group (4.6 months (95% CI, 3.5 to 5.3); p=0.11). Hazard ratio (HR) for 381 disease progression was 1.26 (95%CI, 0.95 to 1.68), and 12-month PFS rates were 21.3% (12.9-31.2) for AXL^{high} compared to 28.6% (23.0-34.4) for the AXL^{neg/low} group 382 383 (Fig. 1A). On multivariate analysis adjusting for age, sex, IMDC and number of 384 previous therapy lines (2 vs >2), high AXL expression was associated with a trend 385 toward reduced PFS (HR = 1.28 (95%CI, 0.96 to 1.71), p=0.098 (Supplementary 386 Table S1). The ORR in patients with AXL^{high} tumors was 15.5% (11/71) vs 27.8% (65/234) in patients with AXL^{neg/low} tumors, indicating an inverse association between 387

AXL expression and response to anti-PD-1 (**Fig. 1A**, p=0.036). For OS, univariate and multivariate analyses indicated no statistical difference (**Fig. 1A** and **Supplementary Table S2**). Additional analysis showed that patients whose tumors exhibited AXL^{low} or AXL^{neg} profiles had similar clinical outcomes and characteristics (**Supplementary Fig. S2A** and **Supplementary Table S3**). No association was observed between AXL staining in immune cells and OS, PFS or ORR (not shown).

394 To further explore the link between AXL expression and clinical outcomes, we 395 exploited the RNA-seq data available for a subset of the cases (n=79). Despite the 396 small number of cases, we recapitulated our findings. A higher AXL expression in the 397 specimens tended to be associated with reduced 12-month PFS (p=0.15) and OS 398 rates (p=0.63) (Fig. 1B). We then interrogated expression data from the Braun et al. 399 study [11]. This cohort included patients with advanced RCC patients enrolled in 400 CheckMate (CM-009, CM-010 and CM-025 trials) who were treated with an mTOR 401 inhibitor (Everolimus), or nivolumab after progression on at least one prior anti-402 angiogenic treatment [11]. The potential effect of high AXL expression on survival 403 and ORR was examined in the anti-PD-1 treated arm. High AXL expression was 404 associated with worsened OS under anti-PD-1 (HR for death, 1.46; 95% CI, 1.00 to 405 2.22, P<0.05) (Fig. 1C). Median OS was 19.8 months in patients with tumors 406 expressing high levels of AXL mRNA vs 35.1 months in cases displaying reduced 407 levels of AXL mRNA. A trend was observed towards shorter PFS under anti-PD-1 408 blockade and lower ORR in tumors with high AXL mRNA expression (19%, 11/58 409 cases) compared to tumors with low AXL expression (24.56%, 28/114), although the 410 differences could not reach statistical significance.

Together, these findings suggest that AXL, especially when highly expressed in cancer cells, may be predictor of tumors with reduced response rate and worse progression following PD-1 blockade.

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415 AXL expression is associated with increased tumor expression of PD-L1

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417 We then explored the TME composition of these tumors by investigating associations 418 between AXL and various markers as assessed by IHC. This included VEGFA, PD-419 L1 and markers of the most common immune cell populations CD3, CD8 (T cells), 420 CD163 (macrophages) and CD20 (B-cells). PD-L1 tumor cell staining was detected in 421 23.9% (76/317) of evaluable ccRCC cases and PD-L1 on TILs in 58,6% (186/317) of 422 cases. We then stratified cases according to AXL expression and PD-L1 expression 423 status in tumor cells, and tested putative associations between the two markers (Fig. **2A**). A significant enrichment of PD-L1 positivity within tumors with AXL^{high} profile 424 425 was observed (p=0.0031). Moreover, the mean % of PD-L1 positive tumor cells was 426 increased in the AXL^{high} group compared to tumors with AXL^{neg/low} profiles (p=0.0019) 427 (Fig. 2A and Supplementary Fig. S2B). In contrast, no significant associations were 428 observed between AXL and PD-L1 TIL expression (not shown, p > .99). AXL 429 expression was associated with a greater proportion of tumor cells positive for VEGF 430 (p=0.002) (Fig. 2B). We observed a trend toward a higher level of tumor-infiltrating 431 CD8+ T cells in AXL^{high} vs AXL^{neg/low} cases, and no association between AXL and 432 CD3, CD163, CD20 densities in the tumor core (Figure 2B-C, Supplementary Fig. 433 S2B). RNA-seq data revealed a trend toward greater inflammation (p=0.072), immune suppression (p=0.105), and monocytic lineage (p=0.052) signatures in 434 AXL^{high} tumors (**Fig. 2D**). 435

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High AXL combined with PD-L1 expression is associated with poor survival in patients treated with PD-1 blockade

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443 We set out to determine the extent to which combining AXL and PD-L1 markers 444 would impact on OS under anti-PD-1 treatment (Fig. 3). PD-L1 positive staining in 445 tumor cell alone (using cutoff 0;0.5 versus >=1) associated with worse 12-month survival rate (70% (58.6-78.8) versus 77.3 (71.5-82.1)) with HR for death 1.51; log-446 447 rank p = 0.02)(Fig. 3A). No association was found with PFS (HR = 1.10, p = 0.5) or 448 the ORR (28.6% in PD-L1⁺ vs 23.9% in PD-L1^{neg} subsets)(Supplementary Fig. 449 S3A). PD-L1 staining in TILs had limited impact on OS (12-month survival rate 73.3%) 450 (66.4-79) vs 78.7 (70.7-84.8)), HR to death 1.34; log-rank p = 0.09), no impact on PFS (p = 0.16), nor on the ORR (25.1% in PD-L1⁺ vs 25% in PD-L1^{neg}) (Fig. 3B and 451 452 Supplementary Fig. S3B).

