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Original article

Expression of tyrosine kinase receptor AXL is associated with worse outcome of metastatic renal cell carcinomas treated with sunitinib

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Abstract

Background: Renal cell carcinoma (RCC) represents 2%–3% of all cancers of the Western countries. Currently, sunitinib, a receptor tyrosine kinase inhibitor, particularly of PDGF and VEGF receptors, is the first-line therapy for metastatic RCC (mRCC), with significant improvement in clinical outcome. However, there is a lack of predictive biomarkers of sunitinib response. Recently, others and our group suggested that the receptor tyrosine kinase AXL may modify the response to sunitinib.

Objective: To study the expression of AXL in a series patients with of mRCC treated with sunitinib and to correlate it with patient's clinic-pathological features and therapeutic response.

Material and methods: Sixty-four patients with mRCC (51 clear cell carcinomas (CCCs) and 13 non-CCCs) were evaluated for AXL expression by immunohistochemistry in the primary tumor.

Results: AXL positivity was observed in 47% (30/64) of cases, namely in 43% (22/51) of CCCs and 61% (8/13) of non-CCC. Considering only the clear cell subtype, the univariate analysis showed that AXL expression was statistically associated with a poor prognosis, with a median overall survival of 13 months vs. 43 months in patients with negative AXL. In this subtype, along with the AXL positivity, other prognostic factors were absence of nephrectomy, Karnofsky performance status, more than 1 site of metastasis and liver metastasis. Moreover, AXL expression was associated with shorter progression to sunitinib. Overall, the multivariate survival analysis showed that absence of nephrectomy (HR = 4.85, $P = 0.001$), more than 1 site of metastasis (HR = 2.99, $P = 0.002$), bone metastasis (HR = 2.95, $P = 0.001$), together with AXL expression (HR = 2.01, $P = 0.048$) were independent poor prognostic factor in patients with mRCC.

Conclusion: AXL expression was associated with worse clinical outcome and may be an important prognostic biomarker in sunitinib-treated patients with metastatic renal cell carcinoma.

Keywords: Renal cell carcinoma; AXL; Sunitinib; Cabozantinib; Prognostic biomarker

1. Introduction

Kidney cancer is the seventh most common cancer in men and tenth most common cancer in woman worldwide [1]. Renal cell carcinoma (RCC) is responsible for 90% of all kidney cancer, and approximately 80% of these are clear cell tumors, with the other 20% being less common RCC subtypes, like papillary and chromophobe tumors [2,3]. In Brazil kidney cancer is the 15th cancer in incidence with approximately 3,700 new cases per year responsible for more than 2,000 deaths per year [4].

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Most clear cell cancer is sporadic while hereditary autosomal dominant syndromes are described as responsible for approximately 2%–3% of all RCCs, being the Von Hippel Lindau (VHL) disease the most common form [5,6]. VHL disease is caused by a germline mutation in the *VHL* gene that predisposes to clear cell RCC and other proliferative vascular tumors [7]. *VHL* gene plays an important role, not only in hereditary but also in sporadic clear cell cancer, since inactivation of VHL leads to elevated levels of the transcription factor hypoxia-inducible factor 1α (HIF-1α) and subsequent overexpression of vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), which promote tumor angiogenesis. Bi-allelic *VHL* gene alterations can occur through *VHL* gene mutations, hypermethylation of *VHL* gene promoter and loss of heterozygosity, which characterizes more than 80% of sporadic cases [6].

In this scenario, the receptor tyrosine kinase inhibitors (TKI), sunitinib, an anti-angiogenic agent, was tested in phase III trial, leading to a longer progressing free survival when compared to interferon (median: 11 vs. 5 mo, respectively, hazard ratio (HR) = 0.42; 95% CI: 0.32–0.54; $P < 0.001$) and an improved overall survival (OS) (median 26.4 vs. 21.8 mo, HR = 0.82, 95% CI: 0.673–1.001, $P = 0.051$) [8,9]. Consequently, sunitinib was approved and established as a standard of care in the treatment of advanced RCC worldwide [5,10–12]. Despite of this rational, TKIs rarely cause durable tumor regressions and most patients will experience disease progression after an initial period of response [13].

