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Identification of a Genomic Region Between *SLC29A1* and *HSP90AB1* Associated with Risk of Bevacizumab-Induced Hypertension: CALGB 80405 (Alliance)

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Abstract

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Purpose—Bevacizumab is a VEGF-specific angiogenesis inhibitor indicated as an adjunct to chemotherapy for the treatment of multiple cancers. Hypertension is commonly observed during bevacizumab treatment, and high-grade toxicity can limit therapy or lead to cardiovascular complications. The factors that contribute to interindividual variability in blood pressure rise during bevacizumab treatment are not well understood.

Experimental Design—To identify genomic regions associated with bevacizumab-induced hypertension risk, sequencing of candidate genes and flanking regulatory regions was performed on 61 bevacizumab-treated patients (19 cases developed early-onset grade 3 hypertension and 42 controls had no reported hypertension in the first six cycles of treatment). SNP-based tests for common variant associations and gene-based tests for rare variant associations were performed in 174 candidate genes.

Results—Four common variants in independent linkage disequilibrium blocks between *SLC29A1* and *HSP90AB1* were among the top associations. Validation in larger bevacizumab-treated cohorts supported association between rs9381299 with early grade 3+ hypertension ($P=0.01$, OR 2.4) and systolic blood pressure > 180 mmHg ($P=0.02$, OR 2.1). rs834576 was associated with early grade 3+ hypertension in CALGB 40502 ($P=0.03$, OR 2.9). These SNP regions are enriched for regulatory elements that may potentially increase gene expression. *In vitro* overexpression of *SLC29A1* in human endothelial cells disrupted adenosine signaling and reduced nitric oxide levels that were further lowered upon bevacizumab exposure.

Conclusions—The genomic region between *SLC29A1* and *HSP90AB1* and its role in regulating adenosine signaling are key targets for further investigation into the pathogenesis of bevacizumab-induced hypertension.

Keywords

Bevacizumab; hypertension; sequencing; *SLC29A1*; *HSP90AB1*

Introduction

Bevacizumab is a recombinant humanized monoclonal antibody that targets human VEGF, preventing its binding to VEGF receptor 2 (VEGFR2); its modes of action are thought to include the inhibition of angiogenesis (1) as well as enhanced antigen presentation. Bevacizumab has been approved by the U.S. Food and Drug Administration for the treatment of metastatic colorectal cancer, advanced nonsquamous non-small cell lung cancer, metastatic renal cell carcinoma, recurrent glioblastoma, advanced cervical cancer, and platinum-resistant ovarian cancer (2).

The development of hypertension is frequently observed during treatment with bevacizumab. Hypertension of all grades has been observed in up to 36% of patients treated with bevacizumab (3), with grade 3–4 hypertension reported in 1.8 to 22% of treated patients (4). Generally, the blood pressure increase can be controlled with standard antihypertensive medications. However, unmanaged hypertension may lead to serious cardiovascular complications and can occasionally be life-threatening. In the setting of severe hypertension, bevacizumab is either held temporarily or discontinued, thereby limiting therapy that may otherwise be beneficial. There are currently no validated biomarkers to predict bevacizumab

toxicity, and the factors that contribute to interindividual variability in blood pressure rise during bevacizumab treatment are not well understood.

Prior studies have evaluated and identified associations between hypertension incidence during bevacizumab treatment and common single nucleotide polymorphisms (SNPs) in candidate genes encoding VEGF (5–9) and VEGFR2 (10); however, the directions of effect for several of these findings are discordant. More recent studies utilizing expanded candidate gene and genome-wide strategies have identified risk variants in *SV2C* (11) and *FIP200* (12) and modest associations in *EGLN3*, *EGF*, and *WNK1* (13). Given the heritable but complex nature of primary hypertension, the genetic architecture underlying bevacizumab-induced hypertension is also likely to be polygenic. Additional examination of genetic variation in non-VEGF pathways and of rare variants with potentially large phenotypic effects may identify novel mechanisms contributing to bevacizumab-induced hypertension.

In the present study, candidate genes were sequenced in bevacizumab-treated subjects with the objective of identifying genes harboring multiple variants associated with severe, early-onset bevacizumab-induced hypertension. Functional studies in human endothelial cells provide evidence for the potential involvement of the most significant genomic region in this dose-limiting toxicity.

Materials and Methods

Patient population

The patient cohort for this study was selected from the bevacizumab arm of Cancer and Leukemia Group B (CALGB) 80405, a phase III trial conducted to determine if the addition of cetuximab to FOLFIRI or FOLFOX chemotherapy prolongs survival compared to FOLFIRI or FOLFOX with bevacizumab in patients with untreated advanced or metastatic colorectal cancer who have *KRAS* wild type tumors. This trial has been previously described in detail (14). CALGB is now part of the Alliance for Clinical Trials in Oncology. A total of 899 patients accrued into the bevacizumab arm and received 5 mg/kg bevacizumab every two weeks followed by FOLFOX or FOLFIRI every two weeks; treatment was continued until disease progression, unacceptable toxicity, or surgery with curative intent. DNA was available from 581 bevacizumab-treated patients who were also enrolled in a pharmacogenetic companion study (CALGB 60501) embedded within CALGB 80405. The protocol was approved by the National Cancer Institute Adult Central IRB and by local institutional review boards. All participants provided written informed consent for pharmacogenetic sample procurement and analysis. All studies were conducted in accordance with guidelines outlined in the Declaration of Helsinki.

Phenotype

Eligibility criteria required that patients with preexisting hypertension must be well controlled (blood pressure < 160/90 mmHg) on a regimen of antihypertensive therapy. Blood pressure was measured prior to randomization, at day 1 of each eight-week treatment cycle, and every two weeks during treatment. Serious adverse events, including hypertension, were reported during each cycle. The severity of hypertension was assessed on a scale of 1–5

according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.

Cases and controls were selected using an extreme phenotype design to compare severe, early-onset hypertension patients to those with no reported hypertension (Fig. 1). Cases were defined as having at least one grade 3 or higher hypertension event during the first three treatment cycles. Forty-eight cases were confirmed by extensive chart reviews, with 25 having consent and available DNA. Controls were defined as having no reported hypertension during the first six treatment cycles while completing a minimum of four uninterrupted cycles with no gaps in adverse event form coverage; a cut-off of six cycles was chosen to limit the number of subjects who develop bevacizumab-induced hypertension only after longer treatment. After identifying 299 potential controls, 53 remained after exclusion for one or more criteria (Fig. 1). Forty-six controls who had both pharmacogenetic consent and available DNA were sequenced.