453 The combination of the two markers AXL and PD-L1 on tumor cells had significant 454 impact on OS since the group of patients whose tumors exhibited both high levels of 455 AXL and PD-L1 tumor cell positivity had the lowest survival rate at 12 months 60.7% 456 (40.4-76.0%), HR to death 1.98 (1.17-3.35), Logrank p = 0.046, compared to the three other groups (AXL^{neg/low}/PD-L1⁺; AXL^{neg/low}/PD-L1^{neg};AXL^{high}/PD-L1^{neg})(Fig. 457 458 **3A**). On multivariate analysis adjusting for age, sex, IMDC risk groups and number of previous therapy lines (≤2 vs >2), high AXL combined with PD-L1 expression was 459 460 associated with a trend to reduced OS (HR = 2.01 (95%Cl, 1.18-3.44), p=0.084 (Supplementary Table S5)). We further interrogated the prognostic value of combining AXL and PD-L1 TC markers specifically in patients with IMDC intermediate-risk/poor-risk RCC. In this setting, a AXL^{high}/PD-L1⁺ TC profile was associated with the lowest overall survival rate at 12 months 56.0% (34.8-72.7%) and HR to death 2.05 (1.21-3.50), p=0.053 (Supplementary Fig. S4).

There was no significant difference between ORR across the different groups (p=0.123), and AXL/PD-L1 TC combination had little if no further influence on PFS compared to AXL alone (**supplementary Fig. S3A**). However, it is noteworthy that the combination of high AXL expression with PD-L1 staining in TILs displayed notable features, with patients in the AXL high/PD-L1+ TIL group showing the worst clinical outcomes particularly in terms of ORR (12%, p=0.04) and PFS, with relatively

472 low PFS rate at 12 months (15.9% (7.0-28.1%), p=0.14) (**Supplementary Fig. S3B**).

We examined the repartition of the different markers in each subgroup, namely, PD-L1 tumor, PD-L1 TIL, VEGF, CD3, CD8, CD163 and CD20. Patients whose tumors were positive for PD-L1 had increased immune cell infiltration. Hence, both AXL^{high} and AXL^{neg/low} cases within the PD-L1⁺ subset demonstrated high immune cell infiltration compared to PD-L1^{neg} groups (**Fig. 4A-B**).

These data indicate that AXL and PD-L1 staining can identify a particular group of patients with poor outcomes when receiving anti-PD-1 treatment.

480

481 *VHL* status does not interfere with clinical outcomes or AXL expression in
 482 advanced ccRCC treated with Nivolumab

483

484 Since *VHL* is the most common genetic alteration in RCC, we characterized *VHL* 485 alterations in the ccRCC cases of the NIVOREN cohort. Point mutations were found

in 76.7% (231/301), LOH in 84.1% (254/302), and promoter hypermethylation in 486 487 19.1% (27/141) of cases. VHL status could be ascertained in 260 cases. 74% of 488 cases (193/260) were found to have a biallelic alteration of VHL which supposes LOF 489 of the gene. Among the remaining cases, 7.7% (20/260) presented with no VHL 490 alteration (+/+), and 18% (47/260) had one VHL alteration (+/-) implying one 491 remaining functional copy. No significant differences in ORR (chi-square p=0.40), PFS (HR 1.00 (0.73-1.37), p=0.98) or OS (0.92 (0.61-1.38), p=0.68) were observed 492 493 between patients with biallelic VHL inactivation (VHL^{neg/neg}) and those presenting with at least one unaltered VHL copy (VHL^{+/+} and VHL ^{+/neg})(Supplementary Fig. S5A-B). 494 495 AXL expression was homogeneously distributed across cases with or without biallelic 496 VHL alterations excluding the possibility that AXL expression is dependent on VHL 497 status (Supplementary Fig. S5C). When stratified based on VHL status, a trend was 498 found towards increased PD-L1 positivity in TILs within tumor specimens harboring a VHL^{neg/neg} profile (Supplementary Fig. S5C), whereas VHL^{+/+}/VHL^{+/neg} tumors 499 500 tended to be enriched with PD-L1⁺ tumor cells (Supplementary Fig. S5C) in line with 501 a previous study [22]. Analysis of the Nivoren RNAseq dataset [24] indicated that 502 $VHL^{+/+}/VHL^{+/neg}$ tumors expressed more PD-L1 (p=0.01) and VHL mRNAs (p=0.03) 503 (Supplementary Fig. S5D). As expected, VHL^{neg/neg} tumors exhibited increased 504 angiogenesis (p=0.02) and hypoxia signatures (p=0.02). $VHL^{+/+}/VHL^{+/neg}$ tumors 505 displayed a higher VEGF tumor expression, suggesting a VHL/HIF-independent 506 regulation of VEGF in this group, as previously proposed [29] (Supplementary Fig. 507 S6A). Together, this data support that despite similar clinical outcomes under PD-1 blockade, VHL^{neg/neg} and VHL^{+/+}/VHL^{+/neg} tumors are biologically different and 508 509 associated with a distinct microenvironment.

