Genetic variants of *VEGF* and *FLT4* are determinants of survival in renal cell carcinoma patients treated with sorafenib

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Abstract

Purpose: Sorafenib is a potent kinase inhibitor. The purpose of this study was to discover genetic determinants of survival in metastatic renal cell carcinoma (mRCC) patients treated with sorafenib.

Patients/Methods: 11,117 germline DNA variants from 56 genes were genotyped in 295 patients from the phase III TARGET study (n=155 from the sorafenib arm, n=140 from the placebo arm). The association between variants and overall survival (OS) was tested by multivariate Cox regression models.

Results: In the sorafenib arm, *VEGFA* rs1885657 (HR=17.3; 95% CI, 5.7-52.7; P=1.4x10^{-4}), *ITGAV* rs3816375 (HR=5.9; 95% CI, 2.1-16.4; P=4.9x10^{-4}), and *WWOX* rs8047917 (HR=4.1; 95% CI, 1.9-8.8; P=3.3x10^{-4}) were associated with shorter OS. In both arms combined, *FLT4* rs307826 (HR=13.8; 95% CI, 3.0-62.6; P=1.2x10^{-4}) and *VEGFA* rs3024987 (HR=3.0; 95% CI, 1.7-5.4; P=8.3x10^{-5}) were associated with shorter OS. *VEGFA* rs1885657 increased luciferase activity in human cell lines. *FLT4* rs307826 increased luciferase activity in human cell lines, increased VEGFR-3 phosphorylation, and led to differences in post-translational VEGFR-3 processing. *VEGFA* rs58159269 (in complete linkage disequilibrium with rs1885657) increased endothelial cell proliferation and endothelial tube formation. Both *FLT4* rs307826 and *VEGFA* rs58159269 led to reduced sorafenib cytotoxicity.

Conclusions: By inducing cellular changes that affect angiogenesis and the activity of sorafenib, genetic variants in *VEGFA* and *FLT4* could influence survival in mRCC patients treated with sorafenib. These are novel clinical biomarkers for sorafenib, and their relevance to other angiogenesis inhibitors should be tested in mRCC.

Statement of Significance

The clinical efficacy of sorafenib is highly variable. Molecular mechanisms underlying the interpatient variability in sorafenib efficacy are poorly understood, and no predictive molecular biomarkers have been validated. This study analyzed 11,117 DNA variants in 56 genes in metastatic renal cell carcinoma patients (mRCC) treated with either sorafenib or placebo. This study represents the most comprehensive germline genetic study of sorafenib.

The combination of clinical and mechanistic studies provides evidence for the clinical validity of genetic markers of overall survival. Functional studies supported the clinical association of shorter survival conferred by variants in *FLT4* and *VEGFA*. *FLT4* rs307826 increased VEGFR-3 phosphorylation, signaling activation and membrane trafficking. *VEGFA* rs58159269 increased the angiogenic potential of endothelial cells through changes in gene expression regulation. Both *FLT4* rs307826 and *VEGFA* rs58159269 increased resistance to sorafenib in cytotoxicity assays. The identification of novel genetic determinants of survival is an important step towards personalization of sorafenib treatment in mRCC.

Introduction

Sorafenib tosylate (sorafenib, Nexavar®) is a vascular endothelial growth factor (VEGF)-pathway inhibitor approved by the U.S. FDA for the treatment of advanced and metastatic renal cell carcinoma (mRCC), unresectable hepatocellular carcinoma, radioactive iodine-refractory differentiated thyroid carcinoma, and is becoming increasingly utilized off-label for FLT3-mutated acute myeloid leukemia. 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 mRCC patients who had failed previous cytokine therapy(2). Based upon results from TARGET and a previous phase II study, the U.S. FDA approved use of sorafenib for the treatment of mRCC(2,3). In mRCC, sorafenib is primarily used after progression on first-line therapy(4).

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, and the host vascular endothelium and pericytes(7,8). Because RCC is dependent on angiogenesis, the VEGF pathway is 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 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 translational approach in reverse, novel mechanistic hypotheses can be produced to advance the field of precision oncology. In this study, 11,117 germline DNA variants in 56 genes were tested for association with overall survival (OS) in 295 mRCC patients from the TARGET study. For variants associated with OS, we employed a series of functional experiments to determine novel mechanism by which DNA variation in angiogenesis genes might affect the biology of RCC and the efficacy of sorafenib.

Results

A total of 11,117 germline variants were tested for association with OS in 295 patients, and a flow diagram of patients and variants is shown in Supplementary Figure SF1. The clinical characteristics of patients are shown in Table 1. Using both $P<0.05$ and false discovery rate (FDR) $q<0.10$ as the threshold 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 the multivariate model testing the association between variants and OS.