Sequencing

Genomic DNA extracted from blood samples was provided by the Alliance Pathology Coordinating Office, and sequencing was performed at the UCSF Genomics Core Facility. Probes were designed to target the whole exome and intronic, UTR, and 50 kb regions upstream and downstream of selected candidate genes (Supplementary Table S1) for a total target size of 85 Mb (64 Mb standard exome + 21 Mb custom regions). Candidate genes were selected based on their documented role in VEGF signaling, endothelial cell biology, nitric oxide signaling or hypertension, as defined by assignment to a curated gene set or pathway in Gene Ontology Consortium (GO, <http://www.geneontology.org/>), Kyoto Encyclopedia of Genes and Genomes (<http://www.genome.jp/kegg/>), or BioCarta (https://cgap.nci.nih.gov/Pathways/BioCarta_Pathways). GO annotations were required to be supported by experimental evidence, computational evidence, author statements, or curator statements. The gene sets and pathways that were examined for candidate gene selection are listed in Supplementary Table S2. Additional sequencing and quality control methods are described in the Supplementary Methods.

SNP-based association testing

Variants were filtered for MAF ≥ 0.10 in the study cohort (threshold chosen based on limited sample size) and LD-pruned at $r^2 > 0.8$ before testing each variant independently for case-control association using logistic regression under an additive genetic model. Tests were adjusted for sex, age, body mass index (BMI) ≥ 25 , preexisting hypertension, and preexisting diabetes, based on published data describing clinical predictors of the toxicity (15). Associations with Bonferroni-adjusted P -values < 0.05 were considered statistically significant; unadjusted P -values are reported. Top SNPs were analyzed by *in silico* methods described in the Supplementary Methods.

Gene-based association testing

Association testing was conducted at the gene level to evaluate combined effects from multiple rare and low frequency variants (MAF < 0.03) in each gene. Only genes containing more than one observed rare variant allele were tested. SKAT-O (16), which combines the

sequence kernel association test (SKAT) and a standard burden test into a single framework and adaptively selects the best linear combination of test statistics, was used to test each gene for case-control association. All tests were adjusted for sex, age, BMI ≥ 25 , preexisting hypertension, and preexisting diabetes. Associations with Bonferroni-adjusted P -values < 0.05 were considered statistically significant; unadjusted P -values are reported.

Replication analysis of top SNP associations

Top SNP associations were tested for replication in two larger, independent cohorts of bevacizumab-treated patients from clinical trials CALGB 40502 (17) and CALGB 90401 (18). Associations with systolic blood pressure (SBP) > 180 mmHg for available SNPs were also looked up in the GWAS results of a third independent cohort in the ECOG-5103 trial (11) (Supplementary Table S5).

rs3734704 and rs6902226 (proxy for rs6929249 at a physical distance of 346 bp and $r^2 = 0.98$ in 1000G EUR) were individually genotyped in both CALGB populations. rs9381299 genotypes were extracted from existing GWAS data. rs6929249 and rs834576 genotypes in CALGB 40502 were imputed using genome-wide genotyping data and sequencing data from the 1000 Genomes Project (Michigan Imputation Server). SNPs were tested for association with early grade 3+ hypertension using logistic regression under an additive genetic model. Tests were adjusted for the same covariates as in the discovery study, where available. Early hypertension was defined as hypertension occurring within the number of treatment cycles equaling the same total exposure of bevacizumab (60 mg/kg) in the first three cycles of CALGB 80405. Associations with Bonferroni-adjusted P -values < 0.05 were considered statistically significant; unadjusted P -values are reported.

Cell culture

Human umbilical vein endothelial cells (HUVEC; C0035C; Thermo Fisher Scientific) were mycoplasma free upon purchase and maintained in endothelial cell growth medium-2 (EGM-2; Lonza) at 37 °C with 5% CO₂. Cells within passages 2–5 were seeded on 24-well collagen-coated plates for experiments.

Pharmacological treatment

HUVEC were serum-starved overnight in endothelial basal medium-2 (EBM-2; Lonza) with 0.5% FBS. Cells were then treated for one hour at 37 °C with adenosine (Sigma-Aldrich) and human VEGF165 (50 ng/mL; Sigma-Aldrich). Where indicated, bevacizumab (provided by Genentech) was added at a 10X molar ratio of VEGF during the 1-hour treatment period.

For inhibitor experiments, cells were pre-incubated for 30 minutes at 37 °C with non-selective adenosine receptor antagonist CGS-15943 (1 μ M; Tocris Bioscience) or ENT1 inhibitor *S*-(4-Nitrobenzyl)-6-thioinosine (NBTI, 1 μ M; Sigma-Aldrich). Inhibitors were added at the same concentrations during the 1-hour treatment period.

Overexpression of SLC29A1 in HUVEC

At approximately 90% confluency, HUVEC were transiently transfected according to the manufacturer's protocol with 0.75 μ L/well Lipofectamine 3000 (Thermo Fisher), 1 μ L/well

P3000 reagent (Thermo Fisher), and 500 ng/well plasmid DNA in EGM-2. Cells were transfected with pcDNA3.1(+) vector containing human SLC29A1 cDNA (ORF clone ID OHu16500D; GenScript). HUVEC transfected with empty pcDNA3.1(+) vector (Thermo Fisher) were used as negative controls. Cells were incubated at 37 °C for 20 hours following transfection, then serum-starved for four hours (instead of overnight as in untransfected cells) prior to pharmacological treatment.

To confirm SLC29A1 overexpression, RNA was isolated using the RNeasy Mini Kit (Qiagen) and reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad), according to the manufacturers' protocols. Quantitative real-time PCR reactions prepared with Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher) were run on the 7900HT Fast Real-time PCR System (Applied Biosystems) at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. qRT-PCR data were analyzed by SDS software v2.4 (Applied Biosystems).