Remarkable efforts are being made to identify biomarkers that may predict response to select and treat more effectively patients with metastatic RCC (mRCC) [14]. *AXL*, a gene that encodes a receptor tyrosine kinase, is involved with a wide variety of cancerous hallmarks such as proliferation, survival, evasion from apoptosis, enhanced angiogenesis, and invasiveness [15,16]. Recently, AXL have been suggested as a biomarker of poor prognosis and a potential target for different types of cancers [17,18], including RCC [19,20]. Previously, our group suggested that in glioblastoma cell lines, AXL can constitute a predictive biomarker for sunitinib response [21,22]. In this study, we evaluated the AXL presence in the primary tumor through its expression in a cohort of 64 patients with mRCC treated with sunitinib and its association with clinical-pathological features and clinical outcome.

2. Materials and methods

2.1. Patients and sampling

Sixty-four patients with mRCC treated with sunitinib between 2008 and 2014 at Barretos Cancer Hospital were included in this study. Patient inclusion criteria comprised a diagnosis of mRCC of any pathologic subtype that had received at least 1 course of sunitinib in any line and had enough tissue samples for immunohistochemistry analysis. We also divided the patients into 2 groups, the clear cell and non-CCC (NCCC), due to different responses to sunitinib [20]. Baseline data including those previously found to have prognostic value [23,24] were collected retrospectively on all patients using uniform database templates to ensure consistent data collections and a SPSS databank was created. Laboratory values were standardized according to the local laboratory. The project was approved by local ethical committees (No. 837/2014).

Paraffin-embedded tumor samples from all 64 patients were retrieved from the Pathology Department of Barretos Cancer Hospital, Barretos, Brazil. All 64 samples were from the primary renal tumor (49 patients the material came from a previous nephrectomy, and 15 patients the material came from a biopsy from the primary tumor), and from these patients we were able to retrieve 41 normal adjacent renal tissue and 14 metastatic renal tissue. All tumor samples were collected before treatment with sunitinib. The tumor tissue were classified according to the latest criteria [25], reviewed by experienced pathologists to select representative sections (tumor and normal) for TMA (tissue microarray) construction. The TMA blocks were done containing 2 representative areas of each primary cancer of RCC tissue, metastatic tissue, and when available the normal counterpart (1 mm diameter core).

2.2. Immunohistochemistry analysis

Representative 4-μm thick sections of TMA were subjected to immunohistochemistry according to the streptavidin-biotin peroxidase complex system. Briefly, FFPE RCC tissue section were deparaffinised in oven at 80°C for 1 hour, following by antigen retriever by PT-LINK–Dako, using commercial buffer Envision flex target retrieval solution high ph. x1, from Dako (pH = 9). The sections were then incubated with anti-Axl antibody (dilution 1:50, Cat #AF154 R&D Systems, Minneapolis, MN) at room temperature for 2 hours. As positive control, we used normal breast tissue, which showed membranous AXL staining of luminal ductal cells with variable cytoplasmic staining, and cell-block of SNB-19 cell line, known in previous studies of our group for high AXL levels [21,26].

The score used was the sum of the percentage of cytoplasm positive cells (negative, 0%–<1% positive cells; 1, 1%–10% positive cells; 2, >10%–50% positive cells; 3, > 50% positive cells) and the staining intensity (0, negative; 1, weak; 2, moderate and 3, strong). Samples with scores 0 to 2 were considered negative for AXL expression and those with score 3 to 6 were considered positive for AXL expression, as previously described for receptor tyrosine kinase immunohistochemistry analysis [27–29].

2.3. Statistical analyses

Cumulative survival probabilities were calculated using the Kaplan-Meier method. Differences between survivals were tested using the log-rank test. Our primary end point was OS due to the retrospective analyses, which hamper a proper progression-free survival. OS was defined as the time from metastatic diagnoses to death as a result of any cause or was censored at the date of last follow-up. Association between OS and prognostic factors were assessed by using the long-rank test in univariable analysis. Correlations between AXL expression and available clinic-pathological data were performed using the Pearson's chi-square test (χ^2 test) or Fisher's exact test. The Cox proportional hazard model was undertaken subsequently in multivariable analyses by using a step-wise procedure with a significance level of 0.2 for entering and removing variables.

The level of significance in the statistical analysis were indicated as $P < 0.05$. The statistical analysis was performed using SPSS software for Windows, version 21.0.

