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ORIGINAL ARTICLE

VEGF receptor subtypes may serve as novel prognostic factors and putative indicators for anti VEGF receptor treatment response in renal cell carcinoma cases

Omer Eronat, Aykut Onursever, Dinc Suren, Mustafa Yildirim, Olcay Kandemir, Haci Ali Kahya

Department of Pathology, Ataturk Research and Teaching Hospital, Ankara, Turkey

Summary

Purpose: *Targeted therapies are novel treatment options for renal cell carcinoma (RCC). Of the target molecules investigated, vascular endothelial growth factor receptors (VEFGRs) were seldom evaluated. The current study investigated the prognostic significance of VEGFRs and IMP-3 as a potential prognostic markers.*

Methods: *Pathological material and clinical files of 100 patients with RCC were retrospectively evaluated. For each case, the clinical outcome and disease stage were assessed and resected materials were histologically reevaluated. VEGFR-2, VEGFR-3 and IMP-3 expression of tumor samples were analyzed with immunohistochemistry. These expressions were compared with prognosis and clinicopathological variables.*

Results: *Five-year overall survival (OS) was 80% in the whole cohort. Mean survival was 20.3±1.9 months in metastatic disease (95%CI:16.4-24.2). Two-year OS was 20% and 5-year OS was zero in the metastatic group. Survival was significantly longer in VEGFR-2 expressing group than in the nonexpressing group (78.7±2.6 vs 63.9±6; 95%CI:73.7-84 and 52.1-75.7, respectively; p=0.031). VEGFR-3 and IMP-3 expressions were not significantly correlated with survival. In the non-metastatic group mean OS was 82.6±2.1 months and 2- and 5-year OS were 96 and 88%, respectively.*

Conclusions: *Since VEGFRs were expressed on all histological subtypes and significantly correlated with survival, assessment of VEGFR-2 and VEGFR-3 on tumor samples might serve as a putative prognostic factor in RCC cases. These expressions might identify a subset of patients that may benefit from antiangiogenic treatments targeting VEGFR receptors.*

Key words: *IMP-3, prognosis, renal cell carcinoma, VEGF receptors*

Introduction

RCC is the most common malignant tumor of the kidney. Its reported incidence is about 3 in 100,000 persons in USA [1]. Although various prognostic models and parameters exist, the biological behavior of any given RCC can not be predicted correctly [2]. Implementation of molecular markers into prognostic parameters is expected to improve the information about tumor behaviors and aid to identify subsets of patients who may benefit more from targeted treatments and increase survival gain.

Pathological parameters that significantly correlated with prognosis of patients have been well studied [3]. Of these Fuhrman grade, tumor stage and histological types as well as other pertinent variables are well known. In recent years, research on molecular biology of RCC revealed that angiogenesis is another important variable in patients with RCC [4].

The probability of achieving cure in RCC pa-

Correspondence to: Omer Eronat, MD. Department of Pathology, Ataturk State Hospital, 57000 Sinop, Turkey. Tel: +90 5327800411, Fax: +90 3682714471, E-mail: eronatomer@gmail.com, omereronat@hotmail.com
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tients is related to tumor stage and grade [5]. Surgical resection is the backbone of treatment in localized RCC, whereas it is only a palliative measure in metastatic cases. The majority of patients with metastasis requires systemic therapy, however treatment options are limited [5]. Despite recent development of therapeutic agents, response rates with biologic and immunologic therapies are usually lower and range between 15-25% [6]. Therefore, administering effective therapeutic agents, correcting staging and defining prognostic parameters are required to ensure the appropriate management of RCC.

Hematogeneous spread and high vascularity are well known pathological features of RCC. Recent molecular evidence showed that these pathological features may be attributed to specific genetic aberrations in RCC [7]. In the majority of RCC cells, von Hippel Lindau (VHL) gene is either deleted or mutated. This gene encodes a protein (pVHL) which downregulates the hypoxia induced factor 1α (HIF-1α) with ubiquitination during normoxic conditions. In hypoxic conditions, this downregulation does not occur, and HIF-1α induces signaling cascade which results in angiogenesis mediated with growth factors such as vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF) and transforming growth factor alpha (TGF-α). When the VHL gene is deleted or mutated, HIF-1α accumulates and results in upregulation of proangiogenic factors, notably VEGF. It would be expected that high VEGF expression or accumulation would be present in RCC. Indeed, recent investigations have supported this observation by measuring VEGF expression either through mRNA, VEGF serum levels or molecular assays [8]. VEGF expression was immunohistochemically observed in the vast majority of RCCs, and several reports noted increased VEGF expression in tumor tissue compared with normal renal tissue [2-12]. All these observations show that VEGF and its receptor (VEGFR) are one of the key mediators of angiogenesis in RCC.