Measurement of cyclic AMP and nitric oxide

Intracellular cyclic AMP levels were measured with a Human cAMP ELISA kit (ab133051; Abcam) following sample acetylation. Total nitrate and nitrite levels, as a measure of nitric oxide, were measured with a colorimetric Nitric Oxide Assay kit (ab65328; Abcam).

Statistical analysis

SNP-based association tests in the discovery sequencing analysis were performed using PLINK/SEQ (<http://atgu.mgh.harvard.edu/plinkseq/>). Gene-based SKAT-O analyses, implemented through the SKAT package (<https://CRAN.R-project.org/package=SKAT>), and logistic regression analyses in replication cohorts were performed using the R statistical environment (19).

To analyze functional data, linear trends across adenosine concentration levels were assessed by the Cochran-Armitage test. Differences between control and treatment groups (CGS-15943-treated vs. vehicle-treated, NBTI-treated vs. vehicle-treated, SLC29A1-transfected vs. empty vector-transfected, bevacizumab-treated vs. vehicle-treated) were analyzed using unpaired t-test or three-way ANOVA, as appropriate. Differences with two-sided *P*-values < 0.05 were considered statistically significant. Statistical analyses and visualization were conducted by the Kroetz laboratory and Alliance statisticians using R (19), on a data set frozen on February 26, 2013.

Results

Patient characteristics and selection

Seventy-one samples (25 cases and 46 controls) were selected for sequencing. Twelve cases developed grade 3 hypertension in the first treatment cycle, ten cases in cycle 2, and three cases in cycle 3. Following quality control procedures, one sample was excluded due to sample contamination (detected by high heterozygosity, low concordance with GWAS data, and discordant gender). A total of 61 European samples (19 cases and 42 controls) with complete sequence and phenotype data were retained for analysis. Demographic and clinical

characteristics of the analyzed cohort are listed in Table 1 and, with the exception of preexisting hypertension, are similar between cases and controls and reflect the larger study population.

Candidate gene analysis

In a targeted analysis of 174 prespecified candidate genes (Supplementary Table S1) for which additional intronic, UTR, and flanking regions were sequenced, association testing was performed on 92,886 coding and noncoding variants (Supplementary Fig. S1). Common variants (MAF ≥ 0.10 , $N = 9,356$) were analyzed in SNP-based association tests (Table 2); no variants reached Bonferroni-corrected statistical significance ($P < 5.3 \times 10^{-6}$). Within the ten strongest associations, four SNPs were located in the same intergenic region downstream of *SLC29A1* and upstream of *HSP90AB1*: rs6929249, rs3734704, rs834576, and rs9381299 (Supplementary Fig. S2). For all four SNPs, the proportion of early grade 3 hypertension in minor allele carriers was consistently higher (58–70%) than in non-carriers (6–24%) (Fig. 2).

To test the possibility that individual genes harboring multiple rare and low frequency variants contribute to toxicity risk, 35,472 variants with MAF < 0.03 in the 174 candidate gene regions were tested using SKAT-O. No gene associations met statistical significance after Bonferroni correction ($P < 2.9 \times 10^{-4}$; Supplementary Table S6).

In silico functional analysis

The four SNPs between *SLC29A1* and *HSP90AB1* identified in the candidate gene analysis are noncoding, and all proxy SNPs ($r^2 > 0.8$) are also in noncoding regions. These variants were examined for overlap with regulatory elements inferred from functional data generated in HUVEC from the ENCODE Project (Supplementary Fig. S3). rs6929249 and rs3734704 are located within 1 kb downstream of the 3' end of *SLC29A1* in a moderately transcribed region enriched for histone modifications linked to gene activation (H3K4me1, H3K27ac, H3K36me3, H3K79me2) and binding of CTCF, which can act as an insulator. rs834576 is in a region approximately 3.8 kb downstream of *SLC29A1* that is also enriched for H3K4me1, H3K27ac, and CTCF as well as additional activating histone modifications (H3K4me2, H3K4me3, H3K9ac, H2A.Z) and binding of RNA polymerase II; the variant site also maps to a CpG island, a feature often associated with promoters. rs3734704 and rs834576 also overlap with DNase I hypersensitivity peaks representing regions of open chromatin and are in genomic segments predicted to be strong enhancers. rs6929249 is in a predicted region of transcriptional transition, and rs9381299 in a predicted region of weak transcription. Similar data from the Roadmap Epigenomics Project for a diversity of cell lines corroborated the ENCODE predictions for HUVEC and support a more global role for the regions containing the rs6929249, rs3734704 and rs834576 variants as enhancers or promoters. In a search of previously published eQTL analyses, the risk allele of rs9381299 was found to be associated with increased *SLC29A1* expression in monocytes ($P = 3.8 \times 10^{-5}$, $\beta = 0.18$) (20).

Replication analysis of top SNP associations

rs6929249 (or a proxy SNP, rs6902226), rs3734704, rs834576, and rs9381299 were tested for association with early grade 3+ hypertension in independent bevacizumab-treated

cohorts within CALGB 40502 and CALGB 90401; imputed rs834576 genotypes were available only in CALGB 40502. Association of rs9381299 with SBP > 180 mmHg was also examined in ECOG-5103 GWAS data, with SBP > 180 being an even more extreme phenotype than grade 3+ hypertension, which typically occurs at SBP > 160. Other SNPs were not available for lookup in ECOG-5103. Descriptions of these studies and clinical characteristics of the cohorts used in this study are summarized in Supplementary Tables S5 and S7.

rs9381299 significantly associated with early grade 3+ hypertension in CALGB 40502 ($P=0.01$, odds ratio (OR) 2.4) and with SBP > 180 in ECOG-5103 ($P=0.02$, OR 2.1) with a concordant direction of effect, but did not associate with grade 3+ hypertension in CALGB 90401 (Table 3). The proportion of hypertension was consistently higher in risk variant carriers for rs9381299: 5% vs. 14% in CALGB 40502 and 7% vs. 16% in ECOG-5103 (Supplementary Fig. S4). rs834576 also associated with early grade 3+ hypertension in CALGB 40502 ($P=0.03$, OR 2.9); the proportion of rs834576 risk variant carriers with hypertension was 16%, compared to 6% of those with the homozygous reference genotype (Supplementary Fig. S4). The associations of rs6929249 and rs3734704 with grade 3+ hypertension were not validated in either CALGB cohort

Functional characterization of ENT1 inhibition and SLC29A1 overexpression

SLC29A1 encodes ENT1, a member of the equilibrative nucleoside transporter family. ENT1 is responsible for regulating circulating levels of adenosine, which increases endothelial nitric oxide synthase (eNOS)-dependent activity via adenosine receptor signaling (21). eNOS also lies downstream of VEGF signaling, and activation of eNOS produces NO, a potent vasodilator. To follow up on our genetic findings and the eQTL association with *SLC29A1* expression, the functional effects of *SLC29A1* expression on VEGF signaling were evaluated in HUVEC.