3. Results

3.1. Patient clinical-pathological features

Overall, 64 patients were included in this analysis that were divided in 2 major subgroups: 51 (79.6%) CCC cases; and 13 (20.4%) NCCC cases (Table 1). Among the CCC subgroup, 4 (6.3%) cases were CCC with sarcomatoid features, and within the NCCC group, 9 cases (14%) were papillary RCC and 4 (6.3%) were RCC, unclassified, where 2 cases had pure sarcomatoid features. Forty-two (65.6%) were male and 22 (34.4%) were female with a median age of 57-years old (range: 30–81). The clinical parameters available for the two groups showed similar profile (Table 1). The median OS for the entire cohort of 64 patients was 25.6 months (95% CI: 11.6–39.7 mo, Fig. 1A). The mean follow up was 18.5 months. The CCC cohort, which compromised 51 patients, the OS was 26.3 months (95% CI: 6.0–46.5 mo) and the NCCC cohort, which compromised 13 patients, the OS was 10.3 months (95% CI: 5.1–15 mo, Fig. 1B).

In the first line therapy 46 (72%) patients received a TKI, and sunitinib was the drug of choice in the 45 patients, followed by 16 (25%) patients that received interferon alfa. One patient received chemotherapy as first line and one patient was not treated with any systemic therapy. Of the 45 patients that received sunitinib as first line, 26 (58%) had progressed while using sunitinib, 11 (24%) had partial response or stable disease, and 8 (18%) the data were missing.

Seventeen patients did not receive sunitinib as first line, but it was giving at point of treatment. Thirteen patients received in the second line, three patients received in the third line and one patient in the fourth line. Of those patients 8 (47%) had progressed disease and 9 (53%) had partial response or stable disease while in use of sunitinib.

Table 1
Major clinical-pathological features of renal cell carcinomas tumors

Variable	Clear cell			Nonclear cell		
	Total patients evaluated	*n*	(%)	Total patient evaluated	*n*	(%)
Age at diagnosis <57 y	51	27	52.9	13	5	38.5
Male	51	33	64.7	13	9	65.6
Time from diagnosis to metastasis <1 y	51	37	72.5	13	11	84.6
Stage IV at diagnosis	51	27	52.9	13	8	61.5
Fuhrman grade III and IV	36	20	55.6	7	4	57.1
Prior nephrectomy	51	43	84.3	13	10	76.9
Anemia	49	14	28.6	12	4	33.3
Neutrophilia	48	7	14.6	12	2	16.7
Plaquetosis	48	3	6.3	12	1	8.3
KPS ≤ 70	45	9	20.0	12	3	25.0
Time from diagnosis to treatment <1 y	50	33	66.0	13	9	69.2
More than one site of metastasis	51	28	54.9	13	10	76.9
Lung metastases present	51	33	64.7	13	8	61.5
Bone metastases present	51	16	31.4	13	5	38.5
Liver metastases present	51	10	19.6	13	3	23.1
Brain metastases present	51	1	2.0	13	1	7.7
Sarcomatoid features	51	4	7.8	13	2	15.4
TKI as first systemic treatment	49	35	71.4	13	11	84.6

KPS = Karnofsky performance status.

3.2. AXL expression in patients with RCC

The immunohistochemistry analysis of the 64 cases showed a wide variety of expression patterns, from negative to highly positive membranous or cytoplasmatic immunostaining (Fig. 2). Negative immunostaining (scores 0–2+) (Fig. 2A) was present in 34 (53.2%) patients and positive immunostaining (scores: 3+ to 6+) (Fig. 2B and C) was present in 30 (46.8%) of 64 cases. Endothelial cells were highly positive and it was used as internal control (Fig. 2). When analyzed all AXL scores cases separately from 0 through 6+, it was seen that 26 (41%) cases had score 0, 8 (13%) had score 2+, 4 (6%) had score 3+, 15 (23%) had score 4+, 4(6%) had score 5+ and 7 (11%) had score 6+ (Fig. 3).

We also evaluated the AXL positivity in the normal renal tissue of 41 patients and in the metastasis tissue of 14 patients. We could observe a rising in AXL positivity from normal renal tissue to tumor renal tissue and from a tumor renal tissue to a metastatic renal tissue, but with no statistical significance (Fig. 4A and B).

