

Genetic Variants of *VEGFA* and *FLT4* Are Determinants of Survival in Renal Cell Carcinoma Patients Treated with Sorafenib



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Abstract

Molecular markers of sorafenib efficacy in patients with metastatic renal cell carcinoma (mRCC) are not available. The purpose of this study was to discover genetic markers of survival in patients with mRCC treated with sorafenib. Germline variants from 56 genes were genotyped in 295 patients with mRCC. Variant-overall survival (OS) associations were tested in multivariate regression models. Mechanistic studies were conducted to validate clinical associations. *VEGFA* rs1885657, ITGAV rs3816375, and WWOX rs8047917 (sorafenib arm), and *FLT4* rs307826 and *VEGFA* rs3024987 (sorafenib and placebo arms combined) were associated with shorter OS. *FLT4* rs307826 increased VEGFR-3 phosphorylation, membrane trafficking, and receptor activation. *VEGFA*

rs1885657 and rs58159269 increased transcriptional activity of the constructs containing these variants in endothelial and RCC cell lines, and *VEGFA* rs58159269 increased endothelial cell proliferation and tube formation. *FLT4* rs307826 and *VEGFA* rs58159269 led to reduced sorafenib cytotoxicity. Genetic variation in *VEGFA* and *FLT4* could affect survival in sorafenib-treated patients with mRCC. These markers should be examined in additional malignancies treated with sorafenib and in other angiogenesis inhibitors used in mRCC.

Significance: Clinical and mechanistic data identify germline genetic variants in *VEGFA* and *FLT4* as markers of survival in patients with metastatic renal cell carcinoma.

Introduction

Sorafenib tosylate (sorafenib, Nexavar) is a VEGF pathway inhibitor approved by the FDA for the treatment of advanced and metastatic renal cell carcinoma (mRCC), unresectable hepatocellular carcinoma (HCC), radioactive iodine-refractory differentiated thyroid carcinoma, and is becoming increasingly utilized off-label for *FLT3*-mutated acute myeloid leukemia

(AML). Sorafenib is a potent inhibitor of VEGFR-2 and VEGFR-3, PDGFR- β , FLT-3, c-KIT, RAF-1, and BRAF (1).

The phase III Treatment Approaches in Renal Cancer Global Evaluation Trial (TARGET) was a double-blind, randomized, placebo-controlled, multicenter study of 903 patients with mRCC who had failed previous cytokine therapy (2). Based upon results from TARGET and a phase II study that preceded TARGET, the FDA-approved sorafenib for the treatment of mRCC (2, 3). Currently, sorafenib is primarily used in mRCC after progression on first-line therapy (4). Recently, immunotherapy with checkpoint inhibitors nivolumab and ipilimumab has proven to be efficacious in patients with mRCC (5, 6).

Clear cell RCC is a malignancy characterized by high vascularization, resulting from molecular mechanisms that abrogate the activity of the *VHL* tumor suppressor gene (5, 6). Sorafenib affects tumor vascular endothelium, the tumor microenvironment, but importantly the host vascular endothelium and pericytes (7, 8). Because RCC is dependent on angiogenesis, the VEGF pathway has been shown to be a viable target for drug therapy (9), and sorafenib has been shown to inhibit angiogenic targets in multiple RCC models (9, 10). As angiogenesis is primarily a host-mediated process (11), germline variants that regulate angiogenic processes are likely to be associated with both disease progression and sorafenib efficacy.

Clinically, response to sorafenib is highly variable (2, 3, 12), and molecular mechanisms underlying the interpatient variability in sorafenib efficacy have yet to be elucidated. While the armamentarium of treatment for mRCC has been expanded over the past decade, there is still uncertainty concerning selection and sequencing of these agents, particularly after patients progress on

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first-line therapy. Moreover, there is a dearth of validated molecular biomarkers to help inform clinicians about disease progression and drug efficacy.

Clinical studies using genetic analyses are ideal to select novel molecular candidates for testing in experimental models. Through this reverse translational approach (i.e., bedside to bench), novel mechanistic hypotheses can be formulated to advance the field of precision oncology. In this study, 11,117 germline DNA variants in 56 genes were tested for association with survival in 295 patients with mRCC from the TARGET study. For variants associated with survival, a series of functional experiments were employed to determine novel mechanism by which DNA variation in angiogenesis genes might affect the biology of RCC and the efficacy of sorafenib.

Patients and Methods

TARGET study and patient characteristics

TARGET was a double-blind, randomized, placebo-controlled phase III trial of patients with mRCC who had received prior cytokine therapy ($n = 903$; refs. 2, 12). Patients were randomized 1:1 to either 400 mg sorafenib orally twice daily or placebo. Patients remained on study until disease progression, discontinuation due to intolerable toxicity, or death. The TARGET primary endpoint was OS, defined as the time from the date of randomization until the date of death, and OS was also used as the primary endpoint for this genetic study. To avoid the confounding effect of crossover of patients from the placebo arm to the sorafenib arm, the OS data used in this study were recorded before patient crossover. PFS was measured from the date of randomization until the date of progression, as defined by the trial protocol (2). The clinical characteristics and median OS of the 295 genotyped patients were comparable to those of the entire TARGET population (Table 1). All patients provided written informed consent to participate in this genetic analysis, approved by the institutional review board at each center.

Genotyping of gene variants

A total of 56 genes were selected for genotyping. The genes, their chromosomal location, and their biological function are

described in Supplementary Table S1. Selection criteria for single-nucleotide polymorphisms (SNP) to be genotyped are described in the Supplementary Methods and Materials. Germline DNA was extracted from peripheral blood (FlexiGene DNA kit, Qiagen) and 1,536 SNPs were genotyped using the Illumina GoldenGate assay. Genotypes were determined using Illumina GenomeStudio software v2011. Variants were excluded if the genotype call rate was $<97.5\%$, the minor allele frequency (MAF) was $<1\%$, or if they deviated from Hardy-Weinberg equilibrium ($P < 0.0001$). GenTrain scores were derived from Illumina GenomeStudio scatter plots to measure variant detection reliability based on genotypic clustering distributions (13), and only SNPs with GenTrain scores >0.4 were included. Additional variants were obtained through imputation using Impute2 version 2.30 (14). Overall, 11,117 variants were included in the final analyses (Supplementary Fig. S1). Imputation methods are included in the Supplementary Methods.

Statistical analyses

The primary study objective was to identify gene variants associated with OS. Univariate log rank tests were conducted to test each variant-OS association. Likelihood ratio tests were performed to examine associations between OS and each of the following factors: country of origin, gender, age, Eastern Cooperative Oncology Group performance status, time since RCC diagnosis, previous systemic treatment with IL2 or INF, MSKCC prognostic risk score, number of metastatic sites, and evidence of liver or lung metastases. Cox proportional hazards regression included both genetic variants identified by the log-rank tests described above and significant clinical covariates ($P < 0.05$). Additive, dominant, or recessive models were used because the mode of inheritance of risk alleles was not known *a priori*. To use an inclusive approach to detect variant-OS associations, they were tested in the sorafenib arm, the placebo arm, and both arms combined.