Cyclic AMP (cAMP) levels, as a measure of adenosine receptor signaling, increased as a function of adenosine concentration ($P=0.0002$; Supplementary Fig. S5). Treatment with the non-selective adenosine receptor antagonist CGS-15943 almost completely blocked adenosine-stimulated cAMP formation ($P=0.005$; Supplementary Fig. S6), demonstrating the selectivity of cAMP as a measure of adenosine receptor signaling. Levels of NO, which acts as a vasodilatory signaling molecule, also increased as a function of adenosine concentration ($P=3 \times 10^{-5}$; Supplementary Fig. S5) and decreased by ~50% when adenosine receptor signaling was blocked ($P=0.002$; Supplementary Fig. S6), indicating that NO is also generated from pathway(s) distinct from adenosine signaling.

HUVEC treated with the ENT1 inhibitor NBTI showed increased cAMP levels compared to vehicle-treated cells ($P=1 \times 10^{-10}$; Supplementary Fig. S7), indicating an increase in adenosine receptor signaling. NBTI-treated HUVEC also showed increased NO levels compared to controls ($P=6 \times 10^{-13}$; Fig. 3A). Addition of bevacizumab resulted in significant decreases in NO levels both in the presence and absence of NBTI ($P=5 \times 10^{-6}$; Fig. 3A), without an effect on cAMP levels (Supplementary Fig. S7). These results are consistent with a specific effect of bevacizumab on VEGF-mediated NO production without affecting adenosine signaling.

HUVEC transfected with *SLC29A1* cDNA were confirmed to have substantially increased *SLC29A1* mRNA expression compared to empty vector-transfected control cells (difference of relative expression: $P = 0.0002$). *SLC29A1*-overexpressing HUVEC, in the presence of adenosine, decreased the generation of cAMP when compared to empty vector-transfected controls at all tested adenosine concentrations ($P < 2 \times 10^{-16}$; Supplementary Fig. S8), indicating a decrease in adenosine receptor signaling when *SLC29A1* expression is increased. *SLC29A1*-overexpressing HUVEC showed decreased NO levels compared to empty vector-transfected cells ($P < 2 \times 10^{-16}$; Fig. 3B). Addition of bevacizumab had no effect on cAMP levels (Supplementary Fig. S8) but reduced NO levels. The inhibitory effect of bevacizumab on NO levels ranged from an 18–44% decrease in empty vector-transfected cells compared to a 50–75% decrease in *SLC29A1*-transfected cells, demonstrating a greater reduction in NO following bevacizumab treatment under conditions of high *SLC29A1* expression ($P = 0.02$; Fig. 3B).

Discussion

The present study used a sequencing analysis to discover genes that potentially contribute to bevacizumab-induced hypertension. Although no SNP- or gene-based associations achieved genome-wide significance, we identified SNPs with modest associations that may have regulatory effects on biological pathways related to the phenotype.

A targeted analysis examined variants within and flanking preselected candidate genes. The top ten variant associations with bevacizumab-induced hypertension included four SNPs located in the same genomic region between *SLC29A1* and *HSP90AB1*. These SNPs are not in strong LD ($r^2 < 0.6$, Supplementary Fig. S2 and S9), suggesting that they could have independent effects on the toxicity. Genetic variation in *SLC29A1* or *HSP90AB1* has not been previously associated with hypertension or other cardiovascular phenotypes. While the discovery of common variants was not the original strategy of this sequencing analysis, other than rs9381299, these SNPs are not tagged on any standard commercial GWAS or exome arrays, demonstrating the added potential of examining variants of all allele frequencies identified from sequencing data in the setting of prospectively identified clinical outcomes.

SLC29A1 encodes ENT1, which regulates adenosine levels. Increased circulating adenosine levels have been observed in ENT1 knockout mice (22), and increased extracellular adenosine concentrations are associated with reduced ENT1 expression in HUVEC (23). ENT1 also regulates eNOS-dependent NO production via adenosine receptor signaling (21). eNOS lies downstream of VEGF and has been implicated in hypertension resulting from VEGF inhibition (24).

To investigate whether *SLC29A1* may influence an endothelial cell's response to bevacizumab, functional studies were performed to determine a potential role for ENT1 and adenosine in the regulation of VEGF-dependent NO production. Under conditions of increased extracellular adenosine from pharmacological inhibition of ENT1, bevacizumab selectively inhibited a portion of NO formation without altering cAMP production, suggesting that the effects of VEGF inhibition are not simply an off-target effect on adenosine signaling. Consistent with these findings, overexpression of *SLC29A1* in HUVEC

caused a decrease in adenosine receptor signaling that is insensitive to VEGF inhibition while NO synthesis from the parallel VEGF pathway has enhanced sensitivity to bevacizumab under these conditions. While these observations must be further validated, we hypothesize that variants causing increased ENT1 expression and activity may reduce extracellular adenosine levels and cause vasoconstriction by impairing the endothelium's ability to maintain a necessary amount of adenosine-stimulated NO production while under conditions of reduced VEGF signaling. Patients with increased ENT1 expression would thereby be more sensitive to changes in eNOS signaling and NO production from VEGF inhibition by bevacizumab. Similar changes in sensitivity to bevacizumab treatment could occur following changes in ENT1 expression or function as a result of epigenetic regulation of *SLC29A1* or drug interactions.