511 Association between AXL, PD-L1 and survival according to VHL status

512 We then guestioned whether AXL staining would yield comparable prognostic and 513 biologic insights in patients with complete inactivation of VHL compared to those with 514 a non-inactivated status. We categorized cases into four groups based on AXL 515 expression >50 and the VHL status. Patients whose tumors exhibited high AXL 516 expression and VHL^{neg/neg} appeared to have the worst median PFS and 12-month PFS compared to cases with AXL^{neg/low} expression within VHL^{neg/neg} subset, though 517 518 the overall difference was not significant in the log-rank tests comparing the four 519 groups (Fig. 5A). In the VHL^{neg/neg} subset, a separation in the PFS curves was evident between AXL^{high} vs AXL^{neg/low} groups after ~5 months and continued to show 520 521 differences in time as supported by the 12-month PFS rates of 19.6% versus 29.9%, 522 respectively (**Fig. 5A**). In contrast, in the $VHL^{+/+}/VHL^{+/neg}$ tumors, there were no signs 523 of such differences. Of note, when interrogating RNAseq data for a subset of 524 patients, a trend in the same direction was observed toward a negative impact for 525 AXL on PFS in the inactivated VHL subset but not in the non-inactivated VHL subset 526 (Fig. 5B). We then conducted a similar analysis using the Checkmate RNA dataset 527 and generated Kaplan–Meier curves for PFS and OS according to tumor VHL status. AXL^{high} expression associated with worsened survival (PFS and OS) compared to 528 529 AXL^{neg/low} in the inactivated VHL group (n=78; P=0.05), whereas no difference was 530 observed in patients conserving at least one WT copy of VHL in their tumor (n=44; 531 p=0.49) (Fig. 5C). Therefore, the influence of AXL on clinical outcomes was mainly 532 apparent in a VHL^{neg/neg} tumors. The results also suggest that both OS and PFS 533 could be impacted by high AXL expression in this setting.

534 Finally, we sought to examine the putative interaction between AXL and PD-L1 535 according to tumoral *VHL* status. The enrichment for PD-L1 positivity in AXL^{high} cases

was restricted to VHLneg/neg tumors (p=0.0003) and accompanied by a significant 536 increase in the % of PD-L1⁺ tumor cells in AXL^{high} VHL^{neg/neg} cases (Figure 5D). In 537 538 VHL^{+/+}/VHL^{+/neg} cases, PD-L1 staining was detected, but the proportion of positive cases was identical in both the AXL^{neg/low} and AXL^{high} groups (p> 0.99). In contrast, 539 540 the relationship between AXL with VEGF was perceptible in both VHL^{neg/neg} and VHL^{+/+}/VHL^{+/neg} subsets (Supplementary Fig. S6B). We observed no particular 541 effects of AXL on the immune infiltrate (CD8, CD3, CD163, CD20) in the VHLneg/neg 542 543 and VHL^{+/+}/VHL^{+/neg} specimens(Supplementary Fig. S6B and S6C).

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Discussion

547 Immunotherapy approaches are increasingly used in clinical practice. Yet many 548 challenges remain to improve efficacy and RR. Most patients exhibit primary or 549 acquired resistance to immunotherapy for reasons that remain unclear. The 550 development of markers predictive for response or resistance is warranted. While 551 recent studies have already shown promising results of immunotherapies as first-line 552 therapy, it will be critical to provide the best appropriate combinatorial therapy for 553 each patient, and this implies the development of tools to capture the unique 554 microenvironment of their tumor.

In this study, patients with ccRCC tumors exhibiting high expression levels of AXL had poorer clinical response (PFS and ORR) after Nivolumab treatment, but not altered OS. The median follow-up in this study was 23.5 months. Outcome measures derive from a still active multicenter phase II study. Extended follow-up should better inform on the impact of AXL on OS. A final analysis at a four-year minimum follow-up

is planned. The findings obtained from the analysis of the CheckMate dataset areconcordant with a role for AXL on OS.

AXL expression was strongly associated with PD-L1 TC positivity on tumor cells, and patients with tumors displaying concomitant PD-L1 TC expression and high AXL expression had the worst OS. We found no significant differences for PFS. Such discordance between PFS and OS should be interpreted with caution since prior work indicates that PFS may not be an ideal predictor of OS in the setting of anti-PD-1 monotherapy [9].

568 The absence of correlation between PD-L1 expression and ICB therapy response in 569 this cohort is interesting. It could be due to possible interference between factors 570 such as AXL and PD-L1 expressed by tumor cells or TILs, or discrepancies in PD-L1 571 expression in different tumor sites. Intriguingly, patients with tumors exhibiting high 572 AXL TC expression and PD-L1 positivity in TILs demonstrated a much lower 573 response rate, suggesting that AXL might play a substantial role in tumors infiltrated 574 with PD-L1 expressing TILs. The combination of AXL and PD-L1 biomarkers could 575 identify various subgroups of patients displaying high or reduced tumor-infiltrating 576 immune cells. The findings support the hypothesis that AXL may be one factor 577 interfering with the predictive value of PD-L1, though it's not clear yet whether such 578 effects could act directly on tumor cells, TILs or indirectly via the TME components, a 579 question that should be addressed in future investigations.

It will be important to explore the connections between AXL, microenvironment components and therapeutic outcomes in the context of other treatment regimens, namely the anti-PD-1/anti-CTLA4 combination emerging as a standard-of-care firstline therapy option [10,30], anti-PDL1-based combinations used to treat patients with advanced RCCs [5,31,32], as well as anti-PD-1 based combinations integrating

agents targeting AXL such as cabozantinib, expected to target VEGFR2, MET, RET
and AXL [5,33]. Recent data have demonstrated activity of cabozantinib after ICB in
m-ccRCC [34]. This treatment might enable to overcome the deleterious effects of
AXL.

