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Pre-Treatment Status and Changes in Autoantibodies, Lymphocytic Populations, Cytokines and VEGF during Sunitinib Treatment of Metastatic Renal Cell Carcinoma (mRCC)

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Abstract

Aims

Immunotherapy has been effective in mRCC. Given the improved efficacy of anti-VEGF therapies in mRCC, their effect on the immune system emerges as a reasonable question.

Methods

Serum autoantibodies (aAbs), Interferon-γ (IFN-γ), Interleukins (ILs), VEGF and blood lymphocytic populations were determined in 43 previously untreated mRCC patients, before and during sunitinib therapy.

Results

81% of patients had at least 1 baseline aAb. During treatment 83% of patients without baseline aAbs developed aAbs, while C3 and IL-6 levels were increased. All changes were observed the first 9 months of treatment but had no prognostic significance. Baseline low VEGF and IL-6 levels were associated with improved Progression-Free (PFS) and Cancer-Specific (CSS) Survival.

Conclusions

Sunitinib treatment can result in aAbs development but this does not improve prognosis. Baseline VEGF and IL-6 were correlated with outcome.

Keywords: Auto-antibodies; Cytokine levels; Lymphocytes; Vegf; Sunitinib; Renal cell carcinoma

Introduction

Renal Cell Cancer (RCC) accounts for approximately 3% of all cancers. Half of the patients with localized disease can be cured after surgery, but advanced or mRCC has a poor prognosis with only a minority of patients achieving long-term survival.

Systemic therapy of mRCC has been largely ineffective. Immunotherapy using interferon-α (INF-α) and interleukin-2 (IL-2) has long been the only effective treatment and high dose intravenous IL-2 has been the only treatment that can cure some patients with mRCC [1-3]. Furthermore, immunotherapy with vaccines has shown some promise in the adjuvant setting [4] and new targeted molecules such as anti-PD-1, that prevents the cancer cell-induced suppression of T cells, are currently tested in phase III trials [5,6]. Therefore, induction of an anti-tumor-reactive immune response and its augmentation has been an area of intense research in advanced RCC. Studies have shown that RCC patients experience an immune system dysfunction, through a shift from a Th1-type CD4+ T-cell mediated response (producing INF-γ and favouring the development of effective antitumor immunity), to Th2-type cytokine response (IL-4, IL-5, IL-10) that typically biases humoral immunity [7]. Consequently, enhancing the antitumor activity of the immune system in RCC patients is a rational therapeutic strategy.

RCC is a highly vascularised tumour and angiogenesis is a critical step in the development and metastasis of the tumour. Therefore, angiogenesis has become a major therapeutic target for this disease. RCC is the first malignancy in which inhibition of angiogenesis, mediated either by bevacizumab [8,9], a monoclonal antibody against VEGF, or Tyrosine Kinase Inhibitors (TKIs) of its transmembrane receptor (VEGFR) has become the treatment of choice in the advanced setting. Sunitinib is a multiple TKI of VEGFR-1, -2 and -3, but also of platelet-derived growth factor receptor (PDGFR a,b), c-KIT, Fms-Like Tyrosine Kinase-3 Receptor (FLT3) and the receptor encoded by the ret proto-oncogene (RET) [10]. It has demonstrated significant antitumor activity by improving the response rates (RRs) as well as PFS compared to IFN-α and has become the drug of choice in first line treatment of mRCC [10-12].

Despite the considerable efficacy of sunitinib, a significant percentage of patients will not benefit from therapy. There is, therefore, a need to identify factors, which might predict response (or resistance) to therapy. Clinical models, which have been mainly used in the cytokine era, have failed to clearly identify patients who should not be offered novel therapies [13]. Biological markers have also been studied. Soluble