Variation in other nucleoside transport and adenosine signaling pathway genes may have similar effects on the toxicity. Changes in ENT1 expression have been reported to directly affect adenosine deaminase and adenosine A_{2A} receptor expression (25), and decreased SLC29A1 expression and ENT1 transport activity have been described during preeclampsia (26,27), a complication of pregnancy that is characterized by high blood pressure. Such changes are accompanied by observations of increased adenosine concentrations, increased adenosine-generating enzyme activity (26), reduced A_{2A} receptor expression and enhanced A_{2B} receptor signaling (27,28), and upregulation of soluble VEGFR1 (sFlt-1) (29,30), which binds to VEGF as bevacizumab does. Although the association of decreased SLC29A1 expression with hypertension contradicts our hypothesized mechanism, our findings still support the idea of dysregulated adenosine transport having an effect on blood pressure. Expression and activity of these proteins and of other nucleoside transporters should be considered in future studies of bevacizumab-induced hypertension, as multiple aberrations of the adenosine signaling pathway could result in a more sensitive response. Changes in adenosine transport rates, receptor binding affinities, or enzyme kinetics modulated by genetic effects or drug interactions should also be considered during functional studies of bevacizumab treatment.

Although there was more bioinformatic evidence supporting the association of the identified *SLC29A1-HPS90AB1* SNPs with SLC29A1 expression and function, *HSP90AB1* is also of biological interest. This gene encodes a constitutively expressed, cytosolic isoform of Hsp90, a molecular chaperone that facilitates normal folding, stability, activation, function, and proteolytic turnover of many key regulators of cell growth and survival, including eNOS (31). Reduced Hsp90 expression in primary HUVEC has been observed during preeclampsia (32), and hypertension has presented as a common adverse event during early clinical trials of Hsp90 inhibitor treatment (33,34). Hsp90 binds to eNOS and enhances its NO-synthesizing activity (35); at least in part, VEGF itself signals the binding of Hsp90 with eNOS (36,37). Disruption of Hsp90 signaling, either by chemical inhibition or mutagenesis, has been shown to attenuate VEGF-stimulated binding of Hsp90 to eNOS, NO production, and endothelium-dependent relaxation of isolated blood vessels (35,37–39). Thus, variants reducing the expression or function of Hsp90 may have similar effects as increased ENT1 function. Specifically, reduced baseline eNOS activity and NO production could amplify the effects of reduced VEGF signaling during bevacizumab treatment and contribute to the observed dysregulation of vascular tone and increased blood pressure. Future studies should

explore this possibility and the complex interaction between VEGF, Hsp90 and adenosine signaling.

Based on data from our *in silico* analyses, the identified SNPs are located in putative regulatory regions in HUVEC. Three of the four SNPs are in moderately transcribed regions just downstream of *SLC29A1* that are enriched for epigenetic marks, RNA polymerase II binding, and other genomic features associated with transcriptional activity. Together, these data suggest that this region may be part of an unannotated *SLC29A1* splice variant or contain an enhancer that regulates expression of a nearby gene. Variants that alter CTCF binding sites may disrupt insulator activity and permit promoter-enhancer interactions in this region. Detailed analysis of the ENCODE data used for our *in silico* analyses found an abundance of disease-associated SNPs in predicted strong enhancers (40), supporting a role for the regulatory SNPs identified in these studies in the bevacizumab-induced hypertension phenotype. The fourth SNP, rs9381299, was found to be associated with increased *SLC29A1* expression in monocytes but not in other more relevant tissues like the artery. Functional studies in vascular endothelial cell models are needed to better assess the SNP effects on both *SLC29A1* and *HSP90AB1* expression and function.

These identified SNPs were tested for association in larger, independent bevacizumab-treated cohorts. The association of rs9381299 and rs834576 with early grade 3+ hypertension in CALGB 40502 and rs9381299 with SBP > 180 mmHg in ECOG-5103 supports the discovery results. Other SNPs were not validated. Limited statistical power, differences in eligibility, clinical trial design, bevacizumab dosing, and adverse event collection, or demographic and clinical differences between study populations may explain why some associations failed to replicate. In particular, CALGB 90401 did not have preexisting hypertension data available for adjustment of the models, which might contribute to the lack of replication in this cohort. Examination of all of these SNPs in additional bevacizumab-treated cohorts is warranted.

A major limitation of this study is sample size. Due to lack of consent or available DNA and a rigorous phenotypic review, many potential subjects could not be sequenced. An extreme phenotype design was utilized to enrich sampling of causal variants with large effect sizes and to increase statistical power (41). Such an approach has been used in cohorts of similar sizes to identify novel variants associated with complex disease and pharmacogenetic traits, such as *P. aeruginosa* infection (42), warfarin dosing (43), drug-induced long QT interval syndrome (44), gemcitabine/carboplatin-induced myelosuppression (45), and clopidogrel response (46). Considering sample size, we recognize the limitations of the study design and approach these analyses largely as a discovery study. Replication and sequencing are needed in additional cohorts to extend these current findings.

Another challenge of this study lies in the difficulty of identifying true drug-induced hypertension. We limited our phenotype to hypertension that occurred in the first three treatment cycles, as bevacizumab-induced hypertension has been shown to develop early (15), thus removing later cases of hypertension that are more likely to be attributed to other factors. Preexisting hypertension was significantly correlated with on-treatment hypertension ($P = 0.002$, OR 6.7), with 14 of 19 (74%) cases having preexisting hypertension compared to

12 of 42 (29%) controls. This association is possibly confounding, and genetic models were adjusted for preexisting hypertension to minimize this effect. However, this association has been previously shown (47,48) and may possibly be informative. Hypertensive patients were required to be normotensive upon study initiation, and even while on antihypertensive medications many still developed the toxicity. This suggests that some patients may be genetically predisposed to an especially sensitive response to bevacizumab, such that even pharmacologically controlled hypertension is further exacerbated upon bevacizumab treatment. Although the present study had too few subjects to test this hypothesis, a future study stratifying by the class of antihypertensive medication could elucidate a more specific mechanism of the toxicity. The inclusion of subjects from ECOG-5103 as a replication cohort provides some insight regarding the contribution of the identified SNPs in baseline hypertension versus bevacizumab-induced hypertension. The association of rs9381299 with bevacizumab-induced hypertension was replicated in ECOG-5103, a cohort that excluded all patients with preexisting hypertension, supporting our original discovery results with unbalanced preexisting hypertension status. *Post hoc* analyses of the 19 cases of bevacizumab-induced hypertension used in the discovery analysis matched for preexisting hypertension status with a new control set of 49 subjects receiving at least four cycles of bevacizumab gave a similar effect size and direction as the original results for the three SNPs that could be evaluated (data not shown), further supporting our discovery findings. Future genomic and functional studies are needed to more carefully define the involvement of *SLC29A1* and *HSP90AB1* in these phenotypes.