In addition to VEGF, prognostic markers identifying the more aggressive tumors would help in management decision-making. Insulin-like growth factor 2 mRNA binding protein 3 (IGF2BP3, also known as IMP-3 or KOC) is an oncofetal RNA-binding protein that regulates targets such as insulin-like growth factor-2 (IGF-2) and ACTB (beta-actin). In recent analyses, in addition to renal tumors, it was shown to be correlated with aggressive and advanced solid malignant tumors such as neuroendocrine, skin, liver, bladder, breast, ovary, and soft tissue sarcomas [13,14].

The current study aimed to investigate the immunohistochemical expression of VEFGR-2, VEFGR-3 and IMP-3 in RCC tumor samples, and to look whether prognostic and pathological parameters correlate significantly with the expression of these biomarkers.

Methods

Patient selection

In the current study, we retrospectively retrieved cases that were coded as nephrectomy for tumor in the archives of Pathology department of Ataturk Training and Research Hospital between 2008 and 2013. In addition to histological material, the clinical files of all patients were evaluated and pertinent clinical information was recorded. Patient outcomes were categorized as no evidence of disease (NED), alive with disease (AWD) and died of disease (DOD). Patients that were lost to follow up, whose clinical or histological material were not available, were excluded from the current study.

Histological assessment

All slides and pathological reports were reevaluated retrospectively for histological subtype, primary tumor classification, nuclear grade, tumor size, lymph node involvement, vascular invasion and surgical margins. Based on histological and clinical information, TNM stage grouping was reassessed for all cases. During histological assessment, representative tumor samples were selected for immunohistochemical analysis.

Immunohistochemical evaluation

Immunohistochemical studies were performed on 5-μm sections of formalin-fixed, paraffin-embedded archival tissue. Immunohistochemical primers, manufacturers, clones, dilutions and incubation times in the current study were as follows: VEGFR-2 (Abcam, Cambridge-UK (ab39256): polyclonal IgG, 200μg/ml) 1:200 in phosphate buffered saline (PBS) for 60 min; VEGFR-3 (Novocastra, Newcastle-UK, Clone: KLT9, IgG2b kappa, 200μg/ml) 1:50 in PBS for 60 min; and IMP-3 (DAKO, California-USA, Clone: 69.1 Monoclonal Mouse Anti-Human IMP-3 IgG2a, kappa, 200μg/ml) 1:100 in PBS for 60 min.

After primary antibody incubation, the sections were incubated in biotinylated goat anti-polyvalent solution for 20 min, washed in PBS, and submerged for another 20 min in streptavidin-peroxidase solution. To identify the immunoreaction, diaminobenzidine chromogen (DAB) was used for 3 min, followed by counterstaining with Harris hematoxylin.

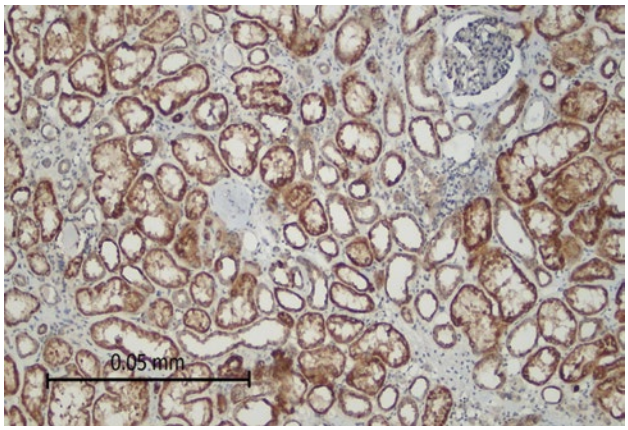

Figure 1. VEGFR-2 expression in renal tubular epithelium (x100).