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VEGFR-3 (sVEGFR-3), basic Fibroblast Growth Factor (bFGF) and IL-8, that can promote angiogenesis independently of VEGF, have shown correlation with clinical response in preliminary studies [14-16]. From the retrospective analysis of phase II and III trials of pazopanib used in the metastatic setting, high levels of IL-6 and IL-8 were associated with shorter PFS in untreated patients, while high concentrations of IL-6 were predictive of improved relative PFS benefit from pazopanib compared with placebo [17]. The immunosuppressive effect of VEGF has been suggested by *in vitro* studies, showing suppression of the ability of Dendritic Cells (DCs) to induce T-lymphocyte proliferation [18] and the cytotoxic ability of T cells isolated from ascites from ovarian cancer patients [19]. Furthermore, clinical data are also supporting the immunosuppressive action of VEGF [20,21]. Based on these data, it is logical to assume that anti-VEGF therapies would enhance antitumor immune response. Existing data on the effect of anti-VEGF agents on anti-tumor immune response is limited, is mainly retrospective and has been the subject of controversy. A German study in mice showed that sorafenib but not sunitinib had a detrimental effect on DC phenotype and inhibited cytokine secretion and function. In the same study, sunitinib significantly reduced the number of Tregs that constitute a major immunosuppressive burden in cancer immunotherapy [22]. These results are in sharp contrast with others showing that sorafenib and bevacizumab, but not sunitinib, reverse the inhibitory effects of VEGF on DC differentiation *in vitro* [23], while Ko et al. showed that sunitinib impairs the proliferation and function of human peripheral T cells and prevents T-cell-mediated immune response [24]. Finke et al. measured the changes in INF-γ and IL-4 as representative cytokines of type-1 and type-2 immune response, respectively, in RCC patients on sunitinib monotherapy. After only one cycle of sunitinib the immune response was reversed from type-2 to type-1 and the number of Tregs was decreased [7]. The same result was observed in sunitinib-treated RCC patients via the reduction of Myeloid-Derived Suppressor Cells (MDSC) [25].

Lately, a French study showed that sunitinib-induced Tregs reduction in peripheral blood as well as in the tumor microenvironment in mRCC patients was correlated with better Overall Survival (OS). Interestingly, this association was mainly observed in patients with high baseline levels of Tregs, reversing by this way the natural unfavourable prognosis of these patients [26]. The development of autoimmunity during cytokine therapy was shown to predict a survival benefit in patients with mRCC as well as in patients with melanoma [27,28]. Franzke et al. found that autoimmunity resulting from cytokine treatment predicted long-term survival in 329 patients with mRCC [27]. More specifically, the evaluation of thyroid aAbs during cytokine therapy appeared to be a useful prognostic marker of survival for patients with RCC who benefited by this treatment. The thyroid aAb formation resulting from cytokine therapy was statistical strongly correlated with thyroid dysfunction [27]. Knowing that hypothyroidism is a common adverse event of sunitinib, the underlying mechanism expectedly drew scientific interest and also provided a rational to further investigate sunitinib in advanced thyroid cancer [27]. Different mechanisms for hypothyroidism have been investigated and proposed, such as destructive thyroiditis through follicular cell apoptosis, inhibition of peroxidase activity or marked capillary regression, but to our knowledge the titres of thyroid aAbs have not been assessed [29-32].

Against this background of uncertainty regarding the effect of the new TKIs on the immune system and the predictive value of such an effect, we conducted a prospective study in patients with mRCC who were treated in first line with sunitinib in order to address these issues. We measured immune parameters before and during therapy with sunitinib until disease progression. More specifically, we studied aAbs, lymphocytic populations, cytokines and VEGF and we correlated these results, with patients' characteristics and with prognosis.

Methods

Patients

Treatment-naïve patients with histologically confirmed advanced RCC not amenable to surgery, receiving first line therapy with sunitinib in our institution, were prospectively evaluated. Sunitinib was administered at 50 mg daily on a 4 weeks on-2 week off schedule, until disease progress according to the RECIST criteria or until unacceptable toxicity. Patients with known immunological disease or hepatitis, or under immunosuppressive drugs were excluded. Adjuvant interferon was allowed, provided that an interval >1 year had elapsed. All patients gave their informed consent prior to entering the study, which was approved by the Institutional Review Board.