Hypertension also occurs during treatment with other VEGF pathway inhibitors (15), including aflibercept, a soluble decoy receptor that binds VEGF, and small molecule VEGF receptor tyrosine kinase inhibitors (e.g., sunitinib, sorafenib). The frequency of all-grade hypertension during treatment with these small molecule inhibitors ranges from 15 to 67%, with a greater incidence observed during treatment with more potent inhibitors such as axitinib, regorafenib, and cediranib, where incidence of grade 3–4 hypertension has been reported as high as 43% (49). Genetic associations with hypertension during sunitinib treatment have also been reported and include SNPs in *VEGFA*, *KDR*, and *NOS3* (eNOS) (50). These pharmacogenetic findings and the correlation of potency and hypertension incidence suggest that the hypertension is primarily an on-target effect of VEGF and eNOS inhibition, as is hypothesized for bevacizumab-induced hypertension. Therefore, our results may also be extended to the study and treatment of other VEGF inhibitor toxicities.

A better understanding of the genetic architecture of bevacizumab-induced hypertension will advance the understanding of how genetic variability influences the action of angiogenesis inhibitors and the pathogenesis of drug-induced hypertension. Furthermore, identifying genetic predictors of this toxicity could support the development of improved and novel strategies to predict and treat pharmacologically induced hypertension and aid in the selection of appropriate treatment for cancer patients. The identification of sub-populations of patients at risk for bevacizumab toxicity will support more aggressive monitoring of blood pressure and prophylactic antihypertensive treatment in these patients. Since bevacizumab treatment that may otherwise be beneficial is held or discontinued upon development of hypertension, it is clinically important to manage complications to prolong effective therapy. Blood pressure elevation itself has been proposed as a marker for VEGF

inhibitor efficacy (51); if true, this group may be at a therapeutic benefit if their hypertension can be well controlled.

In this study, potential regulatory bevacizumab-induced hypertension risk variants were identified near genes not previously associated with this toxicity. This is the first study to date using sequencing to examine bevacizumab-induced hypertension. While our association results were modest, the region between *SLC29A1* and *HSP90AB1* as well as other genes in the adenosine signaling pathway should be prioritized for follow-up in higher-powered replication studies and in functional studies to define the role of these genes in regulation of vasodilation upon bevacizumab exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

Bevacizumab use in the treatment of cancer can lead to the development of hypertension in some patients. High-grade hypertension can lead to cardiovascular complications and require delay or discontinuation of therapy. Biomarkers for the prediction of bevacizumab-induced hypertension could inform personalized treatment decisions and minimize the number of patients experiencing this dose-limiting toxicity. A sequencing analysis of candidate genes identified a putative regulatory genomic region between *SLC29A1* and *HSP90AB1* containing several variants associated with hypertension in bevacizumab-treated colorectal cancer patients with extreme toxicity phenotypes. Functional experiments in human endothelial cells further suggest that the expression of *SLC29A1* determines in part the cellular response to inhibition of VEGF signaling. The proposed association of novel genes with bevacizumab-induced hypertension may lead to improved prediction and management of this toxicity in patients at elevated hypertension risk.

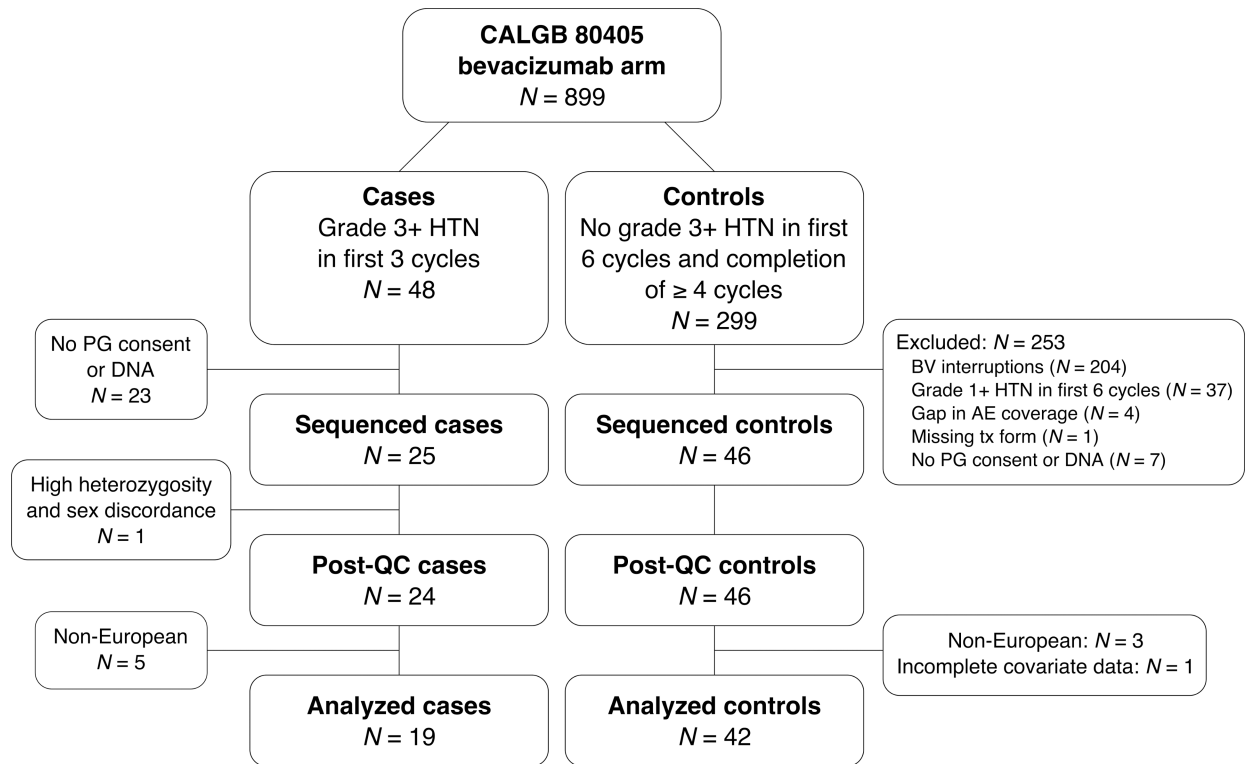


Figure 1. Subject selection from bevacizumab-induced hypertension extreme phenotypes in CALGB 80405

Abbreviations used: HTN, hypertension; PG, pharmacogenetic; BV, bevacizumab; AE, adverse event; tx, treatment; QC, quality control.

