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VEGF and VEGFR polymorphisms affect clinical outcome in advanced renal cell carcinoma patients receiving first-line sunitinib

M Scartozzi*[,1], M Bianconi[2], L Faloppi[2], C Loretelli[3], A Bittoni[1], M Del Prete[2], R Giampieri[3], E Maccaroni[2], S Nicoletti[3], L Burattini[1], D Minardi[4], G Muzzonigro[4], R Montironi[5] and S Cascinu[1]

[1]Department of Clinical Oncology, AOU Ospedali Riuniti, Polytechnic University of the Marche Region, Via Conca 71, Ancona 60126, Italy; [2]Postgraduate School in Medical Oncology, Polytechnic University of the Marche Region, Ancona, Italy; [3]Department of Clinical Oncology, Polytechnic University of the Marche Region, Ancona, Italy; [4]Department of Clinical Urology, AOU Ospedali Riuniti, Ancona, Italy and [5]Institute of Pathological Anatomy, Polytechnic University of the Marche Region, Ancona, Italy

Background: Currently, sunitinib represents one of the therapeutic strongholds for renal cell carcinoma, but the criteria for treatment selection are lacking. We assessed the role of vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR) polymorphisms in the prediction of the clinical outcome in metastatic renal cell carcinoma (mRCC) patients.

Methods: A total of 84 tumour samples from mRCC patients receiving first-line sunitinib were tested for VEGF and VEGFR single-nucleotide polymorphisms (SNPs). The SNP results were correlated with progression-free survival (PFS) and overall survival (OS).

Results: Median PFS was 8.22 months, although whereas median OS was 32.13 months. The VEGF A rs833061 resulted significant in PFS (17 *vs* 4 months; $P<0.0001$) and OS (38 *vs* 10 months; $P<0.0001$). The VEGF A rs699947 was significant for PFS (18 *vs* 4 months; $P=0.0001$) and OS (37 *vs* 16 months; $P<0.0001$). The VEGF A rs2010963 was significant in PFS (18 *vs* 8 *vs* 2 months; $P=0.0001$) and OS (31 *vs* 36 *vs* 9 months; $P=0.0045$). The VEGR3 rs6877011 was significant in PFS (12 *vs* 4 months; $P=0.0075$) and OS (36 *vs* 17 months; $P=0.0001$). At multivariate analysis, rs833061, rs2010963 and rs68877011 were significant in PFS, and rs833061 and rs68877011 were independent factors in OS.

Conclusions: In our analysis, patients with TT polymorphism of rs833061, CC polymorphism of rs699947, CC polymorphism of rs2010963 and CG polymorphism of rs6877011 seem to have a worse PFS and OS when receiving first-line sunitinib.

The metastatic renal cell carcinoma (mRCC) therapy scenario has radically changed in recent years, and currently the therapeutic strongholds are mostly represented by tyrosine-kinase inhibitors (TKIs) directed against the vascular endothelial growth factor (VEGF) signalling pathway. One of these new molecules, approved for first-line mRCC treatment, is Sunitinib (Motzer *et al*, 2000; Lam *et al*, 2005).

Nevertheless, in spite of interesting activity profile, a large proportion of patients, ranging from 60 to 70%, are still refractory to sunitinib and, therefore, they are exposed to potentially relevant toxicities without any clinical benefit (Motzer *et al*, 2007, 2009).

Hypoxia and compensatory hyperactivation of angiogenesis are particularly important in RCC, given the highly vascularised nature of kidney tumours and the specific association of mutation in *VHL*,

*Correspondence: Dr M Scartozzi; E-mail: marioscartozzi@gmail.com

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a critical regulator of the hypoxic response, with the onset of RCC (Pantuck *et al*, 2003).

The VEGF family members are secreted, dimeric glycoproteins of ~40 kDa, consisting of five members, VEGF A, B, C, D and placental growth factor (PLGF), and binding to specific receptors (Valtola *et al*, 1999; Jia *et al*, 2004; Rini *et al*, 2008; Stuttfeld and Ballmer-Hofer, 2009; Zhang *et al*, 2009).