Cell isolation

Peripheral blood (7 ml) was collected prior to the initiation of sunitinib treatment and at 3-4, 6-9, >12 months and at progression or discontinuation of treatment. Following centrifugation at 1750 rpm for 10 minutes at room temperature, serum was removed and stored at -80°C. Cells were separated over a Histopaque (Sigma Chemical Co, St Louis, MO) density gradient for 30 min at 400 g at 16°C. The dense layer enriched for mononuclear cells, was collected and washed.

Flow cytometric analysis

Peripheral blood mononuclear cells were stained with monoclonal Abs and analyzed by flow cytometry to determine cell type and immunophenotype. Flow cytometry analyses were performed on a three-colour fluorescence FACSCalibur cytometer using CellQuest software (Becton-Dickinson, CA, USA). At least 5000 gated events/condition were analyzed. CD45-positive (+) and EpCAM+ cells were measured as a percentage of the total number of cells. Immune subpopulations were measured as a percentage of the total number of CD45+ cells and a percentage of the total number of CD3+ cells. Activation marker expression was assessed on gated CD3+ cells by plotting CD4+ or CD8+ cells versus the given activation marker. CD4+CD25+hi cells were defined as the CD4+CD25+ T cells with CD25 level of expression higher than that of CD4-negative (-) CD25+ cells, according to Hoffmann et al. [33].

VEGF was determined by an Enzyme-Linked Immunosorbent Assay (ELISA) according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). T3, T4 and TSH were measured using an Enhanced Chemiluminescence Immunoassay (ECLIA) and chemiluminescence in the Cobas Modular Elecsys (ROCHE USA) and in the ADVIA Centaur (SIEMENS USA) machine. Anti-dsDNA, anti-Tg and anti-TPO were measured with ELISA. ANA, AMA, ASMA, cANCA, pANCA were calculated using indirect Immunofluoresence Assays (IFA). Titres defining positivity were 1/80,1/40,1/20,1/80 and 1/50 respectively. C3,C4,RF were measured with a nephelometer.

Statistical analysis

All analyses were performed with the SPSS for Windows v14 software (SPSS 13.0 Inc., USA). Correlation coefficients between non categorical variables were calculated according to Spearman's rank correlation coefficient (rho) and two-tailed significance levels were calculated. Independent correlations were assessed using linear regression analysis. Related samples were analyzed using the paired

Wilcoxon test. Nonparametric Kruskal-Wallis H and Mann-Whitney U tests were used to compare medians of cell populations. VEGF levels and percentages of lymphocytic populations were also studied in relation to PFS and OS as categorical variables after they were dichotomized at the level showing the highest statistical significance. Tumour assessment was made by spiral computed tomography at baseline and every 3 cycles based on RECIST criteria. CSS and PFS were calculated from the day of initiation of treatment until the date of last follow-up, death from renal cancer (for CSS) or progression (for PFS). Patients dying from other causes were censored at the time of death. Survival curves were produced with the Kaplan-Meier method and differences according to categorical variables were compared with the log rank test. The association of continuous variables with survival was assessed using cox regression analysis. Changes of aAbs during treatment were categorized as follows: 0: No baseline aAbs, no aAbs during treatment; 1: aAbs at baseline, which remain unchanged during treatment; 2: occurrence of new aAb positivity during treatment; 3: aAbs at baseline, which became negative during treatment. All p values were two-sided and 5% was chosen to denote significance.

Results

Between May 2007 and March 2011, data from 43 patients with mRCC were available. Their baseline characteristics are shown in Table 1.

Baseline autoantibodies, lymphocytic populations and cytokines

The frequency of positive baseline aAbs is shown in Table 2. In 41 patients who had estimation of all aAbs at baseline, 8 (19%) had no aAbs, 11 (27%) had 1, 15 (36%) had 2, 6 (15%) had 3, and 1 (2%) had 4. The most frequent aAb detected was ASMA (63% of patients). Fifty-one % of our patients had positive ANA, but only 2 of these patients had anti-dsDNA antibodies. No patients had positive cANCA, while only 3 had pANCA. All patients with pANCA also had AMA, but there was no correlation with ANA or ASMA positivity. One patient had both anti-TPO and anti-Tg aAbs, but no hypothyroidism. This patient also had ANA, but no correlation with other aAbs was observed. Only one patient had biochemical hypothyroidism (TSH > 5) but had neither anti-TPO nor anti-Tg Abs. Finally, no correlation between ANA, AMA or ASMA was observed. Baseline determinations of lymphocytic populations were available for 37 patients, while cytokine data were available for 25 patients (Table 2). There was no correlation of any lymphocytic population with positive baseline aAbs or baseline cytokines or with baseline VEGF levels. On the contrary, several correlations between cytokines and aAb positivity or lymphocytic populations were found (Table 3).