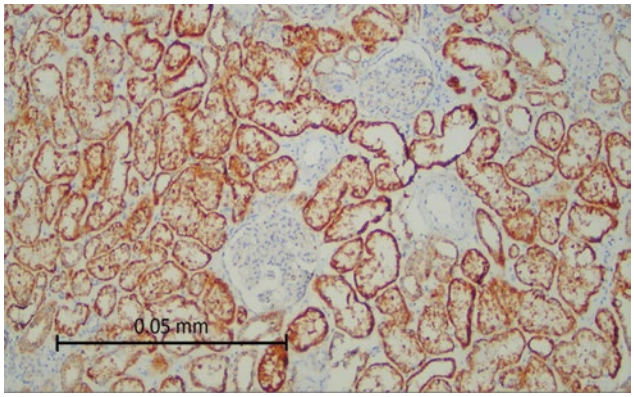

Figure 2. IMP-3 expression in renal tubular epithelium (x100).

Semi quantitative evaluation of immunohistochemical expression

The expression level of all markers was divided into subcategories. Staining was scored as follows: 0 indicated no staining; 1+ indicated less than 10%; 2+ indicated 10 to 75%; 3+ indicated greater than 75%. Score 0 was accepted as negative, and scores 1, 2 and 3 as positive. For each marker, cytoplasmic reactions in the tumor cells were evaluated as positive staining. If no accompanying membranous or nuclear staining were available, appropriate external positive controls were used: Placental tissue for VEGFR-2 and VEGFR-3, and tonsils for IMP-3 served as positive control. We observed cytoplasmic reaction with all three markers in renal tubular epithelium (Figures 1 and 2). No expression was observed in glomerular epithelium, vascular structures, perirenal fat tissue or other non-neoplastic tissues.

Statistics

Correlations of expression levels of VEGFR-2, VEGFR-3, and IMP-3 with clinicopathologic features were evaluated using Fisher's exact probability test and chi-square test. A p value less than 0.05 was regarded as statistically significant. Statistical analyses were performed using the SPSS software (version 16.0, Chicago, IL, USA).

Table 1. Clinicopathologic characteristics

Characteristics	*N (%)*
Gender	
Female	29 (29)
Male	71 (71)
Male/Female ratio	2.44:1
Age, median (range)	
Female	60.7 (46-78)
Male	57.5 (30-81)
Histologic subtype	
Clear Cell	74 (74)
Papillary Type 1	10 (10)
Papillary Type 2	6 (6)
Chromophobe	8 (8)
Multilocular Cystic	2 (2)
Tumor size	
Median (range), cm	6.02 (0.8 -17.5)
< 4	40 (40)
4-6,9	34 (34)
7-10	16 (16)
> 10	10 (10)
Fuhrman nuclear grade	
Grade 1	7 (7)
Grade 2	26 (26)
Grade 3	51 (51)
Grade 4	16 (16)
pT stage	
pT1a	37 (37)
pT1b	33 (33)
pT2	17 (17)
pT3a	9 (9)
pT3b	2 (2)
pT3c	0
PT4	2 (2)
Distant metastasis	
M0	95 (95)
M1	5 (5)
Regional lymph node involvement	
Nx	96 (96)
N1 and N2	4 (4)

Results

One hundred cases were retrieved and studied. There were 48 radical, 29 simple, and 23 partial nephrectomy specimens. Of the patients 29

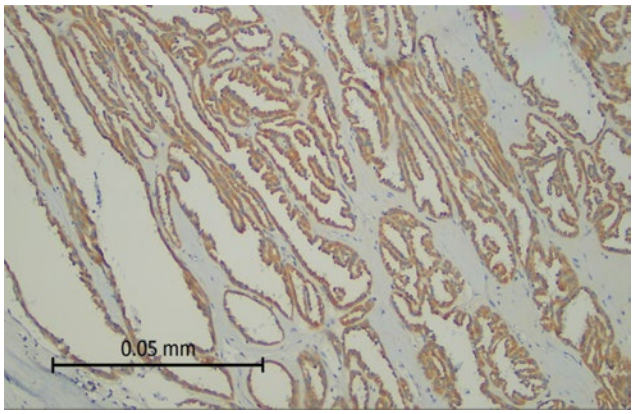

Figure 3. VEGFR-2 expression in papillary type 1 RCC (x100).