FDR q -values were calculated to account for multiple testing (15). FDR was employed in lieu of correcting the family-wise error rate (i.e., Bonferroni correction) to better account for the correlation among tests resulting from the LD among variants and

Table 1. Clinical characteristics of genotyped patients in the TARGET study

	All TARGET patients ($n = 903$)		Genotyped TARGET patients ($n = 295$)	
	Sorafenib ($n = 451$)	Placebo ($n = 452$)	Sorafenib ($n = 155$)	Placebo ($n = 140$)
Male gender, n (%)	315 (70)	340 (75)	114 (74)	108 (77)
Median age, year (range)	58 (19–86)	59 (29–84)	59 (19–80)	58 (31–82)
ECOG performance status, n (%)				
0	219 (49)	210 (46)	83 (54)	81 (58)
1	223 (49)	236 (52)	72 (46)	58 (42)
2	7 (2)	4 (1)	0	0
Missing	2 (<1)	2 (<1)	0	1 (<1)
Number of metastatic sites, n (%)				
1	62 (14)	63 (14)	26 (17)	26 (19)
2	131 (29)	129 (29)	42 (27)	40 (29)
>2	256 (57)	258 (57)	87 (56)	74 (53)
Missing	2 (<1)	2 (<1)	0	0
Lung or liver metastatic sites, n (%)	377 (84)	382 (85)	133 (86)	120 (86)
Previous cytokine use, n (%)	374 (83)	368 (81)	141 (91)	122 (88)
Median duration of disease, year (range)	2 (<1 –19)	2 (<1 –20)	1.7 (<1 –19.5)	1.5 (<1 –16)
MSKCC prognostic risk, n (%)				
Low	233 (52)	228 (50)	73 (47)	68 (49)
Intermediate	218 (48)	223 (49)	82 (53)	72 (51)
Missing	0	1 (<1)	0	0

Abbreviations: ECOG, Eastern Cooperative Oncology Group; MSKCC, Memorial Sloan Kettering Cancer Center.

collinearity among the three genetic models. Variant–OS associations were regarded statistically significant if $P < 0.05$ and $q < 0.10$. These significance thresholds were used as features to select variants for subsequent functional *in vitro* studies. An interaction test (Wald test) between the two arms (placebo vs. sorafenib) was also run on variants that passed the above thresholds, and the interaction P value cutoff for significance was set at 0.1. The restricted mean OS was used to report the OS for each genotype, and was selected in lieu of median OS because the median was undefined for many variants, either because more than half of the patients were alive at the date of the final event, or the last event was censored (16).

Cell culture

Human embryonic kidney cells (HEK-293; ATCC) were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin. Human clear cell mRCC cells (Caki-1; a kind gift from Dr. William Kim, University of North Carolina, Chapel Hill, NC) were cultured in McCoy 5A media (Iwakata and Grace Modification) containing L-glutamine, 10% FBS, and 1% penicillin/streptomycin. Human telomerase-immortalized microvascular endothelial cells (TIME; ATCC) and human liver parenchyma endothelial cells (LPEC; a kind gift of Dr. Lee Ellis, MD Anderson Cancer Center, Houston, TX) were cultured in endothelial basal media supplemented with the EGM-2MV BulletKit. Human umbilical vein endothelial cells (HUVEC; PromoCell GmbH) were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin. Cell lines used in this study were either purchased within 6 months of their use as part of *in vitro* experiments, or were authenticated by STR Mapping using Applied Biosystems GeneMapper IDv3.2.

VEGFR-3 phosphorylation assays

The effect of T494A on VEGFR-3 expression was tested using an SDS-PAGE gel by immunoprecipitation and Western blotting. VEGFR-3 WT and T494A were expressed in HUVECs upon retroviral transfection. The constructs were Strep-tagged to allow separation from the endogenous VEGFR-3 in the HUVECs. Streptactin–sepharose–precipitated samples were run on an 8% SDS-PAGE and Western blotted with anti-VEGFR-3 antibodies (AF743, R&D Systems) as reported previously (17, 18).

The effect of T494A on VEGFR-3 phosphorylation was investigated in VEGFC–stimulated HUVECs. After a 2-hour starvation, 494T and 494A retrovirally transfected cells were stimulated with mature VEGFC ($\Delta\text{N}\Delta\text{C}$ -VEGFC) at a final concentration of 50 ng/mL for 20 minutes. Strep-tagged VEGFR-3 proteins in the lysates were precipitated with streptactin sepharose. They were then loaded onto an 8% SDS-PAGE gel and Western blotted for phospho-tyrosines (4G10) and total VEGFR-3. A Student t test was used to compare means from cells with the WT versus T494A cells ($n = 3$).

VEGFR-3 pulse-chase experiments to evaluate VEGFR-3 processing

VEGFR-3–transfected HUVECs were starved for 2 hour in met-/cys-deficient DMEM without serum, after which, 1 mL of labeling medium (met-/cys-deficient DMEM supplemented with 20 μL EasyTag EXPRESS 35S/ml) was added for 2 hours. Cells were washed with warm PBS and 5 mL of chase medium was added (DMEM 10% FCS+1 mmol/L cold L-methionine+ 2 mmol/L cold L-cysteine). At the indicated time points, cell plates were placed on ice, the medium was removed, and the

cells washed with PBS before being lysed. Analysis was performed by precipitating Strep-tagged VEGFR-3 from the samples with streptactin sepharose. The precipitated samples were resolved on a reducing SDS-PAGE, and signals captured with a phosphoimager plate and a Typhoon scanner. The 125-kDa gel band appears at 130-kDa band due to the double strep tag.

Viability assays in sorafenib-treated cells transfected with *FLT4* rs307826 (A>G, T494A)

FLT4 cDNA with the WT allele (A allele) was introduced in a pCMV6-XL5 expression vector, and the variant rs307826 G allele (leading to T494A) was introduced using site-directed mutagenesis. Sanger sequencing was performed to confirm mutagenesis. VEGFR-3 WT and T494A were expressed in HUVECs upon retroviral transfection, while HEK-293 cells were transfected with using Lipofectamine 2000. After 24 hours, cells were treated with ascending concentrations of sorafenib from 0.5–50 $\mu\text{mol/L}$ (in DMSO 0.1%) based upon pharmacologically relevant concentrations of sorafenib in humans (6–15 $\mu\text{mol/L}$; refs. 19, 20). After 72 hours, cells were either stimulated with VEGFC in DMEM media (200 ng/mL) or DMEM alone for 1 hour prior to the addition of alamarBlue (Thermo Fisher Scientific), which was used to assess cell viability. Three independent experiments in triplicate were performed at each concentration in VEGFC–stimulated and unstimulated HUVEC and HEK-293 cells. IC_{50} values were obtained using a four-parameter nonlinear regression model to 1.0 to assess \log_{10} sorafenib concentration versus average percent viability. A one-way ANOVA, followed by pairwise comparisons and a Dunnett correction for multiple testing, was used to assess differences between IC_{50} values.