Correlation of assessed parameters with baseline patients' characteristics

The presence of lung metastases was correlated with the presence of aAbs: all but 1 patient with lung metastases (96%) had at least one aAb compared to 62% of patients with no lung metastases (p=0.011). Among individual autoantibodies, positive ASMA was correlated with lung metastatic site (79% vs. 44%, p=0.041). Finally, this metastatic site was also associated with higher median C3 (163 vs. 135, p=0.003) and C4 (33 vs 29, p=0.039). Anaemic patients had higher median VEGF (950 vs. 433, p=0.015). Higher IL-6 levels were associated with inferior Performance Status (PS) (p=0.011), anaemia (p=0.005) and higher MSKCC (p=0.021) and Heng's (p=0.016) score (Table 1). No correlation with the other baseline features was observed.

Correlation of assessed parameters with response and survival

Fourteen patients were not evaluable for response. Among evaluable patients, 2 (7.1%) achieved a complete response (CR), 8 (28.6%) partial response (PR), 8 (28.6%) stable disease (SD) and 11 (35.7%) had progressive disease (PD). At the time of analysis 14 patients were still on treatment and the median number of treatment cycles was 5 (0-25). Median follow-up was 35 months (range for surviving patients 6-59). Survival data was not available for one patient. During follow-up 30 patients progressed and 27 died: 25 due to renal cancer, 1 due to pancreatitis and 1 due to myocardial infraction probably related to treatment. Median PFS was 12 months (95% CI: 1-23.7) and median CSS was 31.1 months (95% CI: 11.2-50). Tumour responses were not correlated with baseline VEGF, aAbs, cytokines or any lymphocytic population.

Longer PFS and CSS were associated with absence of bone disease (p<0.001 for both), < 2 metastatic sites (p<0.001 for both), PS 0 (p<0.001 for both) and lower MSKCC (p=0.006/p=0.044) and Heng's (p=0.022 for both) score. Study of immunological factors showed that low baseline VEGF (<700 pg/ml) and IL-6 levels (<7 pg/ml) were associated with longer PFS (20.4 vs. 4, p=0.035; 21.8 vs. 4, p=0.011) and longer CSS (34.1 vs. 6, p=0.023; 37 vs. 4.2, p=0.007) (Figure 1). In addition, higher CD3-CD56+ populations (lower limit set at 9%) were also associated with superior PFS (18.3 vs. 4.2; p=0.013). Associations of IL-6 levels with PFS or CSS were confirmed when this factor was studied as continuous variable.

Changes of assessed parameters during treatment with sunitinib

Twenty-nine patients had at least one measurement apart from that of baseline and were included in this analysis. One patient (3%) neither had nor developed aAbs, 16 (55%) retained their baseline aAbs, 5 (18%) lost their baseline aAbs, while 7 (24%) developed additional aAbs during therapy. Therefore, among the 23 patients with positive baseline aAbs, 16 (70%) showed no change as opposed to 7 (30%) who lost them (n=5) or developed additional positive aAbs (n=2) (p< .001). Among patients with no baseline aAbs (n=6), 5 (83%) developed positive aAbs during treatment, while only one patient remained aAb-negative throughout treatment (p<.001). Incidence of ANA at 3 months did not correlate with those at 6-9 months and beyond 12 months. On the contrary, there was absolute concordance of the incidence of positive ANA recorded at 6-9 and beyond 12 months (p<0.001). Among 26 patients with baseline and during treatment thyroid function and aAbs results, one had baseline thyroid dysfunction without thyroid aAbs and another one had aAbs without thyroid dysfunction. After approximately 3 months on sunitinib 13 patients (50%) developed sub-clinical hypothyroidism. Only in 2 of them anti-Tg Abs were formed.

