

Correlation between SNP rs604723 in *GRAF3* and hypertension

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Approved:

A handwritten signature in dark ink, appearing to be 'JMT', is written over a light blue horizontal line.

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Abstract:

Patients with hypertension (high blood pressure) have increased risk for cardiovascular diseases, stroke, myocardial infarction, diabetes, and kidney failure. Only 51.8% of hypertensive patients have their blood pressure (BP) under control, and control is difficult to achieve since so little is known about the molecular and genetic regulation of BP. A thorough understanding of the pathways that regulate BP can lead to more effective and personalized antihypertensive therapies. We previously established that a protein called GTPase Regulator Associated with Focal adhesion kinase 3 (GRAF3) helps maintain normal BP by inhibiting RhoA in smooth muscle cells (SMCs). This in turn reduces the SMC contractility and BP. Two previous genome wide association studies for BP and cardiovascular disease identified two single nucleotide polymorphisms (SNPs) within the first intron of the *GRAF3* gene, rs633185 and rs604723, which are associated with a 2 mm Hg increase in BP in humans. I performed an allelic discrimination assay on rs604723 and found that this SNP is more prevalent in non-hypertensive individuals. Additionally, the population homozygous for this SNP has the lowest average systolic blood pressure (126.29 mm Hg) and diastolic blood pressure (76.87 mm Hg). It is also known that risk associated with hypertension varies in different race groups. Our study shows that the minor allele frequency of SNP rs604723 is higher in Caucasians (26.74%) than in African Americans (4.84%). These results provide support for the hypothesis that SNP rs604723 plays a protective role in BP regulation. Future analysis will be aimed at studying SNP rs604723 and other SNPs in the RhoA pathway in more detail, in hopes of someday developing better antihypertensive therapies.

Introduction:

Blood pressure (BP) is a very complex trait that is not regulated by one specific organ system or one signaling pathway. It is under the control of multiple organ systems such as the endocrine, nervous, cardiovascular, and urinary systems. Patients with high BP, which is known as hypertension (HTN), have a higher risk for cardiovascular diseases, stroke, myocardial infarction, diabetes, and kidney failure. Even though 1 of 3 US adults has HTN and only 51.8% have their BP under control¹, very little is known about its regulation at the molecular level and what genes and mechanisms are involved. Therefore, a thorough understanding of the molecular and genetic pathways that regulate blood pressure can lead to more effective and personalized antihypertensive therapies.

A common characteristic of HTN is increased peripheral vascular resistance due to decreased arteriolar vessel diameter. This is a result of increased smooth muscle cell (SMC) contractions within resistance arterioles whose internal diameter is less than 200 microns²⁻⁶. Figure 1 shows a schematic representation of the Ca^{2+} -calmodulin pathway and RhoA pathway that regulate SMC contractions and blood pressure. When an extracellular vasoconstrictor signal such as angiotensin II, endothelin-1, or norepinephrine binds to and activates a G-protein coupled receptor (GPCR) on the cell surface of a SMC, the GPCR activates the intracellular Ca^{2+} -calmodulin pathway. This pathway eventually activates myosin light chain (MLC) protein, which then associates with actin and lead to SMC contraction^{7,8}. Increasing SMC contraction increases vascular resistance due to vasoconstriction, resulting in an increase in blood pressure. Simultaneously, the binding of extracellular signals to GPCR also triggers the guanine

nucleotide exchange factors (GEFs) to activate RhoA, a GTP-binding protein, in the SMCs. This activated RhoA activates Rho-kinase (ROCK) whose function is to inhibit myosin phosphatase from dephosphorylating MLC⁹⁻¹². Consequently, MLC remains phosphorylated and activated which results in more SMC contraction. Indeed, increased ROCK activity has been observed in hypertensive rats and some hypertensive patient populations^{9,13} while ROCK inhibitors have led to reduced blood pressure in hypertensive animal models and patients¹⁴. Therefore, RhoA has been implicated in vasoconstrictor-induced hypertension.