589 Although AXL expression was not significantly associated with tumor infiltration by 590 macrophages, B or T cells, a broader assessment of the TME and immune 591 checkpoints is required. Our RNA-seq analysis suggested potential interactions 592 between AXL expression and increased inflammation, immune suppression and 593 monocytic lineage signatures. Such analysis likely suffered from the small number of 594 cases analyzed. Studies using sizeable cohorts of patients will be needed to validate 595 these findings. Multiplex IHC or IF approaches would be needed to fully characterize 596 tumor and immune cell infiltrates in this cohort, dissect intratumor heterogeneity, and 597 test potential associations with therapeutic outcomes.

598 Our analysis is retrospective and has other potential limitations. A number of cases 599 with FFPE block showing <40% of tumor or more than 40% of necrosis were 600 excluded. The archival tumor specimens were often obtained before anti-angiogenic 601 therapy or at an early-stage disease. This is in line with routine clinical practice for 602 treatment decision-making. However, a longitudinal survey with a multiple sampling 603 scheme may be necessary to fully appreciate the TME composition and monitor the 604 influence of previous treatments. Diseases with specific features (e.g., Sarcomatoid, 605 low grade, IMDC favorable-risk groups) also need further investigations, as the 606 current study was limited by the number of cases in these categories. As reported by 607 Boysen et al. [35], we noted a trend toward a greater sarcomatoid differentiation in 608 AXL positive cases (n=25, p=0.09) as well as an enrichment of high grade tumors 609 (Furhman III and IV) in cases with high AXL expression.

610 Interestingly, AXL association with PD-L1 and worse PFS was observed in tumors 611 harboring VHL^{neg/neg} genetic profile, but it has been unclear if this also occurs in those 612 with VHL+/+/ VHL+/neg status where the number of cases was lower. It is postulated that the TME from $VHL^{heg/heg}$ tumors differs from those displaying $VHL^{+/+}$ or $VHL^{+/heg}$. 613 614 It would be interesting to see if among the identified subgroups, some are enriched 615 for genomic alterations reported with values for predicting anti-PD-1 responsiveness 616 including BAP1 and PBRM1 [11]. This could explain, in part, some of the 617 demonstrated effects on PFS, OS or ORR. Previous studies have demonstrated that loss of PBRM1 functions can accentuate the transcriptional response of HIF in 618 619 VHL^{neg/neg} RCC [36]. We postulate that a molecular link exists between AXL and loss 620 of *PBRM1* in *VHL*^{neg/neg} RCC tumors.

621 Moreover, tumor cells may have intrinsic characteristics which likely affect the TME. 622 AXL expression has been associated with EMT, epithelial-mesenchymal plasticity, 623 and phenotypic changes of tumor cells in various cancer types [37]. Compelling 624 evidence also exists that differentiation of tumor cells to a more mesenchymal 625 phenotype can greatly influence the TME *via* increased secretion of 626 immunosuppressive substances such as TGF-beta, VEGF, PD-L1 or IL-8 [38-43]. In 627 a lung cancer study, Thompson and colleagues observed that NSCLC tumors 628 displaying a more mesenchymal phenotype had reduced clinical responses to PD-1 629 blockade. From this work, the investigators derived an EMT/inflammation-based gene 630 expression signature for predicting clinical response of metastatic NSCLC patients 631 [44]. In a cohort of patients with metastatic urothelial cancer treated with nivolumab, 632 Wang et al. found that particularly in patients with T-cell infiltrated tumors, higher 633 EMT/stromal-related gene expression was associated with lower response rates and 634 poor outcomes [45]. Moreover, Chakravarthy et al. showed that an extracellular

matrix (ECM) transcriptional program involving TGF-β signalling and EMT-related
genes is associated with worse prognosis in melanoma and bladder cancer patients
treated with anti-PD-1 [46]. In melanoma, Hugo and colleagues identified a geneexpression signature associated with innate PD-1 resistance (IPRES) of metastatic
melanoma which involves the upregulation of EMT-related factors, including AXL,
immunosuppressive cytokines, hypoxia and pro-angiogenic factors [47].

641 In our survey, AXL staining in immune cells had no prognostic value. However, 642 because the panel of markers analyzed was limited, we cannot exclude the possibility 643 that a particular immune cell subpopulation expressing AXL contributes to anti-PD-1 644 responsiveness. Others have reported evidence of associations between AXL 645 expressing immune cell populations, and the establishment of an immunosuppressed 646 TME [48–50]. Future studies are warranted to elucidate the interplay between 647 pseudo-hypoxia status, epithelial-mesenchymal plasticity, AXL, microenvironmental 648 changes and their impact on anti-PD-1 responsiveness.

649

In summary, we identified associations between expression of AXL, PD-L1 expression and *VHL* status in advanced ccRCC which could contribute to disease progression under ICB therapy. Furthermore, we demonstrated that the combined assessment of AXL and PD-L1 expression may be useful to predict outcomes and discriminate patients who are more likely to be resistant or to benefit from anti-PD-1 immunotherapy.