The VEGF gene is quite complex, with several alternatively spliced isoforms, and the regulation of expression could differ between normal and tumour tissue. Interestingly, as all identified polymorphisms in VEGF are not in the coding region, alternative mechanisms for their role in gene expression have been proposed. In fact, although many transcription factors bind to the promoter regions of VEGF (Pages and Puyssegur, 2005), none occur at the common polymorphic sites associated with VEGF expression. Nevertheless, single-nucleotide polymorphisms (SNPs) have been reported to cause changes in VEGF expression levels (Pander *et al*, 2007).

The SNPs in the VEGF and VEGF receptor (VEGFR) genes have been also correlated with tumour neoangiogenesis through different biological mechanisms.

Numerous SNPs in the promoter, 5′-, and 3′-untranslated regions (UTRs) are present in VEGF family genes. The 5′- and 3′-UTR contains key regulatory elements that are sensitive to hypoxia (Minchenko *et al*, 1994), and contribute to high variability in VEGF production among tissues (Vaziri *et al*, 2010). For example, 634 G>C SNP in the 5′-UTR of VEGF affects the protein translation efficiency (Schultz *et al*, 1999), and 936 C>T SNP in the 3′-UTR influences the circulating plasma concentrations (Watson *et al*, 2000) and tumour tissue expression of VEGF (Renner *et al*, 2000). However, it is likely that only a small number of these polymorphisms and haplotypes (linearly linked SNPs) actually have a functional effect on VEGF translation, whereas others act as proxies (Koukourakis *et al*, 2004).

Although a growing body of evidence suggested a possible correlation between an altered expression of the angiogenetic pathway and global outcome in colorectal, breast and ovarian patients treated with antiangiogenetic therapy (Schneider *et al*, 2008; Schultheis *et al*, 2008; Hansen *et al*, 2010; Steffensen *et al*, 2010; Hansen *et al*, 2011), data in mRCC are lacking. In a study performed on blood samples and tumour tissue specimens, Kim *et al* (2012) showed a statistically significant difference in patients with SNP −634 for sunitinib-related hypertension. Another study published by Garcia-Donas *et al* (2011) correlated SNPs with response and toxicities in mRCC patients treated with sunitinib. The authors showed that polymorphisms in VEGFR3 and CYP3A5*1 might be able to define a subset of patients with decreased sunitinib response and tolerability.

Based on these premises we evaluated the potential role of VEGF and VEGFR polymorphisms to define specific patients subgroups more likely to benefit from sunitinib therapy in terms of progression-free survival (PFS) and overall survival (OS).

PATIENTS AND METHODS

Patient selection. A total of 84 patients receiving first-line sunitinib treatment for histologically proven advanced renal cell carcinoma were eligible.

Follow-up consisted of physical examination, a complete blood count, chest radiography and US of the abdomen or CT/MRI scanning as clinically indicated.

The VEGF and VEGFR genotyping was performed on formalin-fixed, paraffin-embedded tissue block (~30 mg) of renal cell carcinoma samples in nephrectomy or core biopsies, taken from the neoplasm periphery.

Paraffin wax was removed with xylene and the samples were washed twice with 100% ethanol. DNA was isolated from the deparaffinised tissue using the RecoverAll Total Nucleic Acid Isolation Kit for FFPE Tissues (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. DNA from each sample was then eluted in 120 μl of eluting solution.

The SNPs within each gene were selected using the Pupasuite software (http://pupasuite.bioinfo.cipf.es/index.jsf – version 2.0.0, bioinfo 2008), the CIPF SNP database (dbSNP) generated by the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP) and by review of the medical literature, using the following criteria:

(1) The polymorphism had some degree of likelihood to alter the structure or the expression of the gene in a biologically relevant manner (i.e., affecting ESE sequences, 3′-UTR or promoter region);
(2) The minor allele frequency was above 10% (with the only exception of rs2305948, rs6877011 and rs307822); and
(3) The genetic polymorphism was established and well documented.