Luciferase activity assays of intronic variants

All constructs containing variants in *VEGFA*, *ITGAV*, and *WVVOX* were synthesized and inserted into the pGL4.26 [luc2/minP/Hygro] plasmid upstream of its minimal promoter. Site-directed mutagenesis was used to introduce all variants. Sanger sequencing was used to confirm mutagenesis. Caki-1 cells were transfected using Lipofectamine LTX and a *Renilla* HSV-TK plasmid control reporter. TIME cells were transfected using TransIT-2020 transfection reagent and a *Renilla* SV40 plasmid control reporter. LPECs were transfected using magnetofection with CombiMag magnetic nanoparticles, TransIT-2020, and *Renilla* SV40. Cells were lysed 40 hours after transfection and luciferase assays were performed. Four independent experiments were conducted in triplicate for each construct. Luciferase activity was calculated as the Firefly to *Renilla* luciferase ratio, normalized to the empty vector. A one-way ANOVA followed by pairwise comparisons was used, with Dunnett correction for multiple testing.

Creation of isogenic cell lines of *VEGFA* rs58159269 (T>C)

A custom transcription activator-like effector nuclease (TALEN) pair, engineered to allow single base pair editing of rs58159269, was designed and inserted into a pTAL.CMV.T7.v2 plasmid backbone (Collectis Bioresearch). A donor plasmid containing 250 bp of DNA sequence upstream and downstream of rs58159269, as well as the desired nucleotide substitution, was designed (Collectis Bioresearch), synthesized and cloned into a pUC57-Amp expression vector (GeneWiz, Inc.). TIME cells were transfected with the TALEN pair (10 μg) and donor plasmid (2:1 ratio of TALENs to donor plasmid) using TransIT-2020 transfection

Table 2. Gene variants associated with OS

Variant ID	Chr	Gene	Alleles	Feature	MAF	HR (95% CI)	P	FDR <i>q</i> value	<i>P</i> _{interaction}
Sorafenib arm, n = 155									
rs1885657	6	VEGFA	T>C	Intron	0.17	17.3 (5.7-52.7)	1.4×10^{-4}	0.08	0.17
rs200809375*	10	-	A>ATG	7.5 kb 3' of <i>NRP1</i>	0.22	6.8 (2.6-17.5)	2.7×10^{-4}	0.08	0.87
rs6719561*	2	-	C>T	1.8 kb 3' of <i>UGT1A9</i>	0.34	3.8 (1.6-9.5)	3.3×10^{-4}	0.08	0.006
rs8047917	16	WVVOX	T>A	Intron	0.08	4.1 (1.9-8.8)	3.3×10^{-4}	0.08	0.84
rs3816375	2	ITGAV	A>G	Intron	0.38	5.9 (2.1-16.4)	4.9×10^{-4}	0.05	0.10
Both arms combined, n = 295									
rs307826	5	FLT4	A>G	Exon, missense (T494A)	0.10	13.8 (3.0-62.6)	1.2×10^{-4}	0.09	0.09
rs3024987*	6	VEGFA	C>T	Intron	0.11	3.0 (1.7-5.4)	8.3×10^{-5}	0.09	0.26

NOTE: Five variants were associated with OS in patients from the sorafenib arm. Two variants were associated with OS in both arms combined. All of these seven variants passed the cutoff for statistical significance ($P < 0.05$ and FDR $q < 0.10$). The $P_{\text{interaction}}$ (Wald test) in both arms combined is also reported. *, imputed variants. Abbreviations: Chr, chromosome; MAF, minor allele frequency.

reagent 48 hours prior to FACS for isolation of single-cell colonies. Following expansion of single-cell colonies, DNA was extracted and Sanger sequencing used to verify the appropriate point variant (T>C) at the rs58159269 locus. Three clones for each genotype of TT or CC were selected and determined by Sanger sequencing not to harbor off-target variants in potential region that are targets for the TALEN constructs.

Cell proliferation and sorafenib cytotoxicity assays in isogenic cell lines of VEGFA rs58159269 (T>C)

TT and CC isogenic endothelial cell lines were plated in 96-well plates at 3,000 cells/well in EGM-2 MV medium (Lonza). After 8 hours, cells were quiesced overnight in EBM-2 medium (Lonza) with 1% FBS (Omega Scientific) in addition to hydrocortisone, and ascorbic acid (Lonza) at the same concentration as EGM-2 MV medium. For experiments with sorafenib, media were removed and replaced with EGM-2 MV-containing sorafenib 0–10 $\mu\text{mol/L}$ in DMSO (Biotang, Inc.) after 24 hours. Untreated cells contained DMSO alone at 1:1,000. After 72 hours of proliferation at 37°C 5% CO₂, and 95% humidity, viable cells were stained using a Cyquant Direct Cell Proliferation Assay (Life Technologies). Images were acquired using the EVOS Flc microscope (Life Technologies) using a 10 \times objective and counts were accomplished with FIJI particle analyzer. A two-tailed, unpaired *t* test was used to analyze differences in cell proliferation and cytotoxicity between TT and CC cells.

Endothelial cell tube formation assays in isogenic cell lines

TT and CC isogenic endothelial cell lines were plated, grown, and quiesced, as described above for proliferation studies. Matrigel (EMD Millipore) was set into 96-well plates according to the manufacturer's instructions. Cell viability was assessed using Trypan Blue (Bio-Rad). Cells were then added to each well at a final concentration of 50,000 cells/150 μL in EGM-2 MV media with 2.5% FBS. Images were acquired using the EVOS Flc microscope (Life Technologies) using a 4 \times objective, and number of branch points (nodes) was determined using FIJI angiogenesis analyzer. A two-tailed, unpaired *t* test with Welch correction was used to analyze differences in endothelial tube formation between TT and CC cells.

Results

A total of 11,117 germline variants were tested for association with overall survival (OS) in 295 patients, and a study schematic of patients and variants is provided in Supplementary Fig. S1. The

clinical characteristics of patients are shown in Table 1. Using both $P < 0.05$ and FDR $q < 0.10$ as the thresholds for statistical significance, seven variants were associated with OS (Table 2). Because the Memorial Sloan Kettering Cancer Center (MSKCC) prognostic risk score ($P = 0.05$) and the number of metastatic sites ($P = 0.001$) were associated with OS, they were included in multivariate models testing associations between variants and OS. The MSKCC score is used often in outcome studies in RCC (15–17).

Variants associated with OS in the sorafenib arm

Three variants located in genes were associated with OS (Fig. 1A–C). *VEGFA* rs1885657 (T>C, HR = 17.3; 95% CI, 5.7–52.7; $P = 1.4 \times 10^{-4}$), *ITGAV* rs3816375 (A>G, HR = 5.9; 95% CI, 2.1–16.4; $P = 4.9 \times 10^{-4}$), and *WVVOX* rs8047917 (T>A, HR = 4.1; 95% CI, 1.9–8.8; $P = 3.3 \times 10^{-4}$) were associated with shorter OS. The mean OS was 270 (range, 221–319) days for *VEGFA* rs1885657 CC patients versus 336 (34–377) days for TT/TC patients; 387 (34–497) days for *ITGAV* rs3816375 GG patients versus 371 (35–463) days for AA/AG patients; and 307 (34–377) days for *WVVOX* rs8047917 TA patients versus 355 (35–497) days for TT patients (no AA patients were identified).