656

657 Acknowledgments

This work was supported by INSERM, Université Paris-Saclay, Cancéropôle Ile-deFrance, Sorbonne Université, Université de Paris, la Ligue Contre le Cancer

660 (EL2015.LNCC/SaC to Salem Chouaib), l'Institut National du Cancer and Direction 661 générale de l'offre de soins (grant INCa-DGOS-PRT-K16-181, GETUG-AFU26-662 Nivoren translational program to Laurence Albiges), CARPEM program of the Sites 663 Intégrés de Recherche sur le Cancer (SIRIC) (to Catherine Sautès-Fridman) and the 664 association pour la recherche en thérapeutiques innovantes en cancérologie 665 (ARTIC). Maxime Meylan received a PhD fellowship from ARTIC. We thank Florence 666 Tantot (Unicancer) for participating to the translational research program, Sophie 667 Ferlicot (Hôpital de Bicêtre, Paris-Sud 11) for providing material in preliminary 668 studies, Laurence Cornevin, Roselyne Viel and Pascale Bellaud (Plateforme H2P2,

- 669 Biosit, Univ Rennes, F35000 rennes, France) for outstanding technical assistance.
- 670

671 **References**

672 1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics
673 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185
674 countries. CA: A Cancer Journal for Clinicians. 2018;68:394–424.

- 2. Rini BI, Plimack ER, Stus V, Gafanov R, Hawkins R, Nosov D, et al. Pembrolizumab plus
 Axitinib versus Sunitinib for Advanced Renal-Cell Carcinoma. N Engl J Med. 2019;380:1116–
 27.
- 678 3. Motzer R, Alekseev B, Rha S-Y, Porta C, Eto M, Powles T, et al. Lenvatinib plus
 679 Pembrolizumab or Everolimus for Advanced Renal Cell Carcinoma. N Engl J Med. 2021;
- 4. Choueiri TK, Powles T, Burotto M, Escudier B, Bourlon MT, Zurawski B, et al. Nivolumab
 plus Cabozantinib versus Sunitinib for Advanced Renal-Cell Carcinoma. N Engl J Med.
 2021;384:829–41.
- 5. Xu W, Atkins MB, McDermott DF. Checkpoint inhibitor immunotherapy in kidney cancer.
 Nat Rev Urol. 2020;17:137–50.
- 685 6. Sharma P, Hu-Lieskovan S, Wargo JA, Ribas A. Primary, Adaptive, and Acquired 686 Resistance to Cancer Immunotherapy. Cell. 2017/02/12 ed. 2017;168:707–23.
- 687 7. Upadhaya S, Neftelino ST, Hodge JP, Oliva C, Campbell JR, Yu JX. Combinations take
 688 centre stage in PD1/PDL1 inhibitor clinical trials. Nat Rev Drug Discov. 2020;

8. Giraldo NA, Becht E, Pages F, Skliris G, Verkarre V, Vano Y, et al. Orchestration and
Prognostic Significance of Immune Checkpoints in the Microenvironment of Primary and
Metastatic Renal Cell Cancer. Clinical Cancer Research. 2015;21:3031–40.

- 692 9. Motzer RJ, Escudier B, McDermott DF, George S, Hammers HJ, Srinivas S, et al.
 693 Nivolumab versus Everolimus in Advanced Renal-Cell Carcinoma. N Engl J Med.
 694 2015;373:1803–13.
- 695 10. Motzer RJ, Tannir NM, McDermott DF, Arén Frontera O, Melichar B, Choueiri TK, et al.
 696 Nivolumab plus Ipilimumab versus Sunitinib in Advanced Renal-Cell Carcinoma. N Engl J
 697 Med. 2018;378:1277–90.
- 698 11. Braun DA, Hou Y, Bakouny Z, Ficial M, Sant' Angelo M, Forman J, et al. Interplay of
 699 somatic alterations and immune infiltration modulates response to PD-1 blockade in
 700 advanced clear cell renal cell carcinoma. Nat Med. 2020;26:909–18.
- 12. Miao D, Margolis CA, Gao W, Voss MH, Li W, Martini DJ, et al. Genomic correlates of
 response to immune checkpoint therapies in clear cell renal cell carcinoma. Science.
 2018;359:801–6.
- 13. Hsieh JJ, Le VH, Oyama T, Ricketts CJ, Ho TH, Cheng EH. Chromosome 3p LossOrchestrated VHL, HIF, and Epigenetic Deregulation in Clear Cell Renal Cell Carcinoma.
 Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology.
 2018;JCO2018792549.
- 14. Schoumacher M, Burbridge M. Key Roles of AXL and MER Receptor Tyrosine Kinases in
 Resistance to Multiple Anticancer Therapies. Current oncology reports. 2017/03/03 ed.
 2017;19:19.
- 711 15. Peterfi L, Bjercke T, Yusenko MV, Kovacs G, Banyai D. Cytoplasmic Expression of AXL
 712 Is Associated With High Risk of Postoperative Relapse of Conventional Renal Cell
 713 Carcinoma. Anticancer Res. 2020;40:3485–9.
- 16. Landolt L, Eikrem Ø, Strauss P, Scherer A, Lovett DH, Beisland C, et al. Clear Cell Renal
 Cell Carcinoma is linked to Epithelial-to-Mesenchymal Transition and to Fibrosis. Physiol
 Rep. 2017;5.
- 717 17. Terry S, Abdou A, Engelsen AST, Buart S, Dessen P, Corgnac S, et al. AXL Targeting
 718 Overcomes Human Lung Cancer Cell Resistance to NK- and CTL-Mediated Cytotoxicity.
 719 Cancer Immunol Res. 2019;7:1789–802.
- 18. Guo Z, Li Y, Zhang D, Ma J. Axl inhibition induces the antitumor immune response which
 can be further potentiated by PD-1 blockade in the mouse cancer models. Oncotarget.
 2017/11/23 ed. 2017;8:89761–74.
- 19. Aguilera TA, Rafat M, Castellini L, Shehade H, Kariolis MS, Hui AB, et al.
 Reprogramming the immunological microenvironment through radiation and targeting Axl.
 Nature communications. 2016/12/23 ed. 2016;7:13898.
- 20. Ludwig KF, Du W, Sorrelle NB, Wnuk-Lipinska K, Topalovski M, Toombs JE, et al. SmallMolecule Inhibition of Axl Targets Tumor Immune Suppression and Enhances Chemotherapy
 in Pancreatic Cancer. Cancer research. 2017/11/29 ed. 2018;78:246–55.
- 729 21. Flippot R, Dalban C, Laguerre B, Borchiellini D, Gravis G, Négrier S, et al. Safety and
 730 Efficacy of Nivolumab in Brain Metastases From Renal Cell Carcinoma: Results of the
 731 GETUG-AFU 26 NIVOREN Multicenter Phase II Study. J Clin Oncol. 2019;37:2008–16.
- 22. Messai Y, Gad S, Noman MZ, Le Teuff G, Couve S, Janji B, et al. Renal Cell Carcinoma
 Programmed Death-ligand 1, a New Direct Target of Hypoxia-inducible Factor-2 Alpha, is
 Regulated by von Hippel-Lindau Gene Mutation Status. Eur Urol. 2016;70:623–32.