Further considerations drove the selection of SNPs for our study. A correlation between the presence of a specific allele on a polymorphic site and the expression of the respective protein has been previously documented for VEGF (Formento *et al*, 2009; Chen *et al*, 2011). The SNPs in regulatory sequences, such as introns and 5′- and 3′-UTRs, have been shown to affect mRNA stability, processing efficiency, isoform expression and localisation. Moreover, regulatory motif sequences within the 3′ UTR of mRNAs have been shown to affect the stability of the messenger and/or its translational efficiency. Thus, it can be argued that SNPs in these sequences may influence VEGF and VEGFR gene expression. Also on these bases, we selected the SNPs known to affect VEGF and VEGFR expression and those located in regulatory sequences, for which a putative role in protein regulation can be assumed.

Globally, we assumed that selected SNPs had an impact on protein expression and therefore on biological function.

The selected SNPs were as follows: six polymorphisms in the VEGFA gene (rs10434, G>A; rs2010963, G>C; rs25648, C>T; rs3025039, C>T; rs699947, A>C; rs833061, C>T), two in VEGFC (rs4604006, T>C; rs7664413, C>T), two in VEGFR1 (FLT1) (rs664393, G>A; rs7993418, A>G), four in VEGFR2 (KDR) (rs1870377, A>T; rs2071559, A>G; rs2305948, G>A; rs7667298, A>G) and three in VEGFR3 (FLT4) (rs307805, A>G; rs6877011, C>G; rs307822, G>A). Chromosomal locations, positions and biological effects of investigated VEGF and VEGFR SNPs have been summarised in Table 1.

The SNP genotyping was performed by TaqMan technology using SNP genotyping products (Applied Biosystems). The PCR was performed and genotypes were analysed on the 7300 Real-Time PCR System (Applied Biosystems) using an ABI Prism 7300 Sequence Detection System software (version 1.3.1, Applied Biosystems). Each reaction contained 0.2 μl of total genomic DNA. Laboratory personnel blinded to patient status performed genotyping, and a random 10% of the samples were repeated to validate genotyping procedures.

All SNPs genotyped had to present an overall call rate of ≥90% to be included in our analysis; all samples resulted significant during the analysis and did not need test repetition.

Statistical analysis. Statistical analysis was performed with the MedCalc software version 10.4.8 (Mariakerke, Belgium) for Windows.

The association between categorical variables was estimated by the χ^2 test.

Table 1. Chromosomal locations, positions and biological effects of investigated gene SNPs

SNP ID	Gene	Chr	Chr. position	Position in the gene/effect	Codon exchange	aa exchange
rs10434	*VEGFA*	6	43753212	3′-UTR	—	—
rs2010963	*VEGFA*	6	43738350	5′-UTR	—	—
rs25648	*VEGFA*	6	43738977	Syn; ESE	TCC TCT ⇒	S [Ser] ⇒ S [Ser]
rs3025039	*VEGFA*	6	43752536	3′-UTR	—	—
rs699947	*VEGFA*	6	43736389	Prom	—	—
rs833061	*VEGFA*	6	43737486	Prom	—	—
rs4604006	*VEGFC*	4	177608775	Intronic	—	—
rs7664413	*VEGFC*	4	177608707	Intronic	—	—
rs664393	*FLT1*	13	29071001	3′-UTR	—	—
rs7993418	*FLT1*	13	28883061	Syn; ESE	TAC TAT ⇒	Y [Tyr] ⇒ Y [Tyr]
rs1870377	*KDR*	4	55972974	Missense	CAA CAT ⇒	Q [Gln] ⇒ H [His]
rs2071559	*KDR*	4	55992366	Init. Transcription	—	—
rs2305948	*KDR*	4	55979558	Missense	GTA ATA ⇒	V [Val] ⇒ I [Ile]
rs7667298	*KDR*	4	55991731	5′-UTR	—	—
rs307805	*FLT4*	5	180077487	Prom; TFBS	—	—
rs6877011	*FLT4*	5	180029471	3′-UTR	—	—
rs307822	*FLT4*	5	180028717	3′-UTR	—	—

Abbreviations: aa = aminoacid; Chr = chromosome; ESE = exon splicing enhancer; Prom = promoter region; SNP = single-nucleotide polymorphism; Syn = synonymous substitution; TFBS = predicted transcription factor binding site; UTR = untranslated region.