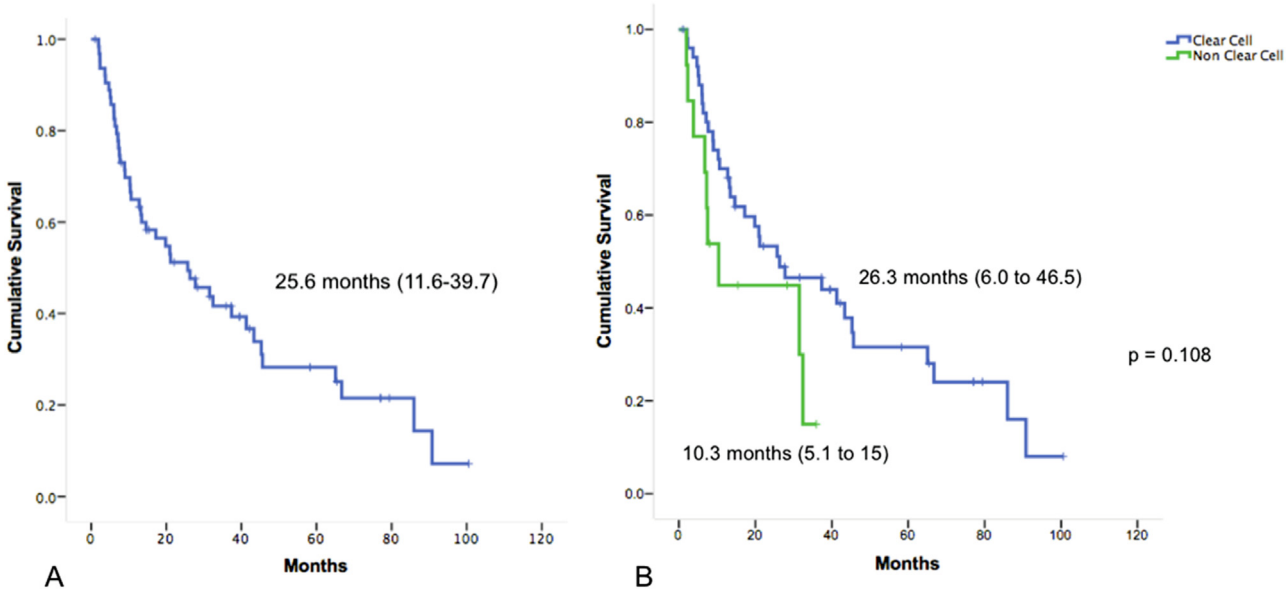


Fig. 1. (A) Overall survival according to initial metastasis diagnosis. (B) Overall survival according to initial metastasis diagnosis and subgroups RCC (clear cell and nonclear cell).

There was no statistical difference ($P = 0.235$) among the distinct histological subtypes. The clear cell subtype depicted a 43.1% (22/51) of AXL positivity, whereas the nonclear cell subtype showed a 61.5% (8/13) of positive AXL expression.

3.3. Association between AXL expression and clinical-pathological features

The univariate analysis between the AXL expression and clinical-pathological features for both histological subtypes combined, demonstrated that AXL positivity was associated with poor prognostic feature, like thrombocytosis, time from diagnostic to treatment less than a year, presence of bone metastasis, presence of brain metastasis and sarcomatoid histology, but none were statically significant besides progression while in sunitinib therapy (Table 2). In multivariate analysis, none of the variables had statically significance correlation with AXL positivity.

When analyze only the patients with CCC, in univariate analysis we also demonstrated that AXL positivity was associated with poor prognostic features, like thrombocytosis, time from diagnoses to treatment less than a year, presence of bone metastasis, presence of brain metastasis and sarcomatoid histology, but only older patients and progression while in sunitinib therapy was statistical significant (Table 2). In multivariate analysis older patients (HR = 4.75; 95% CI: 1.2–17.7; $P = 0.020$) and presence of bone metastasis (HR = 4.78; 95% CI: 1.17–19.4; $P = 0.029$) were significantly associated with AXL positivity.

The overall response while using sunitinib of the entire cohort of 64 patients, 35 (54.7%) patients had progression, 19 (29.7%) had stable or response disease, and 10 (15.6%) patients we were not able to access a response rate. In the univariate analysis, between all patients that used sunitinib and had AXL positivity, 21 (77.8%) patients had disease progression and 6 (22.2%) patients had response or stable