Variants associated with OS in the sorafenib arm

Three variants located in genes were associated with OS (Figure 1A-C). *VEGFA* rs1885657 (T>C, HR=17.3; 95% CI, 5.7-52.7; $P=1.4\text{x}10^{-4}$), *ITGAV* rs3816375 (A>G, HR=5.9; 95% CI, 2.1-16.4; $P=4.9\text{x}10^{-4}$), and *WWOX* rs8047917 (T>A, HR=4.1; 95% CI, 1.9-8.8; $P=3.3\text{x}10^{-4}$) 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 *WWOX* 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=3.3x10^{-4}) and rs200809375 (3' of *NRP1*, ATG insertion, HR=6.8; 95% CI, 2.6-17.5; P=2.7x10^{-4}) were associated with shorter OS. The mean OS was 322 (84-453) days for rs6719561 TT patients versus 384 (34-497) for CC/CT patients; and 322 (34-497) days for patients with an rs200809375 ATG insertion versus 377 (129-455) 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 (Figure 1D-E). *FLT4* rs307826 (A>G, HR=13.8; 95% CI, 3.0-62.6; P=1.2x10^{-4}) and *VEGFA* rs3024987 (C>T, HR=3.0; 95% CI, 1.7-5.4; P=8.3x10^{-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 only or the placebo arm only.

Variants associated with progression-free survival (PFS)

As an exploratory analysis, variants that were significantly associated with OS (Table 2, Figure 1A-E) were also tested for their association with PFS (Supplementary Figure SF2).

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 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

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 (Figure 1D). We tested the hypothesis that T494A might affect the function of VEGFR-3. 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(13), as compared to wild-type (WT) VEGFR-3. This effect was potentiated by VEGF-C stimulation (Figure 2A), and was confirmed in independent experiments (Supplementary Figure SF3).

FLT4 rs307826 (A>G, T494A) increases rates of VEGFR-3 processing

To test the effect of T494A on VEGFR-3 post-translational processing, a pulse chase experiment was conducted in HUVECs. At both the 1 h and 2 h pulse chase time points, there is a clear decrease in mature VEGFR-3 (190 kDa band) for cells with T494A (Figure 2B). Increased VEGFR-3 125 kDa to 170 kDa ratios were observed in cells with T494A compared to WT cells, with and without VEGF-C stimulation (*P*<0.01) (Figure 2C).

FLT4 rs307826 (A>G, T494A) does not affect VEGFR-3 O-glycosylation

Bioinformatic analyses predicted that 494A could be a potent site of O-ß-GlcNAc attachment (Supplementary Figure xx). However, in either HeLa cells or HUVECs, T494A

showed all three VEGFR-3 polypeptide bands, indicating normal glycosylation and proteolytic processing (Supplementary Figure SF4).

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

Based on the association between *FLT4* rs307826 (A>G) and OS (Figure 1D) and results from the VEGFR-3 phosphorylation experiments, we tested the hypothesis that rs307826 might influence the cytotoxicity of sorafenib. In HEK293 cells with and without VEGF-C stimulation, VEGF-C-stimulated cells with the G allele were more resistant to sorafenib than cells with the A allele (mean sorafenib IC_{50} 7.67 versus 2.02 µM, respectively; $P<0.0001$) (Figure 3). In the absence of VEGF-C stimulation, cells with the G allele were also more resistant than cells with the A allele (mean IC_{50} 15.45 versus 7.58 µM, respectively; $P<0.0001$).

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 are of unknown significance, we tested their effect on transcriptional regulation using luciferase assays in human endothelial (LPECs, 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 to the reference T allele. 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 ($P<0.0001$) (Figure 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 to the reference C allele (Figure 4B).

WWOX 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 to the reference T allele. 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) (Figure 4C).

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 to the reference C allele (Figure 4D).

VEGFA rs58159269 (T>C) increases endothelial cell proliferation

Out of the intronic variants increasing luciferase activity (Figure 4A-D), we tested the effect of *VEGFA* rs58159269 in angiogenesis assays. Among the three *VEGFA* variants (Figure 4A), rs58159269 had the strongest luciferase activity in all three cell lines. *VEGFA* rs58159269 is in complete LD (r^2 1.0) with rs1885657, the *VEGFA* variant associated with shorter OS (Figure 1A). Hence, we tested the effect of rs58159269 on endothelial cell proliferation. In isogenic TIME cells transfected with rs58159269 C, endothelial cell proliferation was significantly greater than in cells with the T allele (176±11 versus 103±6 viable cells at 72 h, *P*<0.0001; Figure 5A).

VEGFA rs58159269 (T>C) reduces sorafenib cytotoxicity

Because endothelial cells with rs58159269 C were characterized by increased proliferation, we tested whether increased proliferation would result in reduced cytoxicity of sorafenib. In the same isogenic TIME cells used above, cells with either the T or C allele were responsive to sorafenib. However, the IC_{50} of rs58159269 C cells was nearly double than the IC_{50} of cells with the T allele (IC_{50} 6.43 versus 3.50 μM, Figure 5B).

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

Because endothelial cell proliferation was increased by rs58159269 C, we tested the hypothesis that rs58159269 C would affect endothelial tube formation. Using the same isogenic TIME cells, endothelial tube formation assays showed significantly increased branch points for cells with the C allele when compared to those with the T allele (219.50±27.21 versus 34.35±5.21 branch points, respectively, $P<0.0001$; Figure 5C).