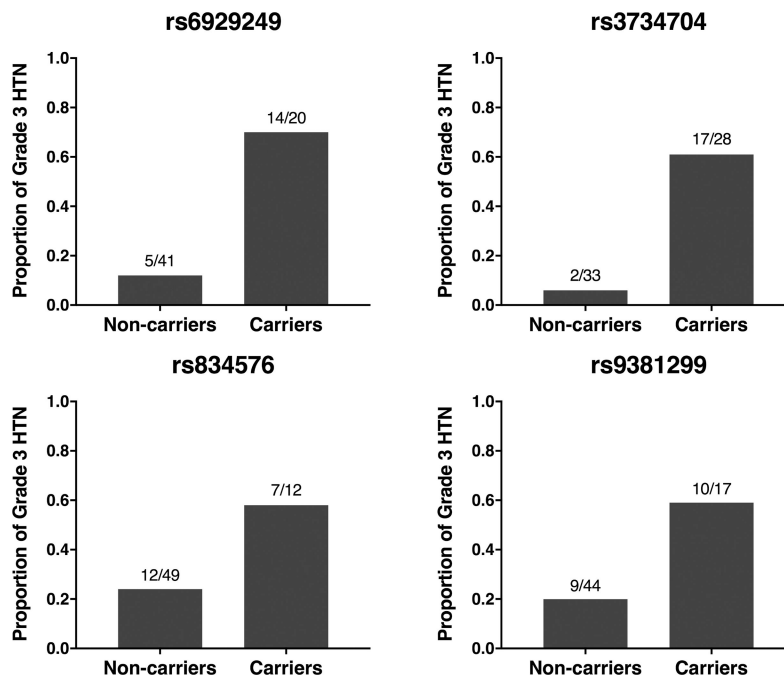


Figure 2. Proportion of early grade 3 hypertension in bevacizumab-treated patients stratified by top *SLC29A1-HSP90ABI* SNP carrier status
 Variant alleles of each SNP are associated with higher incidence of early grade 3 hypertension (HTN) in analyzed subjects. Fractions represent the number of HTN cases over the total number of subjects for each carrier status.

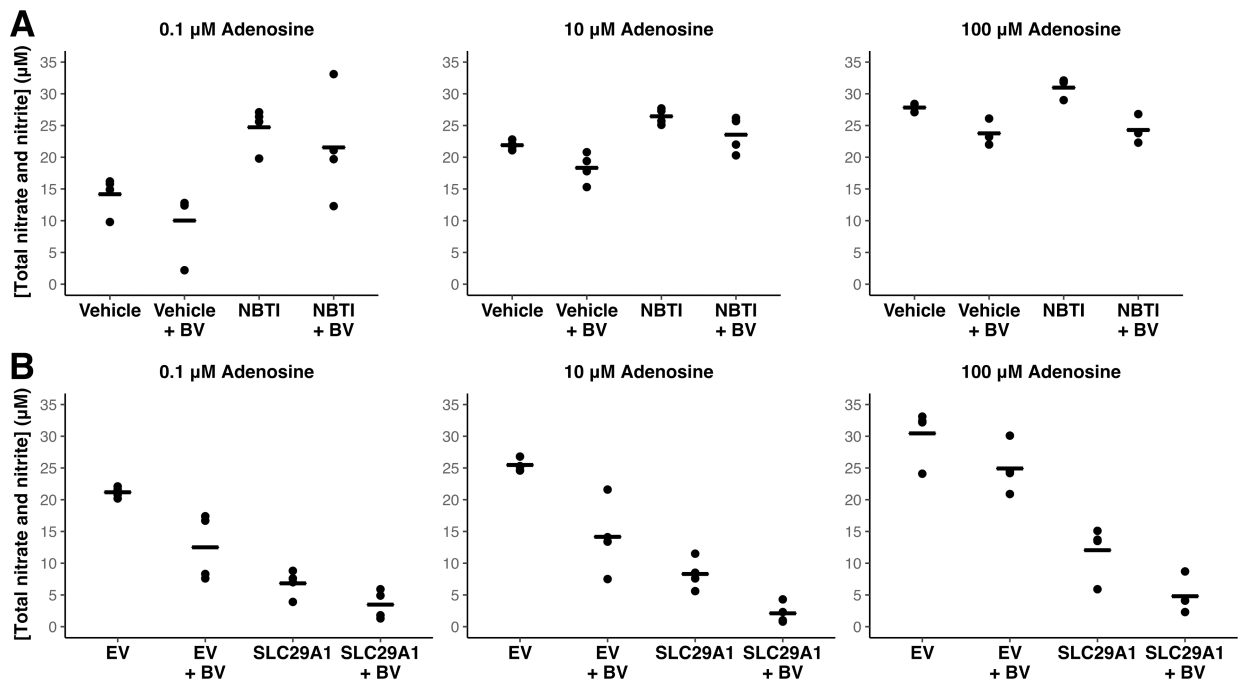


Figure 3. Effect of ENT1 inhibition and SLC29A1-overexpression on nitric oxide levels in HUVEC

HUVEC were incubated in 50 ng/mL VEGF and adenosine (0.1, 10, 100 μM) for one hour.