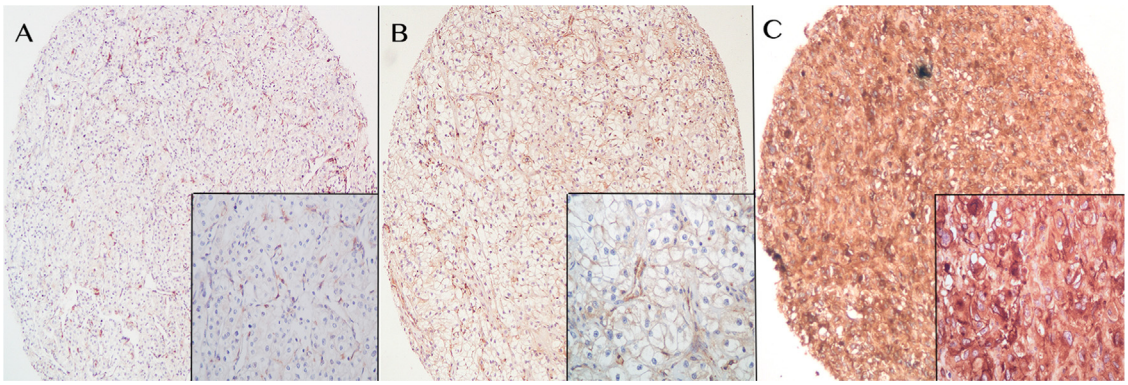


Fig. 2. Representative pictures of IHC analysis of AXL expression in clear cell RCC tissue (×100 scale and in right bottom ×400 scale). Positive marks in endothelial cells. (A) Negative immunostaining. (B) Positive immunostaining score 4+. (C) Positive immunostaining score 6+.

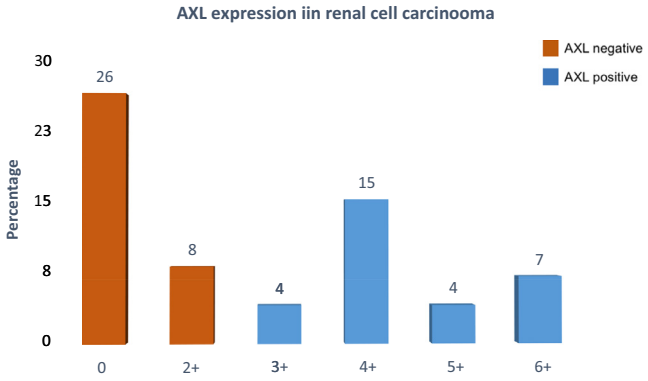


Fig. 3. AXL immunohistochemistry analysis of the 64 cases from 0 through 6+.

disease, whereas between patients with AXL negativity, 14 (51.9%) patients had disease progression and 13 (48.1%) patients had response or stable disease ($P = 0.046$) (Fig. 5A). Among only those with clear cell subtype, patients with AXL positivity, 16 (80%) patients had disease progression and 4 (20%) patients had response or stable disease while using sunitinib, while between patients with AXL negativity, 10 (43.5%) patients had disease progression and 13 (56.5%) patients had response or stable disease ($P = 0.027$) (Fig. 5B).

3.4. AXL expression predicts poor prognosis in patients with RCC

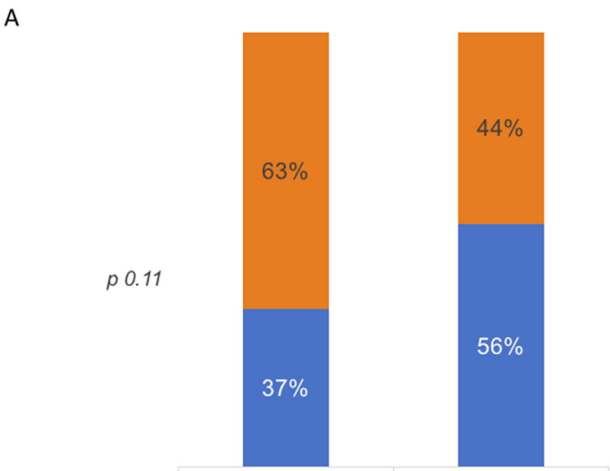
We further evaluated the association of AXL expression with patient outcome. We found that patients with positive AXL expression had a median OS of 41.2 months (95% CI: 10.1–72 mo), compared to 19 months (95% CI: 7.0–32 mo) of patients AXL negative, however, not reaching statistical significance ($P = 0.14$) (Fig. 6A). The multivariate analysis showed that AXL expression was a poor prognostic factor in patients with mRCC (HR = 2.007; CI: 1.006–4.006; $P = 0.048$). In addition of the positivity for AXL, other prognostic factors were absence of nephrectomy, more than 1 site of metastasis and bone metastasis (Table 3). Interestingly, when we stratified the positive AXL expression (3+ thought 6+) there were no difference in OS between groups (data not shown).