Discussion

This study represents the most comprehensive genetic analysis of sorafenib outcome 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 (Figure 1D). *FLT4* encodes VEGFR-3, a transmembrane kinase receptor that is a target of sorafenib, mediating lymphangiogenesis and playing a crucial role in vasculature growth and remodeling(14-16). Inhibition of VEGFR-3 can suppress vascular network formation(17), and preclinical models have shown that VEGFR-3 blockade can inhibit lymphatic metastasis(18).

FLT4 rs307826 (A>G) results in a missense variant that causes 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 signaling and membrane trafficking. T494A occurs in the 5th immunoglobulin (Ig)-like domain of the extracellular domain of VEGFR-3 (Supplementary Figure SF5)(19), which contributes important homotypic interactions that are essential for VEGFR-3 dimerization and activation.

In our mechanistic experiments, T494A increased basal and VEGF-C-stimulated phosphorylation of VEGFR-3 (Figure 2A), suggesting its importance as a driver of increased signaling. Increased phosphorylation could result in a more aggressive form of RCC, possibly due to more rapid metastatic spread secondary to increased lymphangiogenesis. T494A may also increase membrane trafficking of VEGFR-3. Pulse chase experiments revealed increased formation of the proteolytically-processed C-terminal fragment (i.e., increased 125:170 kDa band ratio), as well as increased formation of mature VEGFR-3 (i.e., presence of 190 kDa band) in cells with T494A (Figure 2B-C). Faster decrease of ???? VEGFR-3 with T494A could indicate more rapid endocytosis and degradation. One plausible mechanistic hypothesis could be that T494A renders VEGFR-3 more susceptible to internalization and signaling activation(21,22). VEGFR-3 internalization is crucial to the activation of downstream signaling pathways(21).

Evidence from our clinical and experimental results indicate that, in mRCC patients with T494A, increased VEGFR-3 phosphorylation and membrane trafficking lead to potentiated VEGFR-3 signaling, resulting in a more aggressive tumor due to increased angiogenesis and lymphoangiogenesis. These patients had shorter OS and PFS. Similar to our study, patients with T494A had shorter survival in other studies in mRCC patients treated with angiogenesis inhibitors (sunitinib and pazopanib) (23-25). These clinical studies, now including ours, are all consistent for *FLT4* rs307826 T494A to be a negative determinant of survival in mRCC. One limitation is that these studies cannot establish whether this effect is prognostic and/or predictive of the efficacy of angiogenesis inhibitors. Our experimental studies seem to suggest that T494A could also result into resistance to sorafenib cytotoxicity (Figure 3), and the effect of T494A on the cytoxicity of other angiogenesis inhibitors should be tested in the future.

In addition to *FLT4*, this paper also provides evidence for *VEGFA* as a genetic determinant of survival in mRCC. *VEGFA* encodes for VEGF-A, the most potent pro-angiogenic agent(26). In mRCC, increased circulating VEGF-A levels were shown to be negatively prognostic(12,27). In the present study, two intronic variants in *VEGFA* were associated with shorter OS: rs1885657 in sorafenib-treated patients (Figure 1A) and rs3024987 in both arms (Figure 1E). For both rs1885657 and rs3024987, the luciferase assays in endothelial and RCC cell lines (Figure 4A-B) resulted in increased transcriptional activity of the alleles associated with shorter OS. Although luciferase assays test the activity of a construct and not of the whole gene, these results are suggestive of increased regulatory activity of these variants on *VEGFA* expression. This effect might confer increased angiogenic potential to the tumor. We tested this hypothesis by using angiogenesis models based upon isogenic endothelial cells created by gene editing. Instead of rs1885657, for these assays we used rs58159269 because of its stronger effect on luciferase assays (Figure 4A) and complete LD with rs1885657. Cell proliferation and endothelial tube formation were potentiated by rs58159269, resulting in reduced cytotoxicity of sorafenib (Figure 5A-B). A tube formation assay confirmed that the increased effect on endothelial cell proliferation resulted into potentiated angiogenesis (Figure 5C). These experimental results point towards a mechanism where tumors in patients with *VEGFA* rs1885657 might be more resistant to sorafenib due to a more aggressive angiogenic phenotype. Because *VEGFA* rs1885657 conferred resistance also in cells treated with sunitinib (Supplementary Figure xx), the clinical effects of *VEGFA* rs1885657 should be also tested in mRCC patients treated with other angiogenesis inhibitors.

Additional potentially intriguing associations include *ITGAV* rs3816375 and *WWOX* rs8047917, both intronic variants associated with shorter OS in sorafenib-treated patients. *ITGAV* codes the αv integrin subunit, which can heterodimerize with multiple β subunits. Integrin αvβ3

is upregulated in both tumor and endothelial cells, and has been shown to regulate angiogenesis(32,33). The increased luciferase activity of *ITGAV* rs3816375 G (Figure 4D) generates the hypothesis that rs3816375 could be contributing to *ITGAV* overexpression, increased angiogenesis, and reduced efficacy of sorafenib. For *WWOX* rs8047917, the increased luciferase assay appears to be discordant with the well-described tumor suppressor properties of *WWOX*(34), and additional experimental evaluation is needed.