735 23. Kammerer-Jacquet SF, Crouzet L, Brunot A, Dagher J, Pladys A, Edeline J, et al.
736 Independent association of PD-L1 expression with noninactivated VHL clear cell renal cell
737 carcinoma-A finding with therapeutic potential. International journal of cancer. 2016/09/14 ed.
738 2017;140:142–8.

- 24. Meylan M, Beuselinck B, Dalban C, Vano Y, Rioux Leclercq N, Sautès-Fridman C, et al.
 7000 Kidney ccRCC immune classification (KIC) enhances the predictive value of T effector
 (Teff) and angiogenesis (Angio) signatures in response to nivolumab (N). Annals of
 Oncology. 2020;31:S553.
- 743 25. McDermott DF, Huseni MA, Atkins MB, Motzer RJ, Rini BI, Escudier B, et al. Clinical
 744 activity and molecular correlates of response to atezolizumab alone or in combination with
 745 bevacizumab versus sunitinib in renal cell carcinoma. Nature Medicine. Nature Publishing
 746 Group; 2018;24:749–57.
- 26. Becht E, Giraldo NA, Lacroix L, Buttard B, Elarouci N, Petitprez F, et al. Estimating
 the population abundance of tissue-infiltrating immune and stromal cell populations using
 gene expression. Genome Biol. 2016;17:218.
- 27. Buffa FM, Harris AL, West CM, Miller CJ. Large meta-analysis of multiple cancers reveals
 a common, compact and highly prognostic hypoxia metagene. Br J Cancer. 2010;102:428–
 35.
- 28. Hendry S, Salgado R, Gevaert T, Russell PA, John T, Thapa B, et al. Assessing Tumorinfiltrating Lymphocytes in Solid Tumors: A Practical Review for Pathologists and Proposal
 for a Standardized Method From the International Immunooncology Biomarkers Working
 Group: Part 1: Assessing the Host Immune Response, TILs in Invasive Breast Carcinoma
 and Ductal Carcinoma In Situ, Metastatic Tumor Deposits and Areas for Further Research.
 Adv Anat Pathol. 2017;24:235–51.
- Patard J-J, Rioux-Leclercq N, Masson D, Zerrouki S, Jouan F, Collet N, et al. Absence of
 VHL gene alteration and high VEGF expression are associated with tumour aggressiveness
 and poor survival of renal-cell carcinoma. Br J Cancer. 2009;101:1417–24.
- 30. Tannir NM, Signoretti S, Choueiri TK, McDermott DF, Motzer RJ, Flaifel A, et al. Efficacy
 and Safety of Nivolumab Plus Ipilimumab versus Sunitinib in First-line Treatment of Patients
 with Advanced Sarcomatoid Renal Cell Carcinoma. Clin Cancer Res. 2020;
- 31. Motzer RJ, Robbins PB, Powles T, Albiges L, Haanen JB, Larkin J, et al. Avelumab plus
 axitinib versus sunitinib in advanced renal cell carcinoma: biomarker analysis of the phase 3
 JAVELIN Renal 101 trial. Nat Med. 2020;
- 32. Motzer RJ, Penkov K, Haanen J, Rini B, Albiges L, Campbell MT, et al. Avelumab plus
 Axitinib versus Sunitinib for Advanced Renal-Cell Carcinoma. N Engl J Med. 2019;380:1103–
 15.
- 33. Choueiri TK, Kaelin WG. Targeting the HIF2-VEGF axis in renal cell carcinoma. Nat Med.2020;26:1519–30.

34. McGregor BA, Lalani A-KA, Xie W, Steinharter JA, E Bakouny Z, Martini DJ, et al. Activity
of cabozantinib after immune checkpoint blockade in metastatic clear-cell renal cell
carcinoma. Eur J Cancer. 2020;135:203–10.