Paired Wilcoxon test showed a significant increase of C3 values between baseline and beyond 12 months (p=0.036), as well as between the time points at 3-4 and 6-9 months (p=0.050). There was also a significant increase of median IL-6 levels from baseline to the time point at 6-9 months (p=0.021).

Neither changes in VEGF, cytokines, aAbs and lymphocytic populations nor the absence of any change were associated with tumor response, PFS or CSS. The respective values at PD also did not differ significantly from those measured before progression of the disease. Nevertheless, the low number of patients at these time points limited all these analyses.

Characteristic	N(%)	ASMA	C35	C45	VEGF6	IL-66
Sex						
Male	35 (81)					
Female	8 (19)					
Nephrectomy						
Yes	35 (81)					
No	8 (19)					
Time between diagnosis and sunitinib initiation						
<=12 months	20 (46)					
>12 months	23 (54)					
Histology						
Clear Cell	37 (86)					
Papillary	5 (12)					
Chromophobe	1 (2)					
Performance Status						p=.011
0	24 (56)					3.25
1	14 (33)					4.63
2	4 (9)					22.3
3	1 (2)					
Number of Metastatic sites						
1	24 (56)					
>1	19 (44)					
Site of metastatic disease (Y/N)						
Lung	25/18 (58/42)	79%/44%, p=.041	163/135, p=.003	33/29, p=.039		
Nodes	15 (35)					
Liver	5 (12)					
Renal bed	17 (39)					
Bones	8 (19)					
Brain	4 (9)					
Hb					p=.015	p=.005
<13 for Males, <11.5 for Females	15 (35)				905	22.3
³13 for Males, £11.5 for Females	28 (65)				433	3.35
Ca						
<10	30 (70)					
³10	13 (30)					
LDH						
Normal	33 (77)					
Abnormal	10 (23)					
ALP						
Normal	34 (79)					
Abnormal	9 (21)					
Neutrophilia 1						
Yes	23 (54)					
No	20 (46)					
Thrombocytosis 2						
Yes	3 (7)					
No	40 (93)					
MSKCC3 risk stratification						p=.021
Favorable	9 (21)					2.8
Intermediate	21 (49)					4.5
Poor	13 (30)					23.1
IDC4 risk stratification						p=.016
Favorable	8 (19)					2.4
Intermediate	21 (49)					4.6
Poor	14 (32)					12.6

1: >4,000/mm3; 2: >400,000/mm3; 3: Memorial Sloan Kettering Cancer Center; 4: International Data Consortium; 5: mg/dl; 6: pg/ml; p: Spearman's rank correlation

Table 1. Baseline characteristics of 43 patients with advanced renal cell carcinoma included in the analysis and correlation with autoantibody titers and cytokine levels.

	Positive	Titre [Median (range)]		Median (range [SD])
ANA	21/41 (51%)	1/80 (1/80-1/320)	**CD3+ CD56+**	3.4% (0.32-22.1)
ds DNA	2/41 (5%)	60M (40-80)	**CD3+CD56-**	47.6% (9.3-77.5)
cANCA	0/41 (0%)	NA	**CD3- CD56+**	11.1% (2.1-28.6)
pANCA	3/41 (7%)	1/50 (1/20-1/80)	**CD14+**	20.4% (6.9-47.4)
AMA	4/41 (10%)	1/40 (1/40-1/80)	**nkT**	0.42% (0.03-1.79)
ASMA	26/41 (63%)	1/20 (1/20-1/40)	**CD4+**	65.3% (39-85.7)
RF	4/41 (10%)	14 (11.3-44)	**Tregs**	3% (0.53-10)
antiTg	1/25 (4%)	102.3 IU/ml	**CD4+ HLA-DR+**	6.3% (2.12-21.7)
antiTPO	2/25 (8%)	23 IU/ml (20-26)	**CD8+ HLA-DR+**	13.7% (3.1-46.6)
C3	144 mg/dl (99-187)	VEGF	**692 pg/ml (53-1851 [413])**	
C4	33 mg/dl (16-72)	IL-2	**24.74 pg/ml (0-108.5 [28.8])**	
T3	1.59 nmol/L(1.25-2.8)	IL-6	**4.50 pg/ml (1.8-112 [28.4])**	
T4	117 nmol/L (76-198)	IL-10	**15.81 pg/ml (2.9-42.43 [13.8])**	
TSH	1.18 μIU/ml (0,18-7)	IFN-γ	**17.01 pg/ml (1.3-44.1 [10.3])**	