The evidence from the clinical effects on shorter OS, combined with a mechanistic demonstration of their biological and pharmacological effects, proposes *FLT4* rs307826 and *VEGFA* rs1885657 as two novel biomarkers for mRCC and sorafenib treatment. Their identification and functional validation is an important step towards personalization of sorafenib treatment in mRCC, and these novel gene variants should be evaluated in further clinical studies.

Patients, materials and methods

TARGET study and patient characteristics

TARGET was a double-blind, randomized, placebo-controlled phase III trial of patients with unresectable RCC or mRCC who had received prior cytokine therapy (n=903)(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 primary endpoint was OS, defined as the time from the date of randomization until the date of death, and it 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 biological function of each gene is described in Supplementary Table ST1. Selection criteria for single-nucleotide polymorphisms (SNPs) to be genotyped are described in the Supplementary Methods. 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 (HWE; P<0.0001). GenTrain scores were derived from Illumina GenomeStudio scatter plots to measure variant detection reliability based on genotypic clustering distributions(36), and only SNPs with GenTrain scores >0.4 were included. Additional variants were obtained through imputation using Impute2 version 2.30(37). Overall, 11,117 variants were included in the final analyses (Supplementary Figure SF1). Imputation methods are included in the Supplementary Methods.

Statistical analyses

The primary objective was to identify gene variants associated with OS. Univariate log rank tests were conducted to test each variant-OS association in the sorafenib arm, the placebo arm, and both arms combined. To determine if any clinical or demographic characteristic needed to be modeled along with the genetic variants, likelihood ratio tests were performed to look for 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 IL-2 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 ($P \leq 0.05$). Additive, dominant, or recessive models were used. FDR *q*-values were calculated to account for multiple testing(38). 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 linkage disequilibrium (LD) among variants. Variant-OS associations were regarded statistically significant if $P<0.05$ and $q<0.10$. 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(39).

Cell culture

Human embryonic kidney cells (HEK-293) (ATCC) were cultured in DMEM with 10% fetal bovine serum (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's 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™. HeLa cells and human umbilical vein endothelial cells (HUVECs) (PromoCell GmbH) were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin. Cell lines used in this study were either purchased within six months

of their use as part of in vitro experiments, or were authenticated by STR Mapping using Applied Biosystems GeneMapper IDv3.2.

VEGFR-3 o-glycosylation and phosphorylation assays

The human VEGFR-3 amino acid sequence was analyzed using YinOYang software(40) for potential O-glycosylation by assessing the potent O-ß-GlcNAc attachments. 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 HeLa cells and 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 previously reported.(19,20)

The effect of T494A on VEGFR-3 phosphorylation was investigated in VEGF-C-stimulated HUVECs. After a 2 h starvation, 494T and 494A retrovirally-transfected cells were stimulated with mature VEGF-C (ΔNΔC-VEGF-C) at a final concentration of 50 ng/mL for 20 min. Strep-tagged VEGFR-3 proteins in the lysates were precipitaed with streptactin sepharose. They were then loaded onto an 8% SDS-PAGE gel and western blotted for phospho-tyrosines (4G10) and total VEGFR-3.

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

VEGFR-3 transfected HUVECs were starved for 2 h in met-/cys-deficient DMEM without serum, after which 1 mL of labelling medium (met-/cys-deficient DMEM supplemented with 20 µL EasyTag EXPRESS 35S/ml) was added for 2 h. Cells were washed with warm PBS and 5 mL of chase medium was added (DMEM 10% FCS+1 mM cold L-methionine+2 mM cold

L-cysteine). At the indicated time points, cell plates were place 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 gel appears at 130 kDa band due to the double strep tag. A Student's t test was used to compare means from cells with the WT versus T494A cells (n=3).

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

FLT4 cDNA was introduced in a pCMV6-XL5 expression vector, and the variant rs307826 G allele was introduced using site-directed mutagenesis. Sanger sequencing was performed to confirm mutagenesis. HEK-293 cells were transfected with vectors containing either G or A allele using Lipofectamine® 2000. After 24 h, cells were either stimulated with VEGF-C in DMEM media (200 ng/mL) for 1 h prior to the addition of 0.5-30 µM sorafenib (in DMSO) based upon pharmacologically relevant concentrations of sorafenib in humans (6-15 µM(38,39)). After 72 h, AlamarBlue® was used to assess cell viability. Three independent experiments in triplicate were performed. IC_{50} values were obtained using a four-parameter non-linear regression model that was top constrained to 1.0 to assess $\log_{10}$ sorafenib concentration versus average percent viability. Two-sided t-tests were used to assess differences between IC_{50} values.