Two gene-flanking variants were associated with OS. Both rs6719561 (3' of *UGT1A9*, C>T, HR = 3.8; 95% CI, 1.6–9.5; $P = 3.3 \times 10^{-4}$) and rs200809375 (3' of *NRP1*, ATG insertion, HR = 6.8; 95% CI, 2.6–17.5; $P = 2.7 \times 10^{-4}$) were associated with shorter OS. The mean OS was 322 (84–453) days for rs6719561 TT patients versus 384 (34–497) days for CC/CT patients; and 322 (34–497) days for patients with an rs200809375 ATG insertion versus 377 (129–455) days for patients without the ATG insertion. None of the significant associations in the sorafenib arm were also significant in both arms combined. No significant associations were found in the placebo arm.

Variants associated with OS in both arms combined

Two variants located in genes were associated with OS (Fig. 1D and E). *FLT4* rs307826 (A>G, HR = 13.8; 95% CI, 3.0–62.6; $P = 1.2 \times 10^{-4}$) and *VEGFA* rs3024987 (C>T, HR = 3.0; 95% CI, 1.7–5.4; $P = 8.3 \times 10^{-5}$) were associated with shorter OS. The mean OS was 194 (51–334) days for *FLT4* rs307826 GG patients versus 394 (34–497) days for AA/AG patients; and 348 (39–497) days for *VEGFA* rs3024987 CT patients versus 403 (34–409) days for CC patients (no TT genotypes were found). Neither of the significant associations in both arms combined were significant either in the sorafenib arm or the placebo arm.

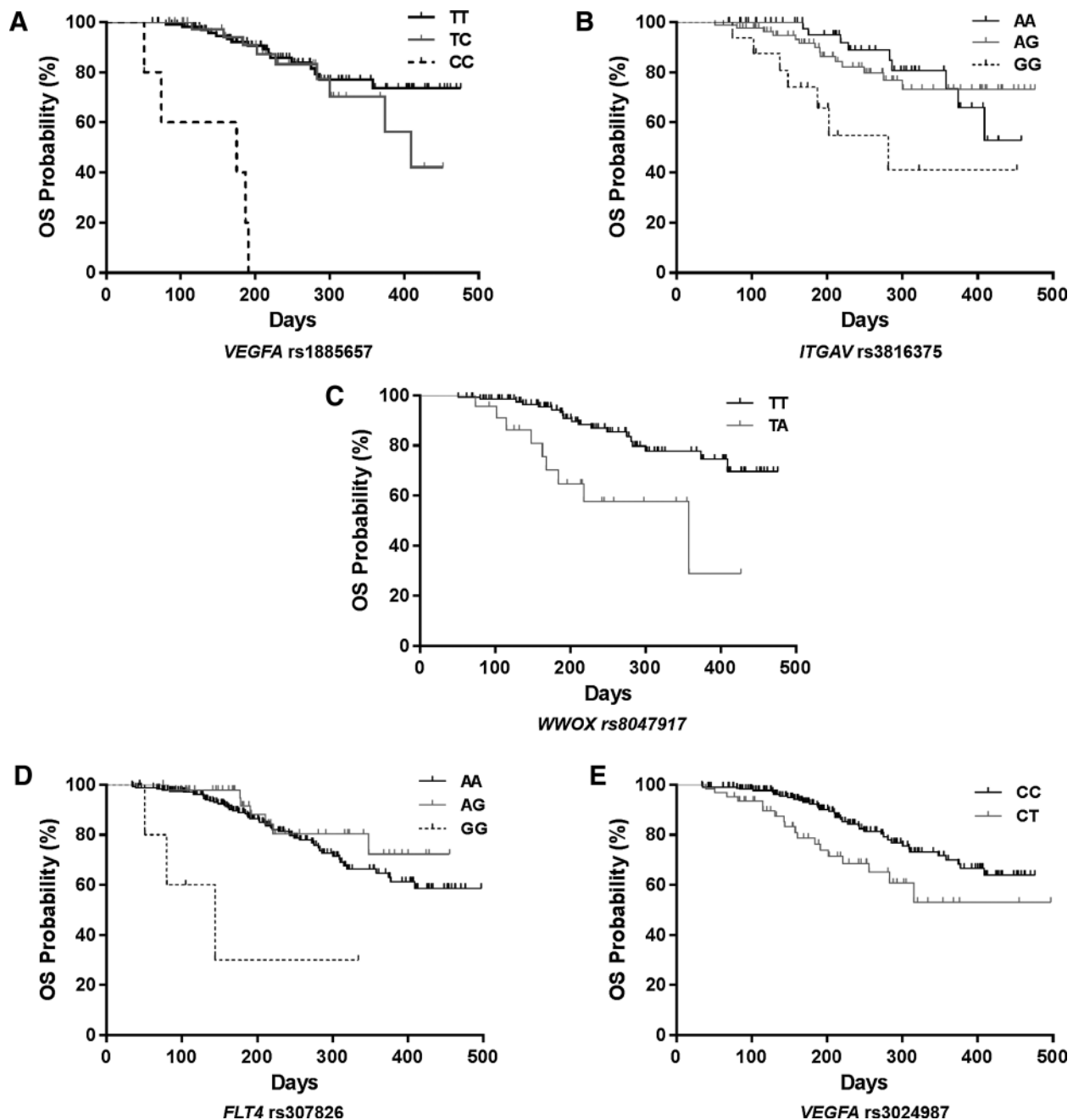


Figure 1.

Kaplan-Meier plots of genetic variants associated with OS. Vertical bars on the survival curves indicate censored observations. **A-C**, Associations observed in the sorafenib arm. **D** and **E**, Associations observed in both arms combined.

Variants associated with progression-free survival

As an exploratory analysis, variants that were significantly associated with OS (Table 2; Fig. 1A-E) were also tested for their association with PFS (Supplementary Fig. S2). *VEGFA* rs1885657 (HR = 4.00, 95% CI 1.57–10.21; $P = 0.004$), rs6719561 (3' of *UGT1A9*, HR = 1.94, CI 1.00–3.76; $P = 0.05$), *WWOX* rs8047917 (HR = 1.77, CI 1.03–3.04; $P = 0.04$), and *FLT4* rs307826 (HR = 2.92, CI 1.19–7.19; $P = 0.019$) were also associated

with PFS. However, *ITGAV* rs3816375, *NRP1* rs200809375, and *VEGFA* rs3024987 were not associated with PFS ($P > 0.05$).