When we stratified by histological subtype, the CCC AXL negative cases ($n = 22$), exhibited an OS of 43.3 months (95% CI: 18.1–68 mo), whereas in the AXL positive cases ($n = 29$) was of 13.4 months (95% CI: 4.0–22 mo), also not reaching a significance ($P = 0.055$) (Fig. 6B). In multivariate analysis, AXL positivity in CCC was associated with poor prognostic along with absence of nephrectomy, Karnofsky performance status less than 70, more than 1 site of metastasis and presence of liver metastasis (Table 4).

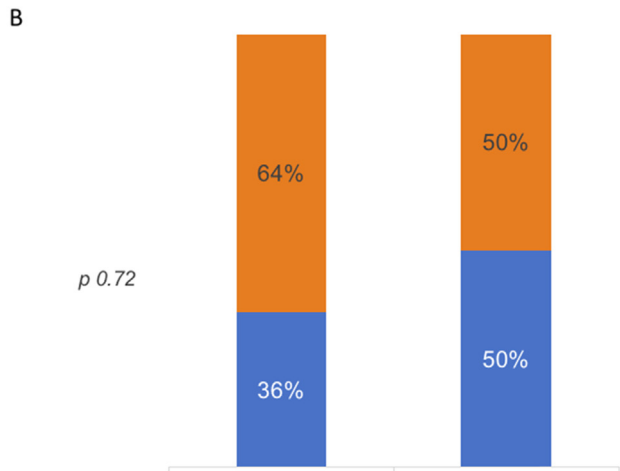
4. Discussion

Despite the advanced systemic treatment in mRCC in the past 10 years and the multiples drugs available [5,12], treatment decision is based in medical expertise rather than in objective predictive biomarkers [30]. With the in vitro evidences that tyrosine kinase AXL expression can be a predictive biomarker to sunitinib [21], our group investigated in the present study whether AXL overexpression in tumor renal tissue is associated with response rate in mRCC patients treated with sunitinib.

We analyzed 64 patients with mRCC treated with sunitinib. Demographic parameters and OS were similar with previous studies [23,31]. We could not correlate increased expression AXL with tumor response to sunitinib. However,



	Normal tissue	Tumor tissue
AXL Negative	26	18
AXL Positive	15	23



	Tumor tissue	Metastatic tissue
AXL Negative	9	7
AXL Positive	5	7

Fig. 4. *AXL* expression in different types of tissue. (A) *AXL* expression in 41 patients that had normal renal tissue and renal tumor tissue. (B) AXL expression in 14 patients that had renal tumor tissue and metastatic tissue.

Table 2
Univariate analysis between AXL expression and clinical-pathological features

Variable		All subtype			Clear cell subtype		
		Positive (*n*)	*P* value (%)		Positive (*n*)	*P* value (%)	
Sex	Male	22	52	0.223	16	48	0.29
	Female	8	3		6	33	
Age at diagnosis	<57 y	12	38	0.133	8	30	0.039
	≥57 y	18	56		14	58	
Hemoglobin concentration, g/dl	>11	5	29	0.10	3	23	0.093
	≤11	23	52		18	50	
Corrected calcium concentration	ULN	10	53	0.71	7	50	1a
	>ULN	6	46		5	50	
Neutrophil count, /mm^3	≤7,500	25	49	0.38	18	44	1
	>7,500	3	33		3	43	
Platelet count	≤450.000	26	46	1[a]	19	42	0.57[a]
	>450.000	2	50		2	67	
Karnofsky performance status	>70	25	56	0.06	18	50	0.37
	≤70	2	25		3	33	
Time from diagnosis to treatment	>1 y	8	38	0.28	5	29	0.13
	≤1 y	22	52		17	74	
Number of metastatic sites	1 site	11	42	0.545	9	39	0.60
	>1 site	19	50		13	46	
Lung metastasis	Absent	13	56	0.3	9	50	0.465
	Present	17	41		13	39.4	
Bone metastasis	Absent	19	44	0.6	12	34	0.059
	Present	11	52		10	63	
Liver metastasis	Absent	24	47	0.95	18	44	0.823
	Present	6	46		4	40	
Brain metastasis	Absent	28	45	0.21[a]	21	42	0.43[a]
	Present	2	100		1	100	
Histological type	Clear cell	22	43	0.235			
	Nonclear cell	8	62				
Sarcomatoid features	Absent	26	45	0.40	19	40	0.30
	Present	4	67		3	75	
Response to sunitinib	Stable/partial response	6	32	0.046	4	24	0.015
	Progression	21	60		19	40	

[a]Fisher's exact test.

we observed that AXL positivity in kidney cancer tissue is associated with shorter survival and a lack of response to the treatment.