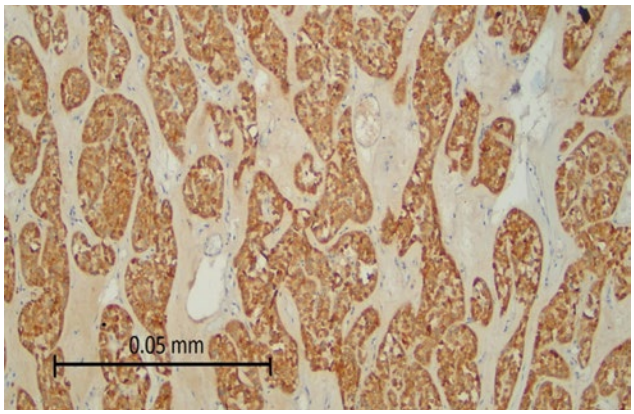

Figure 4. VEGFR-2 expression in chromophobe type RCC (x100)

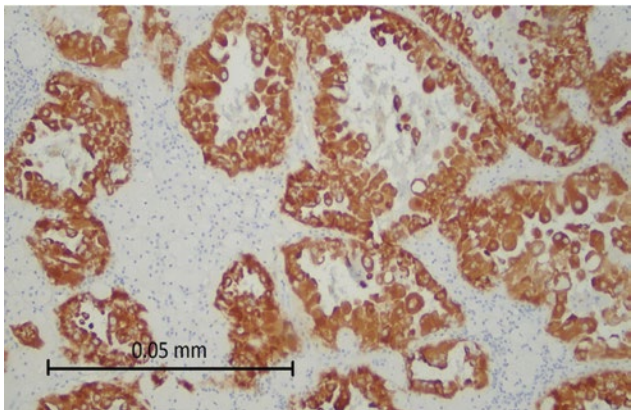

Figure 5. VEGFR-3 expression in papillary type 2 RCC (X100).

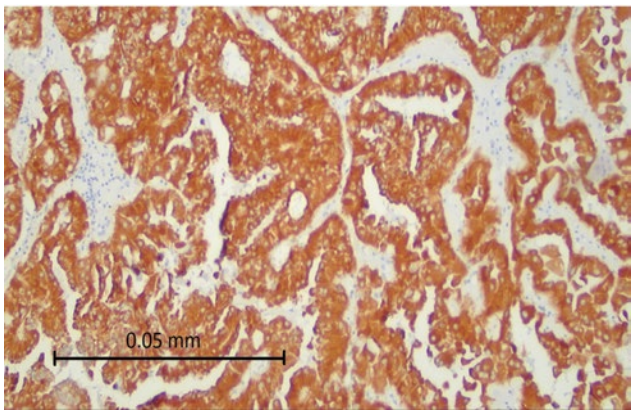

Figure 6. IMP-3 expression in papillary type 2 RCC (X100).

Table 2. The expression levels of VEGFR-2, VEGFR-3, and IMP-3 in histological subtypes

Marker	*Expression*	*Histologic subtype*					*p value*
		Clear cell	*Chromophobe*	*Papillary Type 1*	*Papillary Type 2*	*Multilocular cystic*	
VEGFR2	0	25	0	1	0	1	< 0.001
	1+	20	0	1	0	0	
	2+	19	2	3	2	0	
	3+	10	6	5	4	1	
VEGFR3	0	23	0	1	0	0	< 0.001
	1+	30	1	2	1	0	
	2+	18	2	3	1	1	
	3+	3	5	4	4	1	
IMP3	0	47	0	5	1	1	< 0.001
	1+	16	1	2	0	0	
	2+	9	1	0	0	1	
	3+	2	6	3	5	0	

(29%) were female and 71 (71%) male. Male/female ratio was 2.44. Mean age of female patients was 60.7 years (range 46-78), and of male patients 57.5 years (range 30-81). Histological types of RCCs consisted of 74 clear cell RCC (74%), 10 papillary type 1 (10%), 6 papillary type 2 (6%), 8 chromophobe (8%), and 2 (2%) multilocular cystic cases. The median diameter of tumors was 6.02 cm (range 0.8-17.5). The number of cases with tumor diameter less than 4 cm, between 4-7 cm,

Table 3. Comparison of tumor size and the expression levels of VEGFR-2, VEGFR-3, and IMP-3

Marker	*Expression*	*Tumor size*				*p value*
		< 4cm	*4.1-7cm*	*7.1-10cm*	*> 10cm*	
VEGFR2	0	8	9	7	3	> 0.249
	1+	5	9	5	2	
	2+	14	9	2	1	
	3+	13	7	2	4	
VEGFR3	0	10	6	6	2	> 0.554
	1+	11	13	6	4	
	2+	9	9	4	3	
	3+	10	6	0	1	
IMP3	0	24	17	8	5	> 0.890
	1+	6	9	3	1	
	2+	3	4	2	2	
	3+	7	4	3	2	