Hazard ratios (HRs) for median PFS and OS between groups were estimated from Cox Regression models. The multivariate analysis also included adjustments for other variables such as age ($\geq$65 *vs* $<$65 years), sex, performance status (Eastern Cooperative Oncology Group performance score, 0–1 *vs* 2), haemoglobin at the time of treatment start (less than lower limit of normal *vs* normal), lactate dehydrogenase ($>1.5\times$ the upper limit of normal *vs* normal), corrected calcium (>10 *vs* $<10\,\mathrm{mg\,dl^{-1}}$) and nephrectomy (yes *vs* not).

All polymorphisms were examined for deviation from Hardy–Weinberg equilibrium using the Powermarker v. 3.25 package (http://statgen.ncsu.edu/powermarker).

Linkage disequilibrium (LD) analysis was also performed using the Powermarker v. 3.25 package (www.statgen.ncsu.edu/powermarker). The LD was estimated using r^2, with $r^2=1$ indicating complete LD and $r^2=0$ indicating absent LD.

RESULTS

Hardy–Weinberg equilibrium and LD. The frequencies of the tested genotypes resulted comparable to those reported in Caucasians, with no significant deviation from the Hardy–Weinberg equilibrium.

Linkage disequilibrium was observed for the tumour genotypes rs833061, rs699947 and rs2010963 of VEGF A ($P>0.0001$), correlated with either PFS or OS. No LD was observed for rs6877011 of VEGFR III.

Patient characteristics. For our analysis, 84 patients with histologically proven mRCC receiving first-line sunitinib were available: 65 males and 19 females with median age at diagnosis of 64 years (range 47–85) (Table 2).

In all, 73 patients underwent renal surgery, and for 11 patients, only core biopsies were available. Also, 29 patients were metastatic at diagnosis, and 21 of these patients were resectable. Of the patients, 77 had a clear cell renal cell carcinoma histology, and 7 had other types (2 sarcomatoid and 5 undefined). All patients received sunitinib as first-line treatment with standard schedule (4 weeks on/2 weeks off), and dose reduction was applied in patients with grade 3 and 4 toxicities, as clinically indicated.

No statistically significant differences were found according to genotype for major patients characteristics (PS, tumour burden, and so on).

In the general population, median PFS was 8.22 months, whereas median OS was 32.13 months.

Genotype analysis. All SNPs genotyped presented an overall call rate of $\geq$90%.

A total of 60 patients (71%) were found with a CC or CT genotype of rs833061, whereas 24 patients (29%) had TT genotype. Median PFS was improved for patients showing the CC/CT genotype (17 *vs* 4 months; $P<0.0001$; Figure 1), as also was median OS (38 *vs* 10 months; $P<0.0001$; Figure 2).

In all, 8 patients (10%) had a CC genotype of rs2010963, 39 patients (46%) had a CG genotype and 37 patients (44%) had a GG genotype. Progression-free survival proved statistically significant different among these genotypes with 2 months for CC genotype, 8 months for CG and 18 months for GG ($P=0.0001$; Figure 3). Overall survival was significant (9 *vs* 36 *vs* 31; $P=0.0045$).

A total of 60 patients (71%) had an AA or AC genotype for rs699947 and 24 patients (29%) had a CC genotype. Median PFS was improved for patients showing the AA/AC genotype (18 *vs* 4 months; $P=0.0001$), as also was median OS (37 *vs* 16 months; $P<0.0001$).

An analysis of rs833061, rs2010963 and rs699947 of VEGF A identifies a population of patients who express these polymorphisms in accordance to LD.

In all, 71 patients (85%) expressed a CC genotype of rs6877011 and 13 patients (15%) had a CG genotype. No patients expressed the GG genotype. For patients showing these polymorphisms, PFS was of 12 months for CC genotype and 4 months for CG genotype ($P=0.0075$; Figure 4) and OS was of 36 months for CC and 17 months for CG ($P=0.0001$; Figure 5 and Table 3).