AXL overexpression in tumor tissue has been associated with poor prognoses in a variety of cancers, probably because its involvement in various aspects of cellular

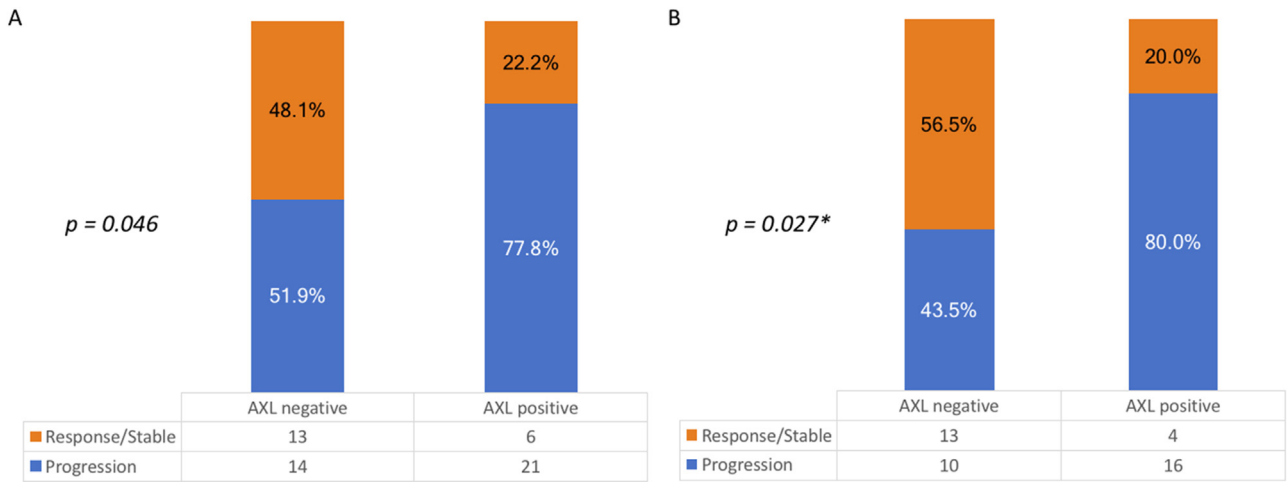


Fig. 5. Overall response to sunitinib according to AXL expression. (A) Entire cohort. (B) Patients with clear cell RCC.