Table 4. Comparison of tumor stage and the expression levels of VEGFR-2, VEGFR-3, and IMP-3

Marker	*Expression*	*pT stage*						*p value*
		pT1a	*pT1b*	*pT2*	*pT3a*	*pT3b*	*pT4*	
VEGFR2	0	8	8	5	2	2	2	> 0.345
	1+	4	9	4	4	0	0	
	2+	13	9	3	1	0	0	
	3+	12	7	5	2	0	0	
VEGFR3	0	10	6	4	3	0	1	> 0.847
	1+	10	12	6	3	2	1	
	2+	9	9	6	1	0	0	
	3+	8	6	1	2	0	0	
IMP3	0	23	16	7	4	2	2	> 0.796
	1+	5	9	2	3	0	0	
	2	3	4	3	1	0	0	
	3+	6	4	5	1	0	0	

between 7-10 cm, and over 10 cm were 40 (40%), 34 (34%), 16 (16%), and 10 (10%), respectively. According to Fuhrman nuclear grade, 7 patients (7%) had grade 1, 26 (26%) had grade 2, 51 (51%) had grade 3, and 16 (16%) had grade 4 tumors. Thirty seven patients had T1a (37%) and 33 T1b (33%) disease. T2, T3a, T3b, and T4 disease was noted in 17 patients (17%), 9 patients (9%), 2 patients (2%), and 2 patients (2%), respectively. Regional lymph node involvement was positive in 4 patients (4%). Distant metastasis was positive in 5 patients (5%). Table 1 shows the results of clinicopathologic features.

All three markers were widely expressed in both papillary and chromophobe histologic subtypes (Figures 3, 4, 5 and 6). The expression rate in clear cell RCC was 66.2% for VEGFR-2, 68.9% for VEGFR-3, and 36.5% for IMP-3. The expression rates for chromophobe, papillary type 1 and type 2 RCC were 100, 90, and 100% for VEGFR-2, respectively, and 87.5, 90 and 100% for VEGFR-3, respectively. The expression rate of IMP-3 was 50% for papillary type 1, 83.3% for papillary type 2, and 100% for chromophobe RCC. Of the two multilocular cystic types, one case had VEGFR-2 and IMP-3 expression, however both cases ex-

Table 5. Comparison of Fuhrman nuclear grade and the expression levels of VEGFR-2, VEGFR-3, and IMP-3

Marker	*Expression*	*Fuhrman nuclear grade*				*p value*
		Grade 1	Grade 2	Grade 3	Grade 4	
VEGFR2	0	4	5	10	8	> 0.265
	1+	0	6	12	3	
	2+	1	8	14	3	
	3+	2	7	15	2	
VEGFR3	0	4	3	10	7	< 0.018
	1+	0	11	16	7	
	2+	2	8	13	2	
	3+	1	4	12	0	
IMP3	0	6	14	23	11	> 0.564
	1+	0	5	11	3	
	2+	1	3	6	1	
	3+	0	4	11	1	