- 35. Boysen G, Bausch-Fluck D, Thoma CR, Nowicka AM, Stiehl DP, Cima I, et al.
 Identification and functional characterization of pVHL-dependent cell surface proteins in renal
 cell carcinoma. Neoplasia. 2012;14:535–46.
- 36. Gao W, Li W, Xiao T, Liu XS, Kaelin WG. Inactivation of the PBRM1 tumor suppressor
 gene amplifies the HIF-response in VHL ^{-/-} clear cell renal carcinoma. Proc Natl Acad Sci
 USA. 2017;114:1027–32.
- 782 37. Lotsberg ML, Rayford A, Thiery JP, Belleggia G, Peters SD, Lorens JB, et al. Decoding 783 cancer's camouflage: epithelial-mesenchymal plasticity in resistance to immune checkpoint 784 blockade. CDR [Internet]. 2020 [cited 2020 Nov 1]; Available from: 785 https://cdrjournal.com/article/view/3689
- 38. Terry S, Savagner P, Ortiz-Cuaran S, Mahjoubi L, Saintigny P, Thiery J-P, et al. New
 insights into the role of EMT in tumor immune escape. Mol Oncol. 2017;11:824–46.
- 39. Lou Y, Diao L, Cuentas ER, Denning WL, Chen L, Fan YH, et al. Epithelial-Mesenchymal
 Transition Is Associated with a Distinct Tumor Microenvironment Including Elevation of
 Inflammatory Signals and Multiple Immune Checkpoints in Lung Adenocarcinoma. Clinical
 cancer research: an official journal of the American Association for Cancer Research.
 2016/02/07 ed. 2016;22:3630–42.
- 40. Mak MP, Tong P, Diao L, Cardnell RJ, Gibbons DL, William WN, et al. A Patient-Derived,
 Pan-Cancer EMT Signature Identifies Global Molecular Alterations and Immune Target
 Enrichment Following Epithelial-to-Mesenchymal Transition. Clinical cancer research : an
 official journal of the American Association for Cancer Research. 2015/10/01 ed.
 2016;22:609–20.
- 41. Dongre A, Rashidian M, Reinhardt F, Bagnato A, Keckesova Z, Ploegh HL, et al.
 Epithelial-to-Mesenchymal Transition Contributes to Immunosuppression in Breast
 Carcinomas. Cancer Res. 2017;77:3982–9.
- 42. Chen L, Gibbons DL, Goswami S, Cortez MA, Ahn YH, Byers LA, et al. Metastasis is regulated via microRNA-200/ZEB1 axis control of tumour cell PD-L1 expression and intratumoral immunosuppression. Nature communications. 2014/10/29 ed. 2014;5:5241.
- 43. Horn LA, Fousek K, Palena C. Tumor Plasticity and Resistance to Immunotherapy.
 Trends Cancer. 2020;6:432–41.
- 44. Thompson JC, Hwang W-T, Davis C, Deshpande C, Jeffries S, Rajpurohit Y, et al. Gene
 signatures of tumor inflammation and epithelial-to-mesenchymal transition (EMT) predict
 responses to immune checkpoint blockade in lung cancer with high accuracy. Lung Cancer.
 2020;139:1–8.
- 45. Wang L, Saci A, Szabo PM, Chasalow SD, Castillo-Martin M, Domingo-Domenech J, et
 al. EMT- and stroma-related gene expression and resistance to PD-1 blockade in urothelial
 cancer. Nature Communications. Nature Publishing Group; 2018;9:3503.
- 46. Chakravarthy A, Khan L, Bensler NP, Bose P, De Carvalho DD. TGF-β-associated
 extracellular matrix genes link cancer-associated fibroblasts to immune evasion and
 immunotherapy failure. Nat Commun. 2018;9:4692.
- 47. Hugo W, Zaretsky JM, Sun L, Song C, Moreno BH, Hu-Lieskovan S, et al. Genomic and
 Transcriptomic Features of Response to Anti-PD-1 Therapy in Metastatic Melanoma. Cell.
 2016;165:35–44.

- 48. Rothlin CV, Ghosh S, Zuniga EI, Oldstone MBA, Lemke G. TAM receptors are pleiotropic inhibitors of the innate immune response. Cell. 2007;131:1124–36.
- 49. Maier B, Leader AM, Chen ST, Tung N, Chang C, LeBerichel J, et al. A conserved dendritic-cell regulatory program limits antitumour immunity. Nature. 2020;580:257–62.
- 823 50. Tanaka M, Siemann DW. Gas6/Axl Signaling Pathway in the Tumor Immune 824 Microenvironment. Cancers (Basel). 2020;12.
- 825
- 826