Table 2. Patient characteristics

Number of patients	84
Gender	
Male	65 (77%)
Female	19 (33%)
Median age (range 47–84)	
>64	41 (49%)
<64	43 (51%)
Surgery	
Yes	73 (87%)
No	11 (13%)
Histology	
Clear cell	77 (92%)
Other	7 (8%)
ECOG	
0	61 (73%)
1	14 (17%)
2	9 (10%)
Stage at diagnosis (AJCC Cancer Staging Manual, 2010)	
I	4 (5%)
II	8 (9%)
III	43 (51%)
IV	29 (35%)
Best response	
CR	2 (2%)
PR	10 (12%)
SD	24 (29%)
PD	48 (57%)

Abbreviations: AJCC = American Joint Committee on Cancer; CR = complete response; ECOG = Eastern Cooperative Oncology Group performance score; PD = progressive disease; PR = partial response; SD = stable disease.

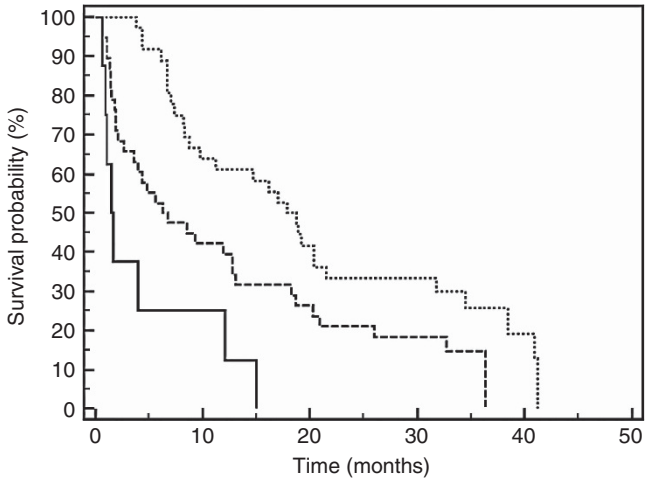

Figure 2. Progression-free survival analysis of rs2010963 ($P = 0.0001$; the lines '——', '——' and '· · · · ·' indicate CC, CG and GG, respectively).

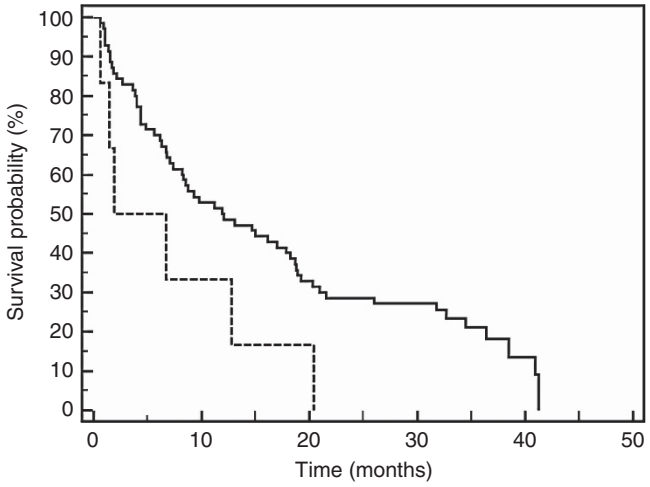

Figure 3. Progression-free survival analysis of rs6877011 ($P = 0.0075$; the lines '——' and '——' indicate CG and CC, respectively).

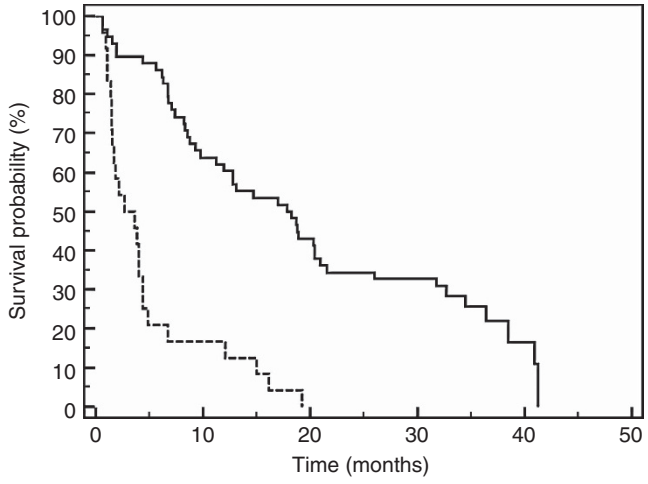

Figure 1. Progression-free survival analysis of rs833061 ($P < 0.0001$; the lines '——' and '——' indicate TT and CC + CT, respectively).