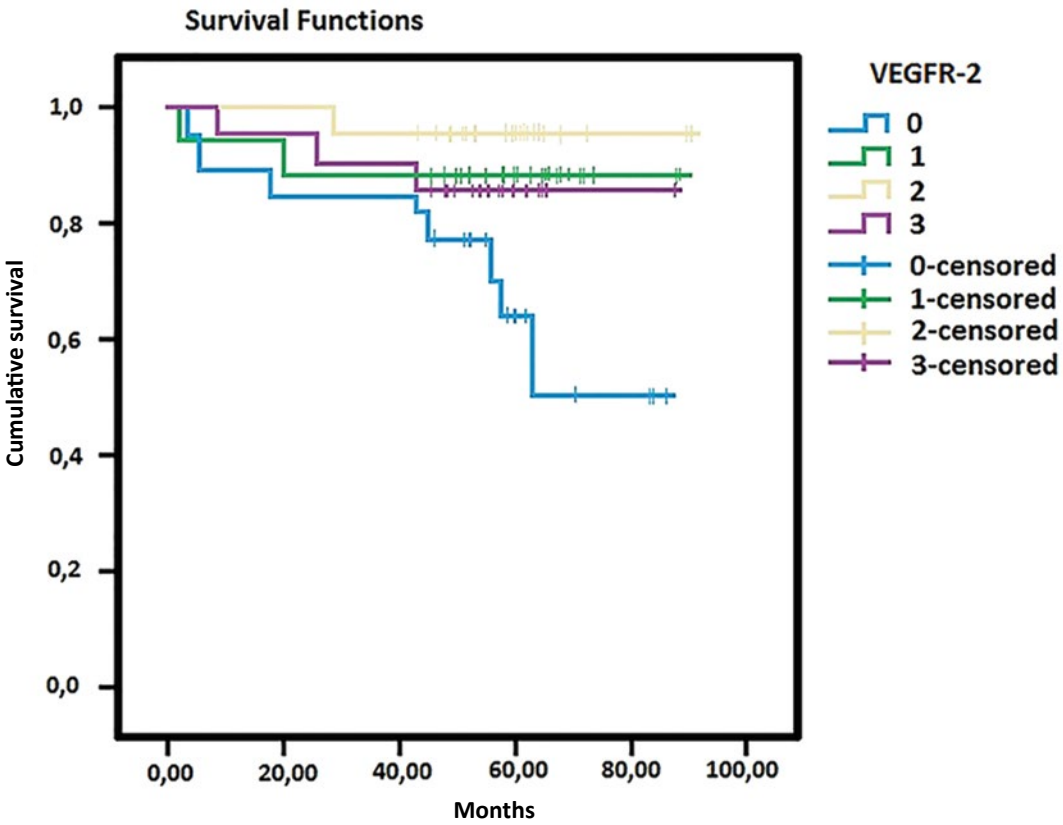

Figure 7. .Relation of VEGFR-2 expression and survival (p=0.031).

pressed VEGFR-3 (Table 2). All four node positive cases expressed VEGFR-3.

No correlation was observed between tumor size, tumor stage and the expression level of VEGFR-2, VEGFR-3 and IMP-3 (Tables 3 and 4).

Mean follow up period was 51.9±16.8 months and mean OS was 78.9±2.6 months. When all of the cases were evaluated as a whole, 5-year OS

was 80%. Mean OS was 20.3±1.9 months in cases with metastatic disease, 2-year OS was 20% and 5-year OS was zero. In the non-metastatic group mean survival was 826±2.1 months, 2-year OS 96% and 5-year OS 88%.

VEGFR-2 expression was significantly associated with survival (p=0.031) (Figure 7). Mean OS was about 15 months more in VEGFR-2 expressing group in comparison to non expressing group (78.7±2.6 vs 63.9±6; 95%CI 73.7-84 and 52.1-75.7, respectively, p 0.054). VEGFR-3 and IMP-3 expressions were not significantly correlated with survival. Fuhrman nuclear grade was significantly correlated with the expression level of VEGFR-3 (p <0.018), and was not significantly correlated with VEGFR-2 and IMP-3 expression (p>0.265, p>0.564, respectively). The results are summarized in Table 5. Metastatic disease, lymph node status, Fuhrman grade, capsular invasion, infiltration of Gerota's fascia, involvement of renal pelvis and adrenals were significantly correlated with OS. T stage, invasion of renal veins, presence of necrosis, infiltration of renal parenchyma were not significantly correlated with OS (p=0.444, p=0.365, p=0.177 and p=0.054, respectively).

Discussion

The median overall survival of RCC patients has been reported to exceed often 2 years [1,2]. Overall, the estimated average 5-year survival rates for patients with RCC are 96% for those presenting with stage 1 disease, 82% for those with stage 2, 64% for stage 3, and 23% for stage 4 [2,14,15]. In the current study, 2-year survival of metastatic cases was 20%, whereas 5-year survival was zero. Our observations are similar to rates reported by other authors [16].

RCC is a heterogeneous disease with diverse histological types with different prognosis. Four subtypes of RCC have been identified (clear cell, papillary, chromophobe, and collecting duct). These histological subtypes have different cell types and growth pattern [17-19]. Of these subtypes, papillary RCC can be further divided into subtypes, each associated with very different prognoses. Type 1 papillary RCCs are low-grade tumors with a chromophilic cytoplasm and a favorable prognosis, and type 2 are generally high-grade tumors with eosinophilic cytoplasm and are associated with a higher risk of metastatic progression and poor prognosis [1,6,16,17]. In the current study, of all cases, 10% were type 1 papillary RCC, and 6% were type 2 papillary RCC. The number of papillary subtypes was limited to perform statistical comparisons.