FLT4 rs307826 (A>G, T494A) increases VEGFR-3 phosphorylation and processing

FLT4 rs307826 is an A>G change that leads to a T494A amino acid substitution in VEGFR-3. *FLT4* rs307826 GG was associated with shorter OS in both arms combined (Fig. 1D). We tested the

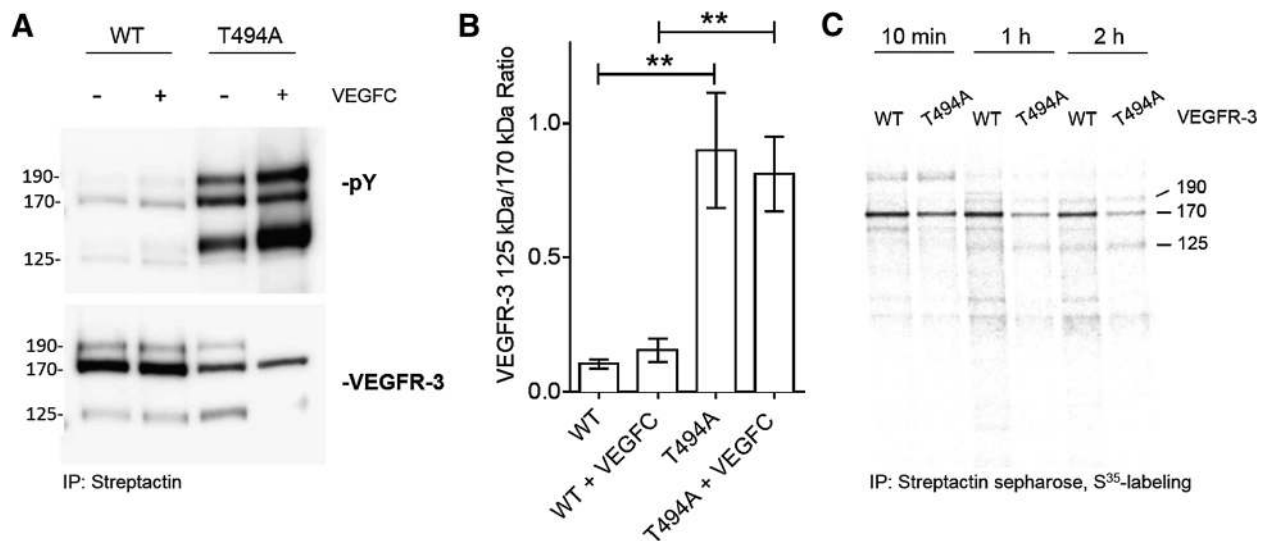


Figure 2.

Effects of *FLT4* rs307826 (A>G, T494A) on VEGFR-3 phosphorylation and posttranslational processing. **A**, VEGFR-3 phosphorylation with and without VEGFC stimulation in HUVECs transfected with either the A (WT) or the G (T494A) allele. Notably, the major precursor of VEGFR-3 (170 kDa) matures to a 190-kDa fully glycosylated cell-surface VEGFR-3, which is then proteolytically cleaved into 125-kDa C-terminal fragment and a shorter N-terminal fragment (not visible). Please note that pY indicates phosphorylated VEGFR-3 forms (top gel). **B**, Quantification of the 125:170 kDa VEGFR-3 ratio bands in the experiments reported in **A**. The mean \pm SEM is shown. **, $P < 0.01$. **C**, Pulse-chase analysis of metabolically labeled and streptactin sepharose-precipitated VEGFR-3 polypeptides.

hypothesis that T494A might affect the function of VEGFR-3. VEGFR-3 is expressed as a 170-kDa precursor that matures to the 190-kDa fully-glycosylated cell-surface VEGFR-3, which is then proteolytically cleaved into 125-kDa C-terminal fragment and a shorter N-terminal fragment (21). In transfected HUVECs, the T494A VEGFR-3 and the wild-type (WT) VEGFR-3 both showed the same polypeptide bands, indicating similar glycosylation and proteolytic processing (Supplementary Fig. S3). In transfected HUVECs, T494A leads to increased phosphorylation of the fully-processed (190-kDa band) and the proteolytically processed C-terminal (125-kDa band) forms of VEGFR-3 (22), when compared with wild-type (WT) VEGFR-3 (Fig. 2A). This effect was potentiated by VEGFC stimulation. This result was also confirmed in independent experiments (Supplementary Fig. S3).

To test the effect of T494A on VEGFR-3 posttranslational processing, a pulse-chase experiment was conducted in HUVECs. Increased VEGFR-3 125-kDa to 170-kDa ratios were observed in cells with T494A compared with WT cells, with and without VEGFC stimulation ($P < 0.01$; Fig. 2B). At both the 1- and 2-hour pulse chase time points, a clear decrease in VEGFR-3 levels was evident in T494A cells (Fig. 2C).

FLT4 rs307826 (A>G, T494A) reduces sorafenib cytotoxicity

On the basis of the association between *FLT4* rs307826 (A>G) and OS (Fig. 1D) and results from the VEGFR-3 phosphorylation experiments, we tested the hypothesis that rs307826 might influence the cytotoxicity of sorafenib. In HUVECs, T494A cells were more resistant than WT cells both in the absence of VEGFC stimulation (mean IC_{50} 11.33 vs. 3.80 μ mol/L, respectively; $P < 0.0001$) and in the presence of VEGFC stimulation (mean sorafenib IC_{50} 7.61 vs. 2.80 μ mol/L, respectively; $P < 0.0001$; Fig. 3A). These results were replicated in human embryonic kidney (HEK-293) cells, where T494A cells were more resistant

than WT cells both in the absence of VEGFC stimulation (mean IC_{50} 15.45 vs. 7.58 μ mol/L, respectively; $P < 0.0001$) and in the presence of VEGFC stimulation (mean sorafenib IC_{50} 7.67 vs. 2.02 μ mol/L, respectively; $P < 0.0001$; Fig. 3B).

Intronic variants associated with OS affect transcriptional activity in luciferase assays

With the exception of *FLT4* rs307826, all other gene variants associated with shorter OS (Table 2) are intronic. Because they were of unknown significance, we tested their effect on transcriptional regulation using luciferase assays in human endothelial (LPEC, TIME) and RCC (Caki-1) cells.

VEGFA rs1885657 C increased luciferase activity by an average of 48% in LPECs ($P = 0.0116$), 57% in TIME cells ($P < 0.0001$), and 30% in Caki-1 cells ($P = 0.0166$), when compared with the reference T allele. rs58159269 and rs943070 were also tested because they are in perfect linkage disequilibrium (LD) with rs1885657 ($r^2 \geq 1.0$ for both variants; Supplementary Table S2). rs58159269 C and rs943070 G also increased luciferase activity. The "triple variant" construct (rs1885657 C/rs58159269 C/rs943070 G) had the greatest increase in luciferase activity (70%–99%) in all cell lines ($P < 0.0001$; Fig. 4A). *VEGFA* rs3024987 T increased luciferase activity in Caki-1 cells (34%, $P = 0.0032$), TIME cells (38%, $P = 0.0002$), and LPECs (32%, $P = 0.0001$), when compared with the reference C allele (Fig. 4D).

WVVOX rs8047917 A increased luciferase activity in TIME cells (63%, $P = 0.0174$), and Caki-1 cells (40%, $P < 0.0001$), but not LPECs, when compared with the reference T allele. rs77533819 and rs7190335 were also tested because of their high LD with rs8047917 ($r^2 \geq 0.96$ for both variants; Supplementary Table S2). The "triple variant" construct (rs77533819 C/rs8047917 A/rs7190035 C) had the greatest increase in luciferase activity (64%–101%) in all cell lines ($P < 0.0001$; Fig. 4C).