Luciferase activity assays of intronic variants

All constructs containing variants in *VEGFA, ITGAV,* and *WWOX* 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. LPEC cells were transfected using magnetofection with CombiMag magnetic nanoparticles, TransIT-2020, and Renilla SV40. Cells were lysed 40 h 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's 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 (Cellectis Bioresearch). A donor plasmid containing 250 bp of DNA sequence upstream and downstream of rs58159269, as well as the desired nucleotide substitution, was designed (Cellectis 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 reagent 48 h prior to fluorescence-activated cell sorting (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 h, 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 was removed and replaced with EGM-2 MV containing sorafenib 0-10 µM in DMSO (Biotang, Inc.) after 24 h. Untreated cells contained DMSO alone at 1:1000. After 72 h of proliferation at 37 ºC 5% CO_2, and 95% humidity, viable cells were stained using Cyquant Direct Cell Proliferation Assay (Life Technologies). Images were acquired using the EVOS FLc microscope (Life Technologies) using a 10x 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 manufactures 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. Cells were treated with sorafenib (0.1 µM, 1 µM, 10 µM). Untreated cells and cells with DMSO (final concentration 1:4000) were used as controls. Cells were then incubated for 4 h at 37 ºC, 5% CO_2, and 95% humidity. Images were acquired using the EVOS FLc microscope (Life Technologies) using a 4x objective, and number of branch points (nodes) was determined using FIJI angiogenesis analyzer. A two-tailed, unpaired t-test with Welch's

correction was used to analyze differences in endothelial tube formation between TT and CC cells.

Author Contributions:

Conception and Design: Crona, Skol, Innocenti

Collection and Assembly of Data: Crona, Skol, Leppänen, Hilliard, Etheridge, Glubb, Innocenti

Data Analysis and Interpretation: All authors

Manuscript Writing: All authors

Final Approval of Manuscript: All Authors

Administrative Support: Crona and Innocenti

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Tables

Table 1. Clinical characteristics of genotyped patients in the entire 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 - no. (%)	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 - no. (%)				
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- no. (%)				
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 - no. (%)	377 (84)	382 (85)	133 (86)	120 (86)
Previous cytokine use - no. (%)	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 - no. (%)				
Low	233 (52)	228 (50)	73 (47)	68 (49)
Intermediate	218 (48)	223 (49)	82 (53)	72 (51)
Missing	0	1 (<1)	0	0

Table 2. Gene variants associated with OS. Five variants were associated with OS in patients from the sorafenib arm. Two variants were associated with OS in both arms combined. All these seven variants passed the cut-off for statistical significance (P<0.05 and FDR q<0.10). *Denotes imputed variants. Abbreviations: Chr, chromosome; Ref, reference. MAF, minor allele frequency.

Variant ID	Chr	Gene	Alleles	Feature	MAF	HR (95% CI)	*P*-value	FDR *q*-value
Sorafenib Arm (n=155)								
rs1885657	6	*VEGFA*	T>C	Intron	0.17	17.3 (5.7-52.7)	$1.4x10^{-4}$	0.08
rs200809375*	10	-	A>ATG	7.5 kb 3' of *NRP1*	0.22	6.8 (2.6-17.5)	$2.7x10^{-4}$	0.08
rs6719561*	2	-	C>T	1.8 kb 3' of *UGT1A9*, 0.6 kb 5' of *HEATR7B1*	0.34	3.8 (1.6-9.5)	$3.3x10^{-4}$	0.08
rs8047917	16	*WWOX*	T>A	Intron	0.08	4.1 (1.9-8.8)	$3.3x10^{-4}$	0.08
rs3816375	2	*ITGAV*	A>G	Intron	0.38	5.9 (2.1-16.4)	$4.9x10^{-4}$	0.05
All Patients (both arms combined, n=295)								
rs307826	5	*FLT4*	A>G	Exon, missense (T494A)	0.10	13.8 (3.0-62.6)	$1.2x10^{-4}$	0.09
rs3024987*	6	*VEGFA*	C>T	Intron	0.11	3.0 (1.7-5.4)	$8.3x10^{-5}$	0.09

Figure Legends

Figure 1. Kaplan-Meier plots of genetic variants associated with OS. Vertical bars on the survival curves indicate censored observations. A-C are associations observed in the sorafenib arm. D-E are associations observed in both arms combined. Vertical bars on the survival curves indicate censored observations

Figure 2. Effects of *FLT4* rs307826 (A>G, T494A) on VEGFR-3 phosphorylation and post-translational processing. A) VEGFR-3 phosphorylation with and without VEGF-C stimulation in HUVECs transfected with either the A (WT) or the G (T494A) allele. **B)** Pulse-chase analysis of metabolically-labeled and streptactin sepharose precipitated VEGFR-3 polypeptides. The 125 kDa band appears at 130 kDa due to the double strep tag. **C)** Quantification of the 125:170 kDa VEGFR-3 ratio bands in the experiments reported in the B panel. The mean±SEM is shown. **, $P \leq 0.01$. Abbreviations: pY, phosphorylated VEGFR-3.

Figure 3. Effects of *FLT4* rs307826 (A>G, T494A) on sorafenib cytotoxicity. HEK-293 cells were transfected with either the A or the G allele, and then treated with sorafenib with and without VEGF-C stimulation. Relative fluorescence units (RFUs) were generated to determine the percentage of viable cells present. The mean±SEM of RFUs is shown.