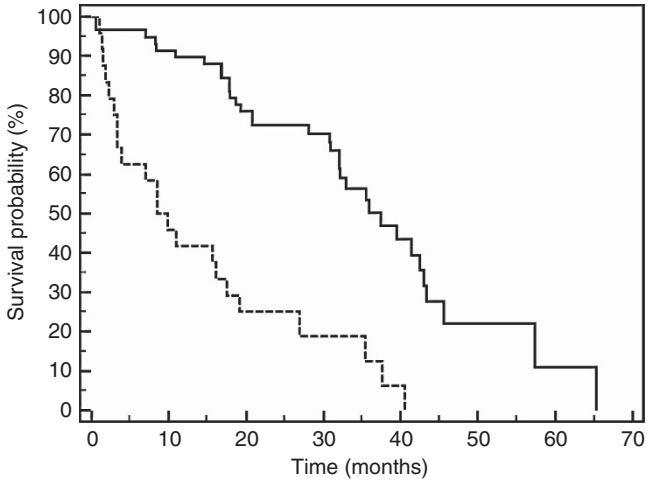

Figure 4. Overall survival analysis of rs833061 ($P < 0.0001$; the lines '——' and '——' indicate TT and CC + CT, respectively).

On multivariate analysis, rs833061 (HR = 0.71), rs2010963 (HR = 0.19) and rs68877011 (HR = 0.35) were significant in PFS. rs833061 (HR = 0.69) and rs68877011 (HR = 0.39) were also independent factors in OS.

Patients expressing all the favourable polymorphisms of rs833061, rs2010963, rs699947 and rs68877011 seem to have better overall response rate compared with those with unfavourable ones (56 *vs* 12%).

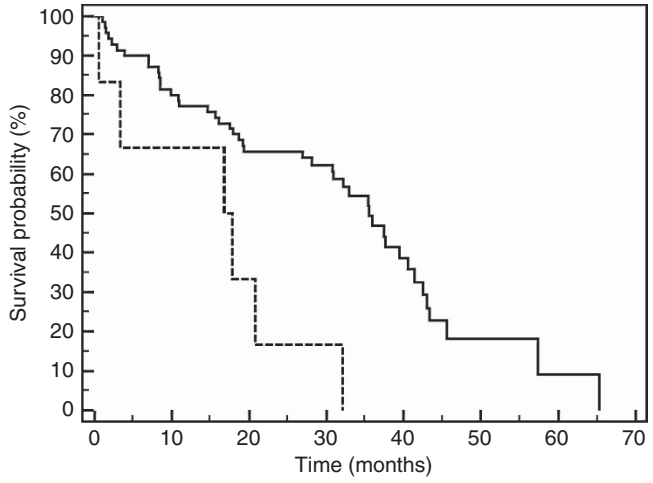

Figure 5. Overall survival analysis of rs6877011 ($P = 0.0001$; the lines '——' and '——' indicate CG and CC, respectively).

DISCUSSION

Targeting the angiogenetic pathway resulted in a complete revolution in the treatment and prognosis of mRCC. However, in mRCC patients treated with anti-VEGF TKIs, PFS and OS may widely vary from patient to patient, ranging from few weeks to years, with no apparent explanation in most of the cases. These observations may not be easily connected to the previous known predictive and prognostic factors and risk categories (Pander *et al*, 2007).

A number of potential mechanisms of action involving both stromal and cancer cells have been hypothesised for sunitinib. Among these mechanisms, 'vascular normalisation' has the most robust clinical evidence (Jain, 2005). Deprimo *et al.* (2007) recently showed how TKIs targeting the RTKs (e.g., sunitinib) produce an increase in VEGF levels and a decrease in soluble VEGFR-2 (sVEGFR-2) and sVEGFR-3 in cytokine-refractory patients with mRCC. Interestingly, these changes in VEGF and sVEGFR were observed during treatment with sunitinib, and levels tended to return to near baseline after 2 weeks off treatment, indicating that these effects were dependent on drug exposure. Furthermore, significantly larger changes in VEGF, sVEGFR-2 and sVEGFR-3 levels were observed in patients exhibiting objective tumour response compared with those exhibiting stable disease or disease progression ($P < 0.05$ for each analyte).