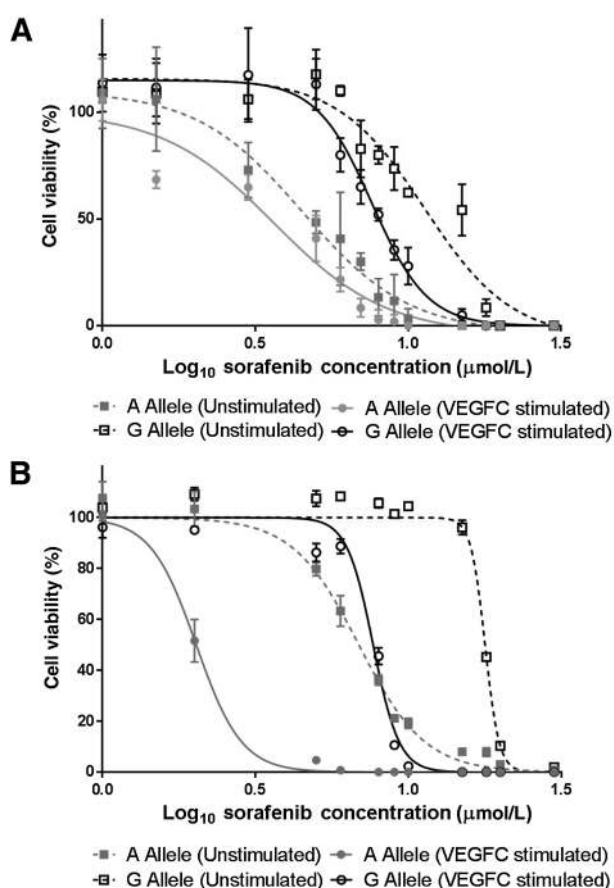


Figure 3.

Effects of *FLT4* rs307826 (A>G, T494A) on sorafenib cytotoxicity. **A**, HUVECs were transfected with either the A or the G allele and then treated with sorafenib with and without VEGFC stimulation. **B**, Results were replicated in HEK-293 cells. All experiments were conducted in triplicate ($n = 9$ at each concentration). Relative fluorescence units were generated to determine the percentage of viable cells present. The mean \pm SEM of relative fluorescence units is shown.

ITGAV rs381637 G increased luciferase activity in Caki-1 cells (57%, $P < 0.0001$), TIME cells (51%, $P < 0.0001$), and LPECs (27%, $P = 0.005$), when compared with the reference C allele (Fig. 4B).

VEGFA rs58159269 (T>C) increases endothelial cell proliferation

Out of the intronic variants that increased luciferase activity (Fig. 4A–D), the effects of *VEGFA* rs58159269 were tested in angiogenesis assays. Among the three *VEGFA* variants tested in the luciferase assays (Fig. 4A), rs58159269 is in perfect LD with rs1885657, which was the *VEGFA* variant associated with OS in the sorafenib arm (Fig. 1A), was predicted *in silico* to affect *VEGFA* (Supplementary Table S2) and had the strongest luciferase activity in all three cell lines (Fig. 4A). Thus, there was clear rationale to test the effect of rs58159269 on endothelial cell proliferation. In isogenic TIME cells transfected with either the rs58159269 reference T allele or variant C allele, endothelial cell proliferation was significantly increased in cells with the C allele, when compared with cells with the T allele (176 ± 11 vs. 103 ± 6 viable cells at 72 hours, $n = 16$, $P < 0.0001$; Fig. 5A).

VEGFA rs58159269 (T>C) reduces sorafenib cytotoxicity

Whether rs58159269 (T>C) might affect differential response to sorafenib was further characterized using a proliferation assay. In the same isogenic TIME cells described previously, cell proliferation was significantly increased in cells with the variant rs58159269 C allele after sorafenib 2.5 µmol/L ($n = 8$, $P = 0.01$) and 5 µmol/L ($n = 8$, $P < 0.0001$), when compared with cells with the reference. And, while TIME cells transfected with either the reference T or variant C allele were responsive to sorafenib, the IC₅₀ of cells with the C allele was nearly double the IC₅₀ of cells with the T allele (IC₅₀ 6.43 vs. 3.50 µmol/L, $P = 0.17$; Fig. 5B).

VEGFA rs58159269 (T>C) increases endothelial cell tube formation

The hypothesis that rs58159269 C would affect endothelial tube formation was also tested. Using the same isogenic TIME cells previously described, endothelial tube formation assays revealed a significant increase branch points in cells with the rs58159269 variant C allele, when compared with cells with the T allele (219.50 ± 27.21 vs. 34.35 ± 5.21 branch points, respectively, $n = 16$, $P < 0.0001$; Fig. 5C).

Discussion

This study represents the most comprehensive genetic analysis of sorafenib outcomes in any tumor type. The most compelling discovery of this study is the clinical identification and subsequent experimental validation of *FLT4* rs307826 as a negative determinant of OS. Patients with the *FLT4* rs307826 GG genotype experienced significantly shorter OS than AA/AG patients in both arms combined (Fig. 1D). *FLT4* encodes VEGFR-3, a transmembrane kinase receptor that is a target of sorafenib, which mediates lymphangiogenesis and plays a crucial role in vasculature growth and remodeling (23–25). Inhibition of VEGFR-3 can suppress vascular network formation (26), and preclinical models have shown that VEGFR-3 blockade can inhibit lymphatic metastasis (27).

FLT4 rs307826 (A>G) results in a threonine to alanine amino acid substitution (T494A). Our experimental results clearly indicate that T494A not only increases VEGFR-3 phosphorylation, but also VEGFR-3 membrane trafficking. T494A occurs in the fifth immunoglobulin (Ig)-like domain of the extracellular domain of VEGFR-3 (Supplementary Fig. S4; ref. 17), which contributes important homotypic interactions that are essential for VEGFR-3 dimerization and activation.

In our mechanistic experiments, T494A increased basal and VEGFC-stimulated phosphorylation of VEGFR-3 (Fig. 2A), suggesting its importance as a driver of increased receptor signaling. T494A may also increase membrane trafficking of VEGFR-3. Increased formation of the proteolytically-processed C-terminal fragment (i.e., increased 125:170 kDa band ratio) in the stimulation assays indicate faster maturation of VEGFR-3 in cells with T494A (Fig. 2B). Pulse chase experiments revealed faster decrease of VEGFR-3-specific bands with T494A, which could indicate more rapid endocytosis (Fig. 2C). One plausible mechanistic hypothesis could be that T494A renders VEGFR-3 more susceptible to internalization and signaling activation, which is important because VEGFR-3 internalization is crucial to the full activation of downstream signaling pathways (28, 29).