SD: standard deviation ; NA : non-applicab3le

Table 2. Baseline autoantibody positivity, distribution of lymphocytic populations and cytokine levels.

	ANA	C3	C4	CD14+	CD4+HLA-DR+
IL-2	n.s.	R^2: -0.798 p=0.002	n.s.	n.s.	n.s.
IL-6	3.4* in –/ve vs. 8.8* in +/ve (p=0.013)	R^2: 0.239 p=0.018	R2: 0.289 p=0.008	R2: 0.343 p=0.007	R2: 0.384 p=0.004
IL-10	n.s.	n.s.	n.s.	n.s.	n.s.
IFN-γ	n.s.	n.s.	n.s.	n.s.	n.s.
VEGF	n.s.	R2: 0.247 p=0.016	n.s.	n.s.	n.s.

n.s.: non-significant; *measured in pg/ml

Table 3. Correlations between baseline cytokines, autoantibodies and lymphocytic populations

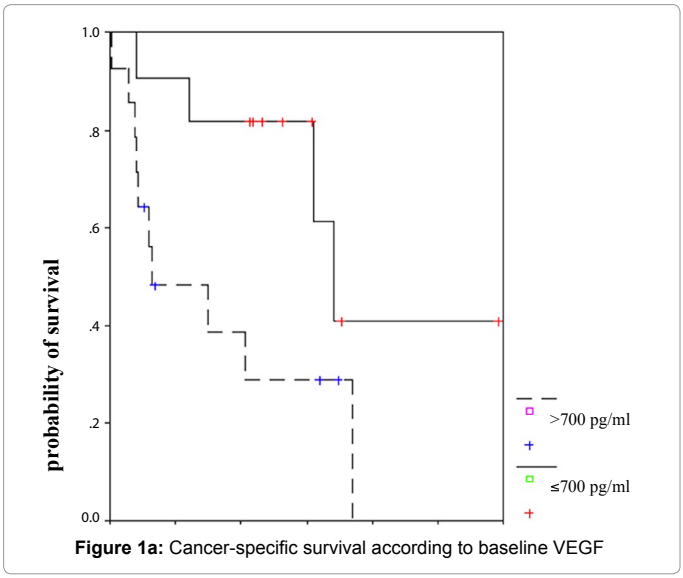

Figure 1a: Cancer-specific survival according to baseline VEGF

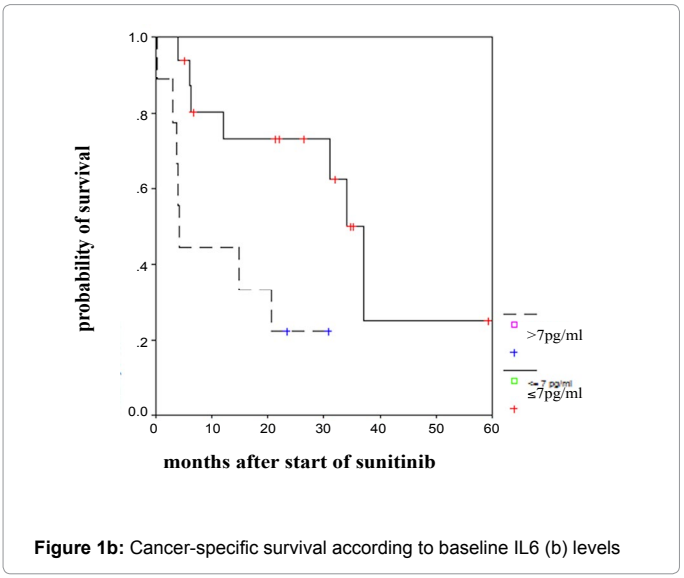

Figure 1b: Cancer-specific survival according to baseline IL6 (b) levels

Discussion

To our knowledge, this is the first study of incidence and changes of aAbs, lymphocytic populations, cytokines and VEGF in patients with mRCC treated with a contemporary standard anti-angiogenic agent, namely sunitinib. Our study is limited by the small sample size; however, there are certain strengths that should be considered: the samples were collected prospectively, the treatment was homogenous and the follow-up period was adequate. Furthermore, our population appears to be representative of mRCC patients managed in everyday practice. Specifically, their distribution according to risk stratification was as expected for an unbiased population, PFS and RRs were in concert with the available efficacy data for sunitinib [11,34], while outcome was correlated with established prognostic factors and with widely accepted risk stratification models. We, therefore, believe, that our study offers useful information, which could serve as the basis for further research.

Traditionally, it has been speculated that enhancing anti-tumor reactive immune response could represent an effective therapeutic strategy in mRCC. The impressive results of IL-2 administration in a minority of patients, was considered by many investigators as a proof-of-concept for the wider application of such strategies in the clinic. Furthermore, retrospective analyses suggested that the development

of autoimmunity was a favorable prognostic marker in patients treated with cytokines [27]. Recently, there has been a change in the treatment paradigm in this disease and anti-angiogenic therapy has become the standard option. Although the mechanism of action is different, modulation of immune response by anti-angiogenic agents cannot be ruled out, especially since VEGF has been shown to suppress anti-tumor immune response [19-21]. Research in this field has been limited and reported results are controversial. Specifically for sunitinib, augmentation, inhibition and no change in immune anti-tumor response have all been suggested [5,21-25].