Figure 4. Effects of intronic variants on luciferase activity. A) *VEGFA* rs1885657 C, rs58159269 C, rs943070 G, and the "triple variant" construct (C/C/G). **B)** *VEGFA* rs3024987 T. **C)** *WWOX* rs77533819 C, rs8047917 A, rs7190335 C, and the "triple variant" construct (C/A/C). **D)** *ITGAV* rs3816375 G. Luciferase activity was tested in Caki-1, TIME and LPEC lines (from

left to right). The mean±SEM of luciferase activity of the experiments conducted in quadruplicate 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 in untreated cells. **B)** Endothelial cell proliferation in cells treated with sorafenib. **C)** Endothelial tube formation in untreated cells. In each panel, the mean±SEM is shown.

Figure 1. Kaplan-Meier plots of genetic variants associated with OS.

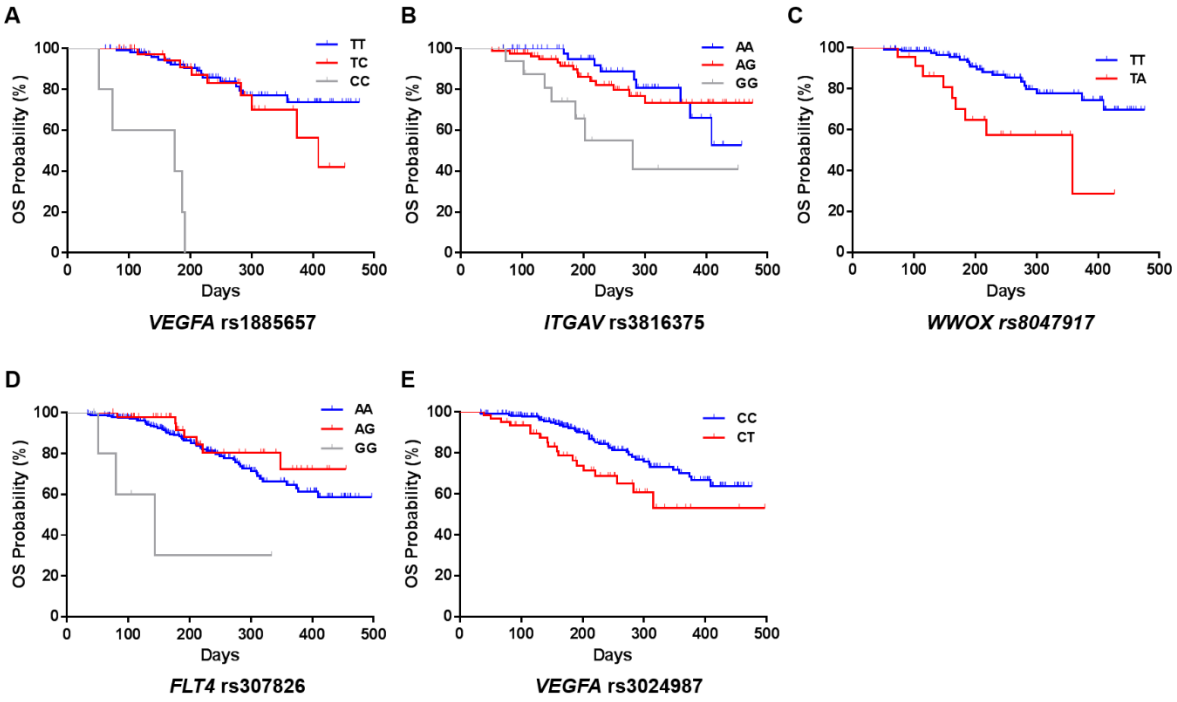


Figure 2. Effects of *FLT4* rs307826 (A>G, T494A) on VEGFR-3 phosphorylation and post-translational processing.

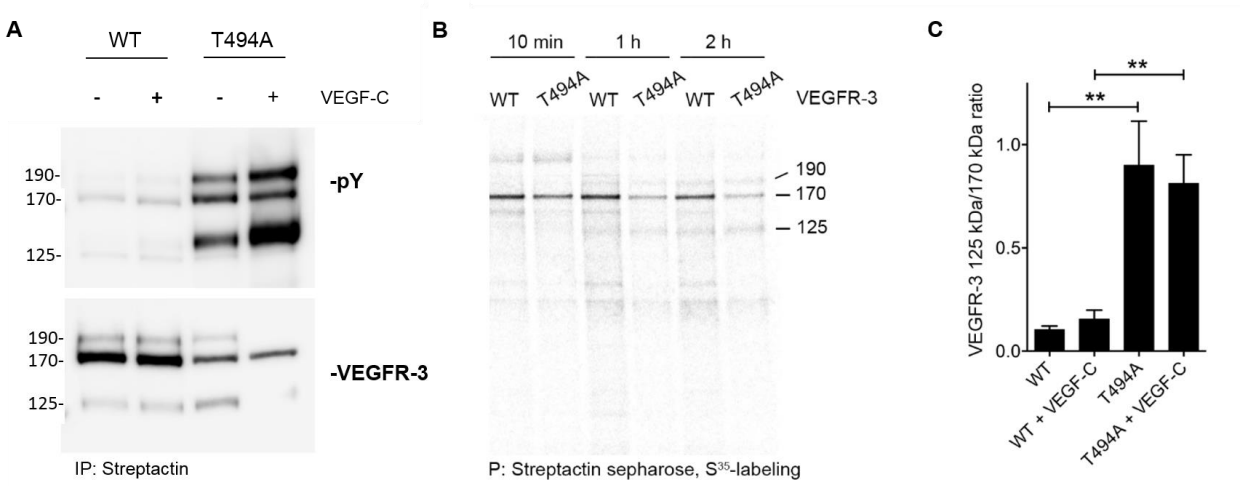


Figure 3. Effects of *FLT4* rs307826 (A>G, T494A) on sorafenib cytotoxicity.