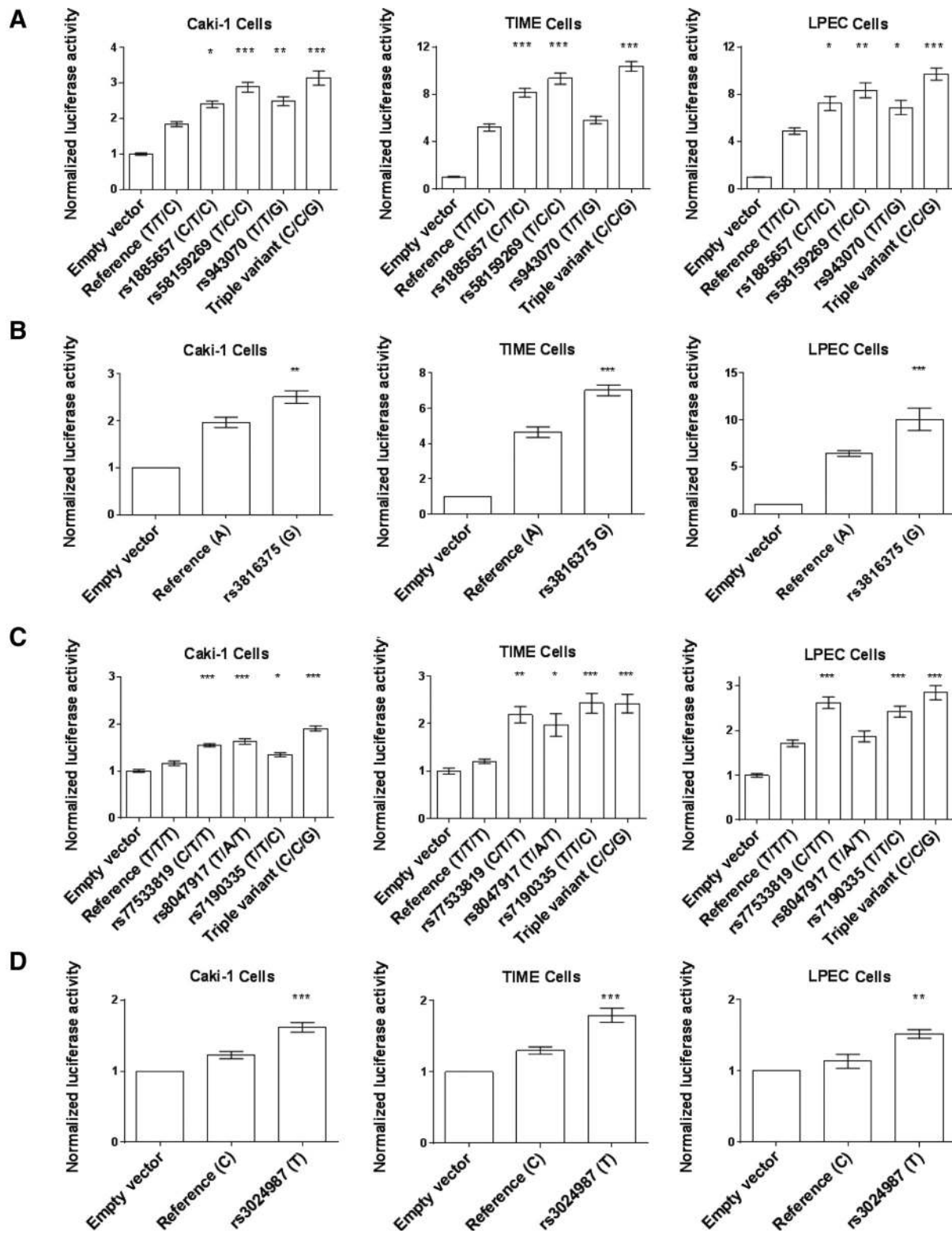


Figure 4. Effects of intronic variants on luciferase activity. **A**, *VEGFA* rs1885657 C, rs58159269 C, rs943070 G, and a "triple variant" construct (C/C/G). **B**, *ITGAV* rs3816375 G. **C**, *WWOX* rs77533819 C, rs8047917 A, rs7190335 C, and a "triple variant" construct (C/A/C). **D**, *VEGFA* rs3024987 T. Luciferase activity was tested in Caki-1, TIME, and LPEC lines (from left to right). The mean \pm SEM of luciferase activity of the experiments, conducted in quadruplicate ($n = 12$ for each construct), is shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

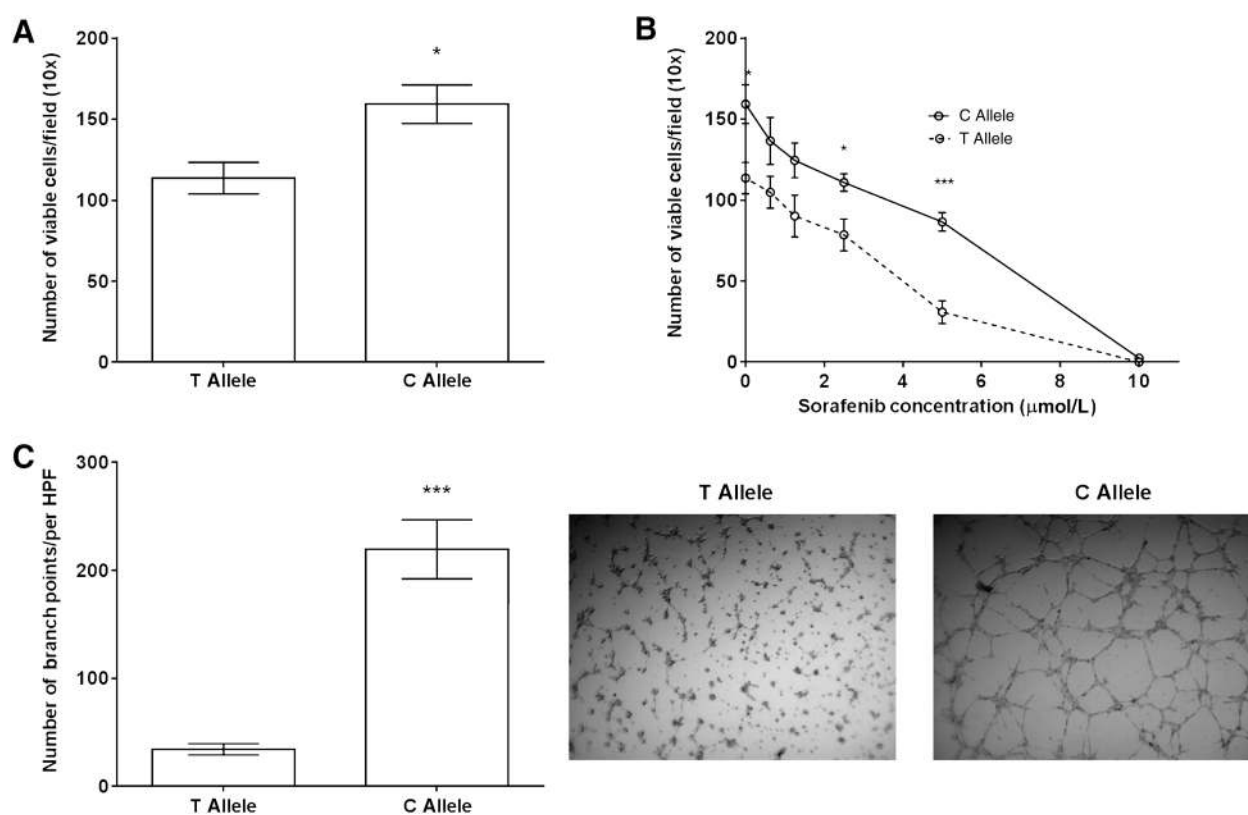


Figure 5.