In our analysis, the CC + CT polymorphism of VEGF A rs833061 proved statistically significant in PFS ($P < 0.0001$) and OS ($P < 0.0001$) along with the AA + AC polymorphism of rs699947 (PFS, $P = 0.0001$; OS, $P < 0.0001$). The CC polymorphism of rs2010963 of VEGF A also showed significant correlation in PFS

Table 3. Polymorphism results in univariate and multivariate analyses

			Univariate		Multivariate	
Polymorphism	**Genotype**	**No. of patients**	**PFS**	**OS**	**PFS**	**OS**
VEGF A						
rs833061	CC + CT	60				
	TT	24				
			$P < 0.0001$	$P < 0.0001$	$P = 0.0197$	$P = 0.0011$
					HR = 0.71	HR = 0.69
rs2010963	CC	8				
	CG	39				
	GG	37				
			$P = 0.0001$	$P = 0.0045$	$P = 0.0201$	$P = 0.5932$
					HR = 0.19	HR = 0.24
rs699947	AA + AC	60				
	CC	24				
			$P = 0.0001$	$P < 0.0001$	$P = 0.9801$	$P = 0.5856$
					HR = 0.69	HR = 0.65
VEGFR3						
rs6877011	CC	71				
	CG	13				
	GG	0				
			$P = 0.0075$	$P = 0.0001$	$P < 0.0001$	$P < 0.0001$
					HR = 0.35	HR = 0.39

Abbreviations: HR = hazard ratio; OS = overall survival; PFS = progression-free survival; VEGF = vascular endothelial growth factor; VEGFR3 = VEGF receptor 3.

($P = 0.0001$) and OS ($P = 0.0045$). rs833061 is located in the promoter region of the VEGF A gene on chromosome 6, similar to rs699947, whereas rs2010963 is located in the terminal 5′-UTR region of the VEGF A gene. We can hypothesise that different SNPs in different regions of the VEGF gene may influence circulating levels of VEGF and thus response to anti-VEGF therapies.

These findings and hypothesis could explain how a certain constitutive variation in VEGF and VEGFR levels could exert a significant difference in tumour outcome during antiangiogenetic treatment. Candidate gene studies exploring associations between VEGF polymorphisms and circulating VEGF levels have yielded controversial results. Eight studies have found significant associations with candidate polymorphisms (rs699947, rs1570360, rs833061, rs2010963, rs3025039, and 2549 18 bp I/D) in the promoter, 5′- and 3′-UTRs of the VEGF gene (Renner *et al*, 2000; Awata *et al*, 2002; Krippl *et al*, 2003; Ferrante *et al*, 2006; Zhai *et al*, 2007; Kamoun *et al*, 2008; Petrovic *et al*, 2008). However, several other studies did not identify any association with these and other VEGF SNPs. Using a hypothesis-free genome-wide approach, Debette *et al* (2011) found associations with 140 SNPs. Of these, 68 SNPs are located on chromosome 6, ~150 kb downstream from the 3′ end of the VEGF gene, far from previously tested candidate SNPs. However, the real effect of SNPs in circulating or tumour tissue VEGF levels needs further studies in order to definitively associate a specific SNP to a specific effect on the corresponding growth factor or receptor.

In our population, we also found a statistical significance in PFS ($P = 0.0075$) and OS ($P = 0.0001$) in the CC polymorphism of rs6877011 of VEGFR 3.

Angiogenic sprouting involves specification of subpopulations of endothelial cells into tip cells that respond to VEGF guidance cues, and stalk cells that follow the tip cells and proliferate to form the vascular network (Gunningham *et al*, 2001). Recent evidence indicates that VEGF induces the membrane-bound Notch ligand delta-like 4 (Dll4) in the tip cells, which leads to the induction of the stalk cell phenotype in adjacent endothelial cells through activation of Notch-1 (Laakkonen *et al*, 2007).

In conclusion, our data show that by analysing polymorphisms of the VEGF and VEGFR genes, we could be able to select proper patients to be treated with sunitinib to improve treatment outcome. Further prospective studies are warranted to confirm our findings.

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