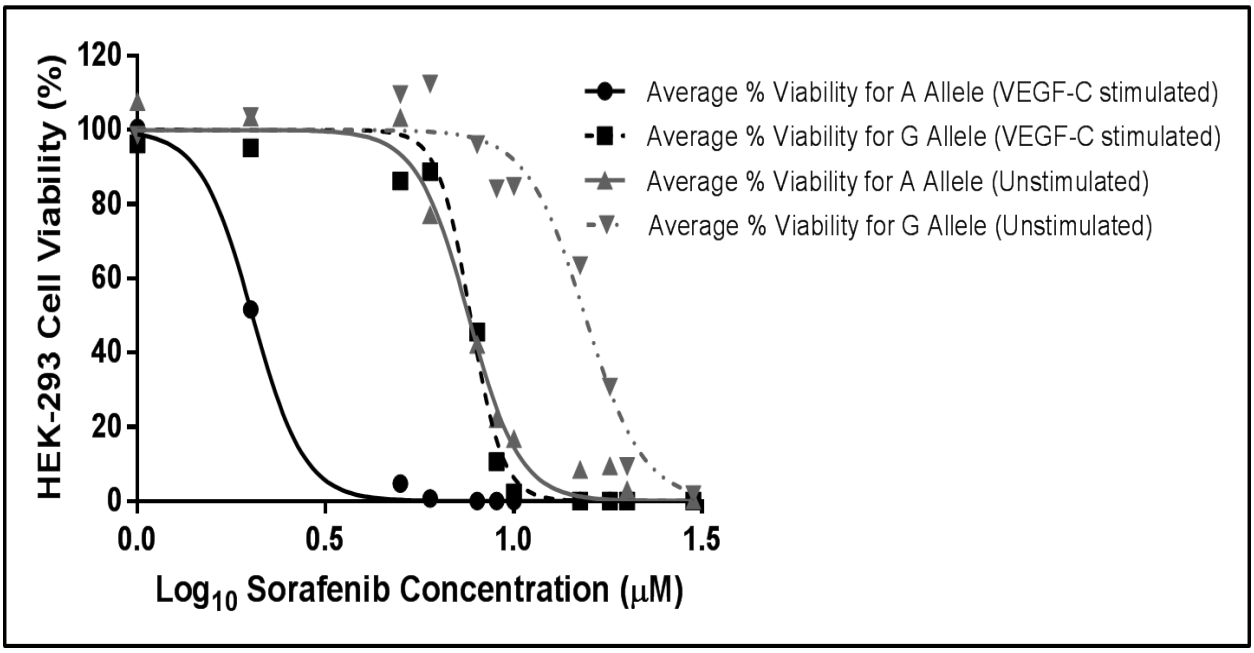


Figure 4. Effects of intronic variants on luciferase activity.