Effects of *VEGFA* rs58159269 (T>C) on endothelial cell proliferation and tube formation. **A**, Endothelial cell proliferation for *VEGFA* rs58159269 in the T versus C cells at 72 hours. Increased cell viability was demonstrated for the variant C allele ($n = 16$; $P = 0.02$). **B**, Endothelial cell proliferation for *VEGFA* rs58159269 in the T versus C cells at 72 hours in the presence of increasing concentrations of sorafenib (0–10 $\mu\text{mol/L}$). The C allele conferred resistance to sorafenib compared with the T allele ($n = 8$ per concentration; $P < 0.0001$ at 5 $\mu\text{mol/L}$). **C**, Matrigel endothelial tube formation assay at 4 hours revealed an increase in tube formation for the C cells ($n = 20$; $P < 0.0001$). In each panel, the mean \pm SEM is shown. HPF, high power field. *, $P < 0.05$; ***, $P < 0.001$.

Evidence from our clinical and experimental results suggests that, in patients with mRCC with T494A, increased VEGFR-3 phosphorylation and membrane trafficking leads to potentiated VEGFR-3 signaling, which gives rise to a more aggressive form of RCC due to more rapid metastatic spread as a result of increased lymphangiogenesis and angiogenesis. In our study, patients with rs307826 (T494A) had shorter OS. A similar effect is also observed on PFS. Similar to ours, other studies of patients with mRCC treated with angiogenesis inhibitors (sunitinib and pazopanib; refs. 30–32), patients with rs307826 (T494A) experienced shorter survival. These clinical studies (now including ours) are all consistent and demonstrate that *FLT4* rs307826 (T494A) to be a negative determinant of survival in mRCC. One limitation of these clinical studies (including ours) is that they did not establish whether the effect of rs307826 is predictive. In our study, the effect of *FLT4* rs307826 is observed in both arms combined and not in either of the two study arms individually, probably due to a reduced sample size leading to loss of power. The sunitinib studies did not have a placebo arm (16, 17), and the preliminary report on the pazopanib study does not mention the results of the placebo arm (28). Our experimental data suggest that rs307826 (T494A) reduces sorafenib cytotoxicity *in vitro* (Fig. 3), and this could imply a possible predictive effect, as suggested by the

$P_{\text{interaction}}$ value of 0.09. Therefore, the potential of *FLT4* rs307826 (T494A) as a predictive marker of other angiogenesis inhibitors should be explored more extensively in future studies. This would be of particular importance, as the limited sample size of our study precludes definitive conclusions on the predictive nature of the genetic variants, in particular when they tend to have a low allele frequency.

In addition to *FLT4*, this article also provides evidence for *VEGFA* as a genetic determinant of survival in mRCC. *VEGFA* encodes for VEGF-A, the most potent proangiogenic ligand (33). In this study, two intronic variants in *VEGFA* were associated with shorter OS: rs1885657 in sorafenib-treated patients (Fig. 1A), also associated with PFS, and rs3024987 in both arms (Fig. 1E). To our knowledge, very few studies have evaluated the germline determinants of sorafenib outcomes (30, 31). One study in Chinese patients treated with sorafenib has identified rs1570360 as a *VEGFA* variant associated with increased PFS (32). rs1570360 is not in LD (using a cutoff of $r^2 < 0.6$ in Asians from the 1000 Genomes Project) with any of the two *VEGFA* variants associated with shorter OS in our study (Table 2).

For both of these intronic variants, luciferase assay data in both endothelial and RCC cell lines demonstrated that the rs1885657 variant C allele and the rs3024987 variant G allele were associated with increased transcriptional activity (Fig. 4A and D). Although

luciferase assays test the activity of a transfected construct and not the entire gene, these results are suggestive of increased regulatory activity of these variants on *VEGFA* expression. Increased *VEGFA* expression might confer increased angiogenic potential to the tumor, so we tested this hypothesis in preclinical models of angiogenesis that leveraged isogenic endothelial cells. Instead of assessing the effects of rs1885657 in these angiogenesis models, *VEGFA* rs58159269 was assessed because it is a variant in perfect LD with rs1885657, *in silico* predictions of its effects on *VEGFA* expression (Supplementary Table S2), and its stronger effect on luciferase activity (Fig. 4A). In our experiments, rs58159269 potentiated cell proliferation and tube formation, and also demonstrated increased resistance to the cytotoxic effects of sorafenib (Fig. 5). These experimental results implicate a mechanism where tumors in patients with either *VEGFA* rs1885657 or rs58159269 might be more resistant to sorafenib due to a more aggressive angiogenic phenotype. Because preliminary evidence suggests that *VEGFA* rs58159269 also conferred resistance to sunitinib (Supplementary Fig. S5), the clinical effects of either *VEGFA* rs1885657 or rs58159269 should also be tested in patients with mRCC treated with other angiogenesis inhibitors (e.g., sunitinib or pazopanib).

Additional potentially intriguing variants include *ITGAV* rs3816375 and *WVVOX* rs8047917, both of which are intronic variants that associated with shorter OS in sorafenib-treated patients. *ITGAV* codes the αv integrin subunit, which can heterodimerize with multiple β subunits. Integrin $\alpha v\beta 3$ is often upregulated in both tumor and endothelial cells, and has been shown to regulate angiogenesis (34, 35). The increased luciferase activity of *ITGAV* rs3816375 G (Fig. 4B) generates the hypothesis that rs3816375 could be contributing to *ITGAV* overexpression, increased angiogenesis, and potentially reduced efficacy of sorafenib treatment. For *WVVOX* rs8047917, the increased luciferase assay data appears to be discordant with the well-described tumor suppressor properties of *WVVOX* (36), and additional experimental evaluation is needed.

The evidence from the clinical effects on shorter OS, combined with a mechanistic demonstration of their biological and pharmacologic effects, proposes *FLT4* rs307826 and *VEGFA* rs1885657 as two biomarkers for mRCC and sorafenib treatment. Their identification and functional validation is an important step toward personalization of sorafenib treatment in mRCC. In addition, if these variants alter the tumor phenotype, leading to increased and abnormal lymphangiogenesis and angiogenesis, these characteristics might hamper the access and homing of immune cells in the tumor microenvironment. Predicting which tumors respond better to vasculature normalization induced by antiangiogenesis agents might open new venues of combinatorial therapies with checkpoint inhibitors. As a general mechanism, a mutual interplay between vascular normalization and induction of type 1 Th lymphocytes in the microenvironment has been recently demonstrated (36).

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These identified gene variants should be evaluated in future clinical studies to assess their impact in patients with other types of malignancies treated with sorafenib (e.g., unresectable HCC, refractory thyroid carcinoma, or *FLT3*-mutated AML), but also in patients with mRCC who are treated with other angiogenesis inhibitors (e.g., sunitinib and pazopanib), as well as with checkpoint inhibitors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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