A) Treatment with the ENT1 inhibitor NBTI (1 μM) increased levels of nitric oxide (NO; $P = 6 \times 10^{-13}$) compared to vehicle-treated cells at all adenosine concentrations. Exposure to bevacizumab (BV; 10X molar ratio of VEGF) decreased NO levels ($P = 5 \times 10^{-6}$). There was no difference in the effect of BV on NBTI-treated versus vehicle-treated cells. **B)** Prior to pharmacological treatment, an expression vector encoding SLC29A1 cDNA was transfected into HUVEC. Empty vector (EV) was transfected into HUVEC as a negative control. SLC29A1 overexpression decreased the release of nitric oxide when compared to EV-transfected HUVEC ($P < 2 \times 10^{-16}$). Exposure to bevacizumab reduced NO levels in both groups and had a greater inhibitory effect in the SLC29A1-transfected cells ($P = 0.02$ compared to BV effect in EV). Points represent means from three or four independent experiments; lines represent group means. For each study, the effects of either NBTI treatment or SLC29A1 overexpression and the comparison of BV exposure at each adenosine concentration were analyzed by three-way ANOVA.

Table 1

Characteristics of extreme phenotype patient subgroups

	Cases	Controls	CALGB 80405 bevacizumab arm	CALGB 80405 total population
Subjects (<i>N</i>)	19	42	899	2334
Female (<i>N</i> , %)	9 (47%)	14 (33%)	364 (40%)	976 (42%)
Age, years (median, range)	59 (31–80)	61 (37–82)	59 (22–85)	59 (21–90)
BMI, kg/m ² (median, range)	25.5 (17.7–40.8)	26.9 (18.6–58.4)	26.8 (14.7–70.7)	27.1 (14.5–70.7)
Preexisting diabetes (<i>N</i> , %)	3 (16%)	4 (10%)	139 (15%)	347 (15%)
Preexisting hypertension (<i>N</i> , %) ^a	14 (74%)	12 (29%)	361 (40%)	932 (40%)

^aDifference between cases vs. controls: *P* = 0.002.

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Table 2

SNP-based analysis of common variants in candidate genes

SNP (ref/var)	Case genotype counts ^a	Control genotype counts ^a	Case MAF	Control MAF	OR (95% CI)	<i>P</i> ^b	Candidate gene ^c	Function
rs6929249 (A/G)	5/13/1	36/6/0	0.39	0.07	37.8 (5.8–247)	1 x 10 ⁻⁴	<i>HSP90AB1</i>	upstream
rs3734704 (A/C)	2/14/3	31/10/1	0.53	0.14	28.9 (4.2–199)	6 x 10 ⁻⁴	<i>HSP90AB1</i>	upstream
rs834576 (C/A)	12/6/1	37/5/0	0.21	0.06	19.3 (2.9–127)	0.002	<i>HSP90AB1</i>	upstream
rs59189065 (G/A)	7/11/1	35/7/0	0.34	0.08	9.9 (2.2–44.3)	0.003	<i>PRKCA</i>	intronic
rs2470417 (C/T)	6/12/1	33/9/0	0.37	0.10	12.7 (2.4–67.5)	0.003	<i>CACNA1C</i>	upstream
rs9381299 (T/C)	9/8/2	35/7/0	0.32	0.08	8.8 (2.0–37.8)	0.004	<i>HSP90AB1</i>	upstream
rs11651806 (C/G)	13/6/0	11/27/4	0.16	0.41	0.1 (0.0–0.5)	0.004	<i>CCL2</i>	upstream
rs72869749 (C/T)	9/9/1	35/7/0	0.29	0.08	26.1 (2.8–239)	0.004	<i>PDE3B</i>	intronic
rs142385484 (C/T)	12/7/0	38/3/1	0.18	0.06	13.9 (2.3–84.2)	0.004	<i>NOS1P</i>	downstream
rs11739214 (G/C)	10/6/3	33/9/0	0.32	0.10	10.1 (2.1–48.9)	0.004	<i>FLT4</i>	downstream

CI, confidence interval

^aGenotype counts: Homozygous reference / heterozygous / homozygous variant.^bUnadjusted *P*-value from logistic regression under an additive genetic model and adjusted for sex, age, BMI, 25, preexisting hypertension, and preexisting diabetes.^cRepresents candidate gene ± 50 kb flanking region containing the sequenced variant. In some cases, this may not be the nearest gene to the variant.

Table 3

Replication analysis of top *SLC29A1-HSP90AB1* SNP associations in independent bevacizumab-treated cohorts

Study	Phenotype	SNP	Case genotype counts ^a	Control genotype counts ^a	Case MAF	Control MAF	OR (95% CI)	<i>P</i> ^b
CALGB 40502	Early grade 3+ hypertension ^c	rs6929249 ^d	19/10/0	278/98/10	0.17	0.15	1.1 (0.5–2.2)	0.81
		rs3734704	11/17/1	222/134/30	0.33	0.25	1.5 (0.8–2.6)	0.20
		rs834576 ^d	23/6/0	355/30/1	0.10	0.04	2.9 (1.0–7.6)	0.03
		rs9381299	16/13/0	303/76/7	0.22	0.12	2.4 (1.2–4.9)	0.01
CALGB 90401	Early grade 3+ hypertension ^c	rs6902226 ^e	8/5/0	214/82/5	0.19	0.15	1.4 (0.4–3.6)	0.57
		rs3734704	6/5/2	164/120/17	0.35	0.26	1.6 (0.6–3.8)	0.29
		rs9381299	8/5/0	225/73/3	0.19	0.13	1.7 (0.5–4.7)	0.36
ECOG-5103	SBP > 180 mmHg	rs9381299	23/16/0	308/79/4	0.21	0.11	2.1 (1.1–3.7)	0.02

CI, confidence interval

^aGenotype counts: Homozygous reference / heterozygous / homozygous variant.

^bUnadjusted *P*-value from logistic regression under an additive genetic model and adjusted for covariates listed in Supplementary Table S5.

^cEarly hypertension is defined as hypertension occurring within the number of treatment cycles equaling the same total exposure of bevacizumab (60 mg/kg) in the first three treatment cycles of CALGB 80405.

^drs6929249 and rs834576 genotypes were imputed. The imputation accuracy for these two SNPs was excellent: rs6929249: $R^2 = 0.95$; rs834576: $R^2 = 0.94$.

^ers6902226 is a proxy for rs6929249 ($r^2 = 0.98$).