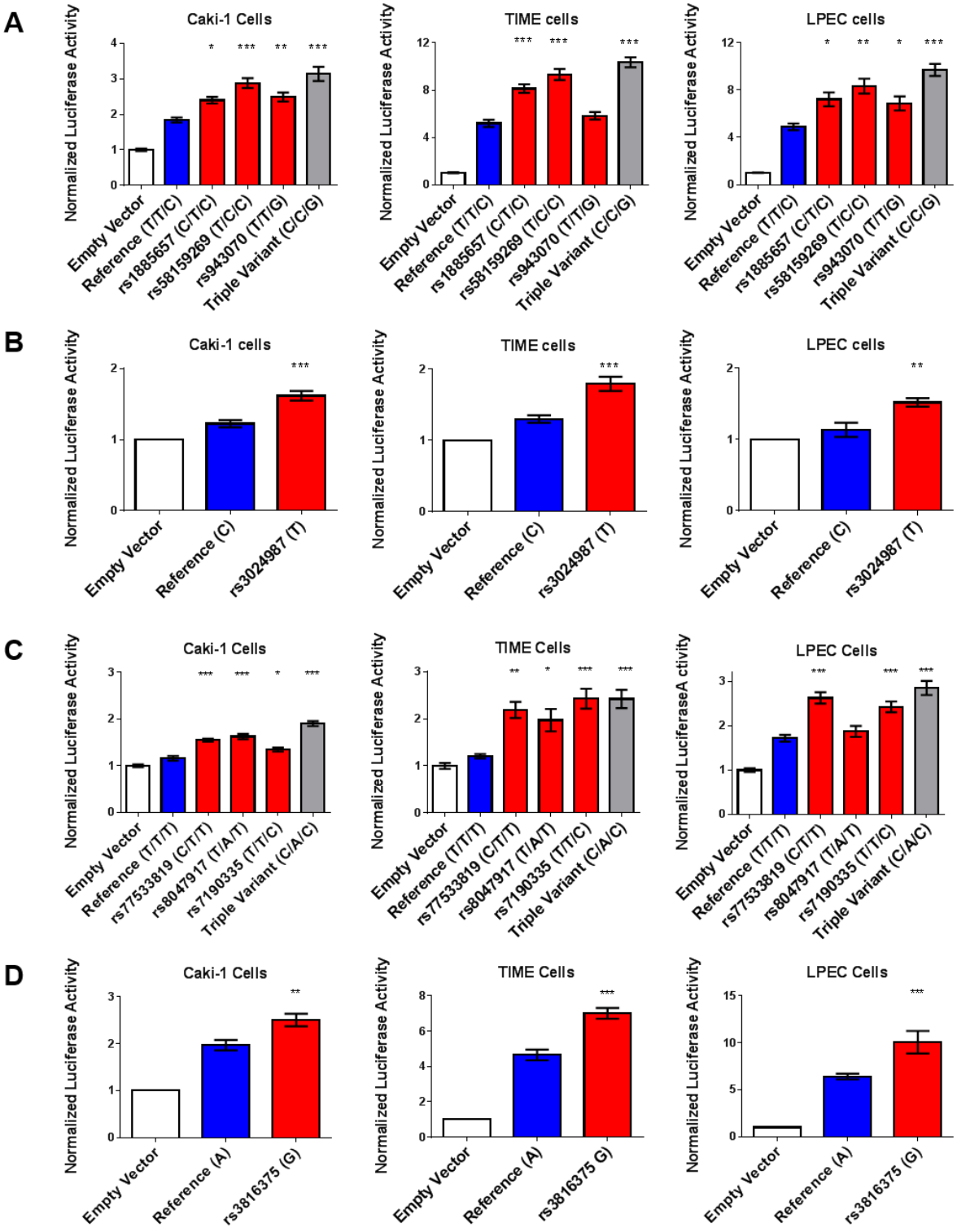
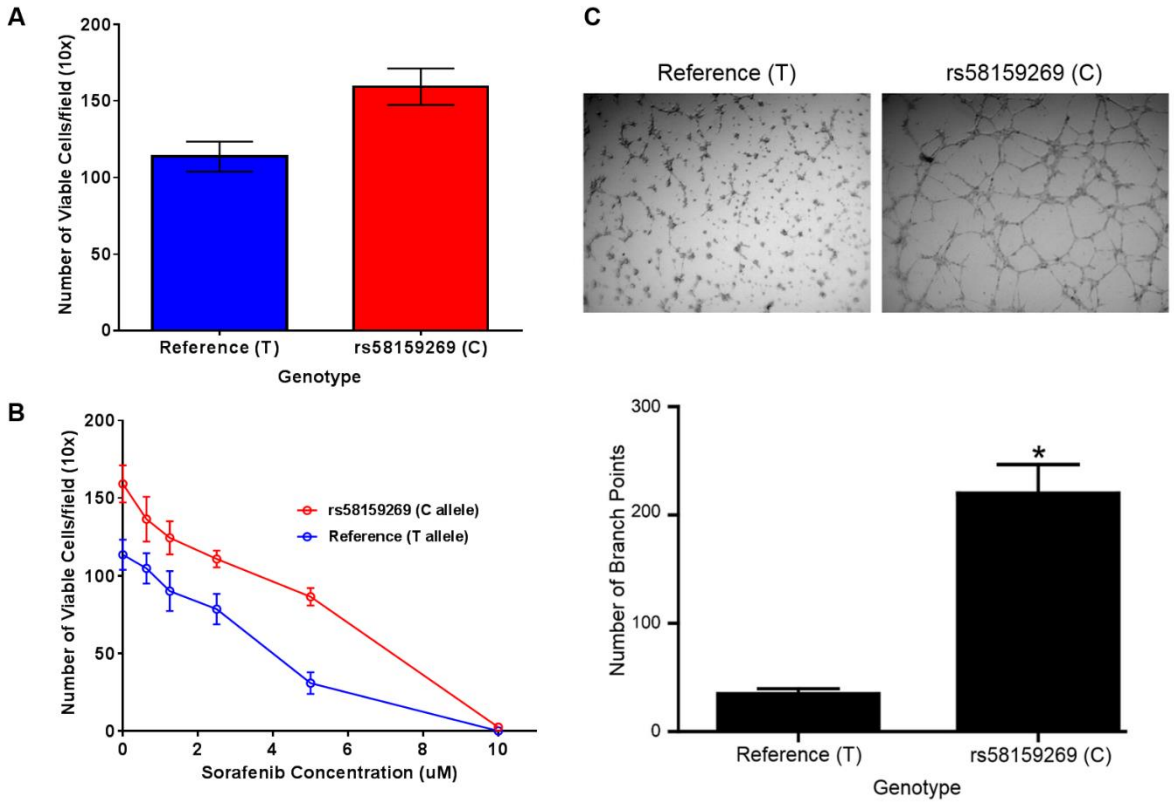


Figure 5. Effects of *VEGFA* rs58159269 on endothelial cell proliferation and tube formation.



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