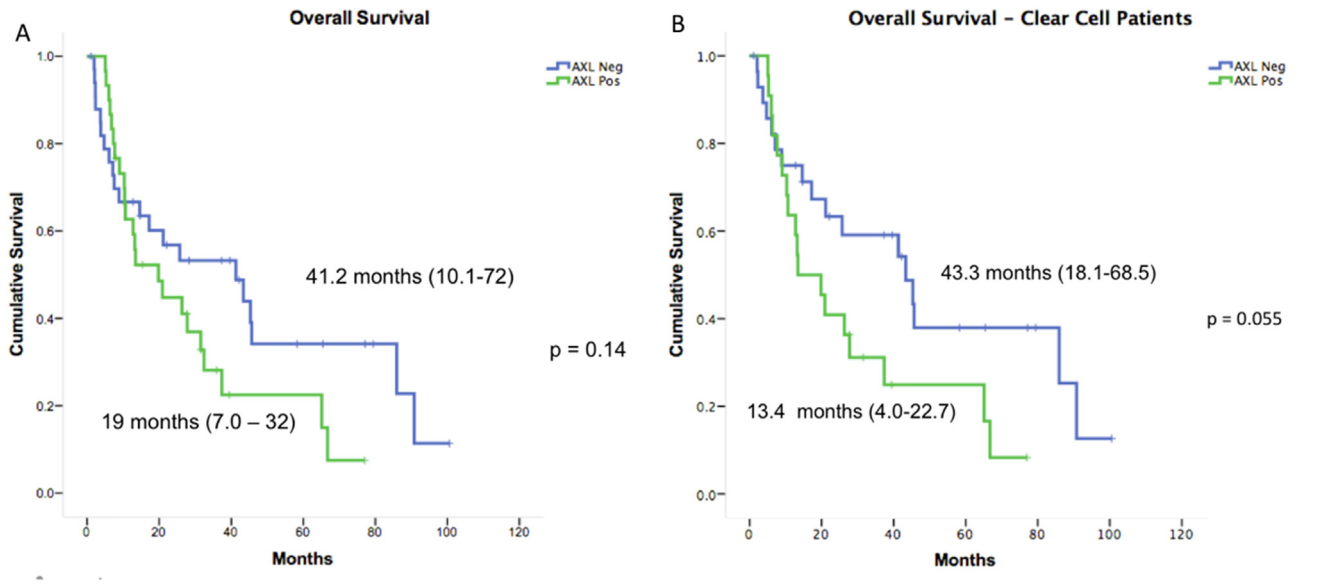


Fig. 6. (A) Overall survival of the entire cohort according to initial metastasis diagnosis and AXL expression. (B) Overall survival only the subtype clear cell RCC according to initial metastasis diagnosis and AXL expression.

signaling and it is implication in a wide variety of hallmarks of cancer [16]. Recent reports showed that high tumor AXL was an independent prognostic factor in patients with RCC [32,33]. Gustafsson et al. [32] in 2009, reported the tumor Axl mRNA was as an independent prognostic factor in cancer-specific survival, and Rankin et al. [33] in 2015 analyzed AXL expression in human RCC tissue within The Cancer Genome Atlas (TCGA) and samples with strong AXL expression was associated patients with reduced survival compared with patients whose samples had weak AXL expression. Our report, represents the first association of AXL positivity by immunohistochemistry with reduced survival in patients with mRCC treated with sunitinib.

AXL has been suggested to promote both intrinsic and acquired resistance to different treatments, from chemotherapy to molecular targets [15]. In RCC xenograft models upregulation of AXL and MET showed resistance to long-term sunitinib therapy, as well as resensitisation to sunitinib after AXL and MET inhibition via treatment with the TKI cabozantinib [34]. Cabozantinib has been demonstrated efficacy in 2 trials in mRCC [35,36]. A large phase III trial demonstrated that cabozantinib increased OS, delayed disease progression, and improved the objective response compared with everolimus after failing one or more anti-VEGF [35]. Recently cabozantinib was compared to sunitinib in first-line therapy in intermediated and poor risk IMDC (International Metastatic Renal Cell Carcinoma Database Consortium) group patients and was observed that cabozantinib had benefit in progression-free survival and overall response rate over sunitinib [36]. None of these 2 trials used a biomarker for predictive response to therapy. Despite of a variety promising new drugs emerging in the scenario of first-line therapy of mRCC, sunitinib might still have its place in some patients, namely in those cases with lack of AXL expression.

Our sample is representative of mRCC treated with sunitinib. However, the results from CCC setting have limited inference due to small exploratory sample ($n = 51$). Additionally, the significance of survival differences in CCC according to AXL staining is tangential. Unfortunately, we were unable to define risk criteria according Motzer et al. [24] and Heng et al. [23]

Table 3
Multivariable analysis—association of overall survival with prognostic risk factors in all subtypes patients

Parameter	All subtypes	
	Hazard ratio (95% CI)	*P* value
AXL positivity	2.007 (1.006–4.006)	0.048
Absence of nephrectomy	4.848 (1.945–12.081)	0.001
Presence of bone metastases	2.952 (1.514–5756)	0.001
More than 2 sites of metastases	2.989 (1.506–5.929)	0.002

Table 4
Multivariable analysis—association of overall survival with prognostic risk factors in patients with clear cell carcinoma

Parameter	Clear cell carcinoma	
	Hazard ratio (95% CI)	*P* value
AXL positivity	5.350 (2.062–13.885)	0.001
Absence of nephrectomy	8.383 (2.774–25.332)	0.000
More than two sites of metastases	3.895 (1.266–13.982)	0.002
Presence of liver metastases	4.208 (1.266–13.982)	0.019
KPS < 80%	2.603 (1.040–6.515)	0.041

for all patients due to paucity of clinical data. Therefore, further studies are warranted to extend and validate the present findings.

5. Conclusion

In the present study we reported that AXL was a prognostic biomarker in patients with mRCC treated with sunitinib. Interesting, AXL constitute also a target of the new drug cabozantinib, which demonstrated efficacy in the treatment in mRCC patients. Further studies are needed to demonstrate that cabozantinib is a target against patients with increased AXL expression.

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