Baseline ANA and ASMA were frequently present in our patients. This positivity rate is higher than that expected for the general population in Greece as well as in other countries [35]. On the contrary, the positivity of other aAbs was low, in line with previous data from RCC patients [27].

In our study, both low baseline VEGF and IL-6 levels were associated with favorable outcome. This is in concert with recent data from other mRCC studies [17,36]. Although the reason for this association is not entirely clear, it could be attributed to the established angiogenic effects of both factors. The angiogenic effects of VEGF have been extensively described but the involvement of IL-6 in the angiogenic process is more complex. IL-6 is a pro-inflammatory cytokine. Therefore, our finding regarding the prognostic association of IL-6 is in line with recent data associating prognosis in RCC with factors involved in the systemic inflammatory response, such as C-reactive protein or neutrophil/lymphocyte ratio [37,38]. IL-6 produced by various types of lymphoid and nonlymphoid cells, fibroblasts, endothelial cells, mesangial cells, and several types of tumour cells, augments maturation of B-lymphocytes, while it also inhibits the activity of Tregs and blocks the cell-mediated mechanisms, which identify and destroy the tumour [39]. It may also promote tumour growth through an autocrine mechanism, acting as a growth and/or anti-apoptotic factor. Apart from its involvement in immune response, it has been shown to stimulate angiogenesis by transcriptional up-regulation of VEGF in a signal transducer and activator of transcription 3 (STAT3)-dependent manner in tumor cells [39]. Activated STAT3 via IL-6 reportedly up-regulates the expression of bFGF and MMP-9 in tumour-associated myeloid cells and endothelial cells that contribute also to tumour angiogenesis [39]. It seems that immunity and angiogenesis has a bidirectional link. Immune system components reportedly have a key role in induction of angiogenesis and vice versa [40]. Interestingly enough, it has been shown that sunitinib acts also by reducing the STAT3 activity and this suggests that Stat3 activity is important for mRCC response to sunitinib [41]. Stat3 pathway inhibition permits the direct proapoptotic activity of sunitinib on tumour cells, the inhibition of angiogenesis and the immunological reactivation [41]. Probably this is the underlying mechanism of action of the anti-VEGFr therapies that reverse the poor prognosis of the high IL-6 patients in pazopanib trial [17]. New therapeutic concepts that target directly IL-6, the first and as it seems the activated part of this pathway, has been studied in phase I/II trials and will be further evaluated [41].