VEGFR-2 and VEGFR-3 expression on tumor samples of RCC cases were assessed in the current analysis to clarify any significant correlation with prognostic parameters. Our results displayed that VEGFR-2 expression patterns were correlated with better prognosis. To the best of our knowledge, studies that evaluated VEGF receptors in RCC cases are very limited to make comparisons [19,20]. Zhang et al. showed that whereas VEGFR-2 has been significantly associated with favorable prognosis, VEGFR-3 has not. However, VEGFR-3 was significantly correlated with the degree of lymph node involvement and metastasis [20]. Del Puerto-Nevado et al. analysed VEGFR-2 expression along with other molecular targets in tumor samples from 48 RCC patients including 23 cases who had sunitinib treatment [21]. Some authors found that VEGFR-2 expression on tumor stroma could be utilized as predictive marker of prognosis [20]. These observations support our findings that VEFGR-2 could be utilized as predictive biomarker for prognosis in RCC cases.

Expression profiles of VEGF and VEGFR might differ among tumor types and stages. Leppert and co-workers published their results of a tissue microarray analysis to investigate the expression of VEGF-A, VEGF-C, VEGF-D, and VEGF receptors VEGFR-1, VEGFR-2, and VEGFR-3 in different histological types of RCC [22]. Papillary RCC showed higher mean expression of VEGF-A and VEGFR-2 in comparison to clear cell type. However, samples from clear cell tumors showed higher expression rates of VEGF-D, VEGFR-1, 2 and 3 within tumor-associated endothelium than papillary tumors. In addition, expression of VEGFR-1 and -2 was shown to be significantly correlated with the presence of distant metastases, whereas VEGFR-3 expression on tumor-associated endothelium was significantly associated with prediction of lymph node metastases [22]. These expression patterns may assist to direct targeted therapies to the appropriate patients with RCC. Based on these observations, it may be postulated that cases with papillary RCC might benefit from therapies targeting the VEGF-A pathway, whereas patients with clear cell RCC may be more likely to benefit from biological agents targeting VEGFR-3.

Sunitinib is one of the antiangiogenic agents targeting VEGFR, and is used in the treatment of metastatic RCC. Despite initial reports that suggested its efficacy in metastatic RCC, today it is well known that about 60% of patients with met-

astatic RCC are refractory to VEGF inhibitors [22-24]. In addition of being ineffective, relevant toxicities in refractory patients is another consideration. Criteria for patient selection are still controversial [24]. Therefore, definition of criteria for which patients would benefit from tyrosine kinase inhibitors (TKIs) as well as sunitinib would be mainstay of an effective treatment with reasonable benefit/harm ratio. Based on our observations, it may be proposed that absence of VEGFR in certain histological subtypes and features might explain why some RCC cases do not respond or become refractory to VEGFR inhibitors.

Several explanations were hypothesized for sunitinib efficacy in RCC [23]. Deprimo et al. showed that TKIs induce an increase in VEGF levels as well as a decrease in VEGFR-2 and VEGFR-3 in patients who are refractory to cytokines (INF-α and IL-2) [24]. In addition, several authors observed that these levels returned to normal in two weeks after discontinuation of sunitinib. Furthermore, in patients with objectively assessed tumor response, VEGF, VEGFR-2 and VEGFR-3 changes were much larger than those of patients whose disease had progressed [22,24]. These observations may indicate that sunitinib treatment leads to modulation of serum levels of VEGF, VEGFR-2 and VEGFR-3. Based on these observations, it may be proposed that VEGF, VEGFR-2 and VEGFR-3 levels could have significant impact on tumor progression in patients receiving antiangiogenic treatments.

IMP-3 was shown to be a significant predictor of metastasis and cancer related deaths in cases with metastatic RCC [25]. In the current study, we did not observe a significant correlation of IMP-3 expression with prognostic parameters of the cases (Furhman grade, tumor size and TNM stage), whereas IMP-3 expression was different among histological subtypes. We believe that utilizing different clones and dilutions in the immunohistochemical analysis would be the basis for differences among studies. Utilization of same clones and same dilutions on immunohistochemical analyses in larger prospective studies would clarify the importance of IMP-3 as predictive factor in metastatic RCC patients.

We conclude that assessment of VEGFR-2 and VEGFR-3 expression on RCC samples might identify a subset of patients that may benefit from antiangiogenic treatment targeting VEGF or VEGFR receptors.

Conflict of interests

The authors declare no conflict of interests.

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