Table 1: Patient characteristics				
	AXL Tumor cell score		All Detients	
Variables	[0;50]	>50	All Patients	Tests
	n=241	n=75	n=316	
Age (years)				
Ν	241	75	316	T-test
Mean (Std)	62.6 (10.9)	61.9 (10.4)	62.4 (10.7)	0.608
Median (min;	64.0 (22.0;	62.0 (41.0;	64.0 (22.0;	
max)	87.0)	86.0)	87.0)	
Gender				
Male	194 (80.5%)	63 (84.0%)	257 (81.3%)	Chi-2
Female	47 (19.5%)	12 (16.0%)	59 (18.7%)	P = 0.497
IMDC group				
Favorable	42 (17.4%)	12 (16.0%)	54 (17.1%)	Chi-2
Intermediate	142 (58.9%)	49 (65.3%)	191 (60.4%)	P = 0.580
Poor	57 (23.7%)	14 (18.7%)	71 (22.5%)	
ECOG				
Missing	8	3	11	
0 or 1	199 (85.4%)	63 (87.5%)	262 (85.9%)	Chi-2
2 or 3	34 (14.6%)	9 (12.5%)	43 (14.1%)	P = 0.656
M Stage				
Missing	14	5	19	
0	83 (36.6%)	28 (40.0%)	111 (37.4%)	Chi-2
1	59 (26.0%)	20 (28.6%)	79 (26.6%)	P = 0.657
х	85 (37.4%)	22 (31.4%)	107 (36.0%)	
Furhman				
Missing data	7	2	9	
1, 11	69 (29.5%)	13 (17.8%)	82 (26.7%)	Chi-2
III, IV	165 (70.5%)	60 (82.2%)	225 (73.3%)	P = 0.049
Brain Metastasis				
Missing	16	3	19	
NO	197 (87.6%)	65 (90.3%)	262 (88.2%)	Chi-2
YES	28 (12.4%)	7 (9.7%)	35 (11.8%)	P = 0.533
Surgery	241 (100%)	75 (100%)	316 (100%)	
Nephrectomy				
NO	9 (3.7%)	3 (4.0%)	12 (3.8%)	Chi-2
YES	232 (96.3%)	72 (96.0%)	304 (96.2%)	P = 0.916
Radiotherapy				
NO	148 (61.4%)	47 (62.7%)	195 (61.7%)	Chi-2
YES	93 (38.6%)	28 (37.3%)	121 (38.3%)	P = 0.845

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Figure legends

831 Figure 1 High AXL expression is associated with a trend to shorter PFS and 832 lower ORR in patients with advanced ccRCC treated with Nivolumab. A, 833 Kaplan-Meier survival curves for PFS and OS of RCC patients when stratified 834 according to AXL H-score into two groups, negative/Low [0;50] vs High >50. 835 Corresponding Objective Response Rates, hazard ratios, median PFS (mPFS), and 836 survival estimates at 12 months post-treatment are shown. **B**, Kaplan–Meier PFS and 837 OS curves, and outcome measures deriving from RNA-seq data available for a 838 subset of cases. Nivolumab-treated RCC patients divided into two groups based on a 839 cutoff \geq highest 1/3 (High) vs \leq lowest 2/3 (Low) AXL mRNA expression to assess the impact of AXL on survival outcomes. C, Kaplan-Meier PFS and OS curves derived 840 from CheckMate RNA-seq data available from the study of Braun [11]. Nivolumab-841 842 treated RCC patients were considered and divided into AXL^{high} or AXL^{low} expression 843 groups as in **B**.

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845 Figure 2 AXL is associated with PD-L1 expression. A. Distribution of PD-L1 (% of cases and % of positive cells) in tumors with AXL^{neg/low} vs AXL^{high} profiles. B 846 Distribution of VEGF, CD8, CD3, CD163, CD20 expression in AXL^{Neg/Low} versus 847 AXL^{high} cases. **C**, A heatmap is shown to illustrate the biomarker composition across 848 849 the tumor specimens according to AXL groups. D, RNA-Seq-derived expression of various gene signatures in AXL^{neg/low} vs ^{high} cases. Violin plots are shown with box 850 representing the 25th to 75th percentiles and central line representing the median 851 852 (Mann-Whitney test).

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Figure 3 High AXL expression plus PD-L1 TC positivity is associated with worse OS in patients treated with Nivolumab. A, Kaplan-Meier estimates for OS according to PD-L1 TC status (pos/neg), or AXL expression and PD-L1 TC status (four biomarker subgroups). B, Kaplan-Meier estimates for OS according to PD-L1 TIL status (pos/neg), or AXL expression and PD-L1 TIL status (four biomarker subgroups).

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Figure 4 biomarker distribution across groups stratified based on PD-L1 TC and AXL expression

A, Bar graphs comparing the biomarker composition in the corresponding subgroups.
 Data are presented as means. Errors bars are s.e.m. Mann-Whitney test. B, A
 heatmap showing the biomarker distribution across the tumor specimens in the
 indicated subgroups.

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Figure 5 AXL association with PD-L1 and outcomes in VHL^{neg/neg} and VHL^{+/+} 868 869 /VHL^{+/neg} tumors A, Kaplan–Meier estimates for PFS in patients divided into four groups based on AXL expression and VHL status. In the VHL^{neg/neg} subset, patients 870 871 with tumors expressing high levels of AXL (red line) trended to worse PFS compared to those with AXL^{Null/Low} expression (grey line). B, Kaplan-Meier PFS curves 872 873 generated after categorization using RNA-seq data on a subset of cases in VHLinactivated (VHLneg/neg) and VHL non-inactivated (VHLneg/+/ VHL+/+) cases. C, 874 875 Kaplan–Meier estimates for PFS and OS as in B, but using the CheckMate dataset [11]. P values from Log rank tests are shown. **D**. AXL expression associated with PD-876 L1 TC expression especially in the VHL^{neg/neg} tumors. Distribution of PD-L1 (% of 877

cases and % of positive cells) across the tumor specimens according to the indicated
subgroups. Data are presented as means. Errors bars are s.e.m. Chi-square and
Mann-Whitney tests were applied.









FIGURE 4