Furthermore, IL-6 was increased during therapy with sunitinib, suggesting that this factor may be involved in the development of resistance to anti-VEGF therapy. As there was no correlation of IL-6 levels with response, we made the assumption that this increase was not due to tumor or tumor endothelial cells' contribution and it could be attributed to sunitinib exposure, thus supporting the immune modulating role of the anti VEGFr therapies. Nevertheless, the type of immune enhancement that we observed was not found to have these parameters, as was found in other studies. High baseline IL-6 was also correlated with positive ANA and high C3, C4, CD14+ and HLA-DR+CD4+ lymphocytes, keeping with its known association with the development of aAbs and activation of CD4+ lymphocytes [42]. During treatment, however, only C3 was increased in parallel to the increase of IL-6, while the sole increase of IL-6 did not predict outcome. Our results indicate that, in the context of anti-VEGF therapy, the pro-angiogenic properties of IL-6 might be more relevant than the immunological ones. Another possible explanation for the prognostic significance of VEGF and IL-6 is the association of their high levels with unfavorable baseline characteristics, such as anemia and poor PS. They were also associated with the poor risk groups according to MSKCC and IMDC criteria. The small number of patients in our study precluded multivariate analyses in order to investigate whether these were independent prognostic factors. Nevertheless, our findings may indicate that long established clinical prognostic factors may represent surrogates of biological properties, such as angiogenesis and underline the importance of considering the incorporation of biological factors in the existing risk stratification models.

During therapy, 24% of our patients developed new aAbs, suggesting that sunitinib augments autoimmunity in some patients. These changes occurred during the first 9 months of treatment and it is in line with the increase of IL-6. The development of new aAbs, however, was not correlated with outcome. In a previous study in mRCC (329 pts) treated with interferon, only anti-thyroid antibody changes were associated with survival [27], while in patients with melanoma treated with adjuvant interferon, the development of any aAb was a favorable prognostic feature [28]. In our study only two patients developed anti-thyroid Abs making any further analyses of this feature meaningless. Regarding the study by Gogas et al. [28] several differences between the two studies, i.e. the origin of the tumor, the setting of therapy (adjuvant vs. metastatic) and the different aAbs studied could account for these different results. It should also be stressed that in both studies showing an association of aAbs development and outcome, interferon, an agent known to augment immune anti-tumor response, was used. The fact that lung metastases, known to respond favorably to cytokines [43], were associated with the presence of immunologically relevant factors further strengthens the hypothesis that the activating effect of cytokines is mainly exerted through the modulation of immune response.

Sunitinib therapy did not induce significant changes in lymphocytic populations, while we did not find correlation of any baseline population or any change during treatment with survival. This is in contrast with a reported association of Tregs reduction with improved survival [25]. The results of that study are limited by the small number of patients (n=35), the relative short period of studying changes in the immunological parameters on therapy (only 3 cycles) and the inclusion of patients treated with both sunitinib and bevacizumab. Furthermore, the difference of patient's percentage that underwent cytoreductive nephrectomy before treatment (96% vs 81% in our population) as well as methodological differences in the detection of Tregs may account for this discrepancy [44].

In conclusion, sunitinib may induce aAb development and increase of C3 and IL-6 levels in patients with mRCC, but these changes were not predictive of outcome in our study. No impact on T-regs or other lymphocytes was observed. Thus, the immune changes that we found cannot support the anti-tumor effect of sunitinb. On the contrary, the baseline levels of VEGF and IL-6 were related to disease prognosis, enforcing the notion that angiogenesis-related factors may be useful in determine response to anti-VEGF therapy in mRCC. This is also supported by data regarding other angiogenesis-related factors, such as sVEGFR-3, bFGF and IL-8 [14-17]. Due to the sample size limitation

our study should be mainly viewed as question generating, especially regarding the potential of the IL-6 pathway as a legitimate target for the treatment of mRCC [45].

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