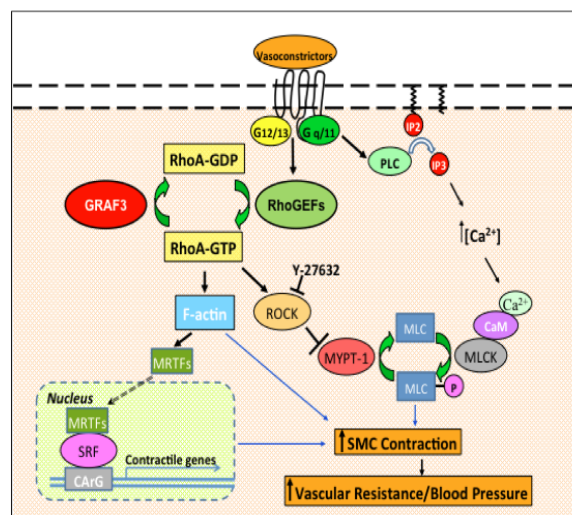


Figure 1: A schematic representation of SMC contractility and blood pressure regulation by the Ca²⁺ - calmodulin pathway and RhoA pathway

Once activated, RhoA has intrinsic GTP-hydrolyzing (GTPase) activity, and it becomes inactivated by hydrolyzing its bound GTP to GDP. The GTPase-activating proteins (GAPs) promote the GTP hydrolysis. Therefore, proper activity of the RhoA-specific GAPs is required to keep RhoA and ROCK activity in control, which in turn can keep SMC contraction and blood pressure in control. Our lab has identified GTPase regulator associated with focal adhesion kinase 3 (GRAF3) to be a RhoA-specific GAP. Immunohistochemical analysis of mouse and human tissues with a GRAF3 antibody showed that *GRAF3* gene is selectively expressed in the SMCs¹⁵. Thus, the lab reasoned that *GRAF3* might play an important role in regulating SMC contractions and BP.

In order to test the association between *GRAF3* gene and blood pressure, the lab had knocked down the *GRAF3* gene via a gene-trap in mice. In a gene-trap mouse, a gene-trap cassette is genetically engineered in the gene of interest, which results in alternative splicing of the mRNA and translation of a dysfunctional, truncated protein. When *GRAF3* is knocked down in this manner, a significant increase in BP is observed in the mice ¹⁵. Specifically, homozygous gene-trap mice showed higher BP than heterozygous gene-trap mice. This indicates the dose dependent phenotype of *GRAF3*. Furthermore, *GRAF3*-deficient SMCs have increased levels of MLC phosphorylation and RhoA activity. The *GRAF3* gene can be returned to its original state by using Cre-Lox recombination. Cre-recombinase removes the gene trap cassette, which has been flanked by Lox P sites. When *GRAF3* gene is reintroduced only in SMC, by crossing *GRAF3* gene trap mice in SMMHC-Cre ER^{T2} mice, BP returns back to normal. Hence, all these results indicate that *GRAF3* helps maintain normal BP by inhibiting RhoA activity in SMC, which in turn reduces the SMC contractility and BP ¹⁵.

After establishing the fact that *GRAF3* gene plays a role in controlling SMC contraction and thus BP, the next step is to identify how the function of *GRAF3* can be compromised. Two previous genome wide association studies have identified a novel BP associated locus containing two single nucleotide polymorphisms (SNPs) in perfect linkage disequilibrium (rs633185 and rs604723) within the first intron of *GRAF3* gene^{16,17}. This means that a statistical non-random association between these two SNPs have been found. Both these SNPs are located in DNase hypersensitivity sites (DHS), which are specific regions of the genome where the chromatin is not in its condensed form, making the DNA more accessible for transcription. Since there is

“open” chromatin, active transcription takes place at these sites. These SNPs are also associated with a significant reduction in BP with each copy of the minor allele. Thus, the lab has hypothesized that these SNPs play a protective role in regulation of BP. However, when the lab cloned both the SNP containing segments into luciferase vectors and tested protein expression, the differing alleles of rs604723 showed differences in expression, whereas the differing alleles of rs633185 showed no differences. Therefore, the SNP rs604723 was chosen for further research. Specifically, my research project focused on determining whether the SNP rs604273 is correlative with HTN and whether it is more frequent in people of a particular race since it is known that risk associated with hypertension varies in different races.

Methods:*Allelic Discrimination Assay*

The Applied Biosystems 7500 Fast Reverse Transcription Polymerase Chain Reaction (RT PCR) machine was used to perform a Taqman Allelic Discrimination Assay for the SNP rs604723 on 510 human gDNA samples, obtained from the Heart Healthy Lenoir Study. Allelic Discrimination Assay is a PCR-based assay that genotypes the two possible variants at the SNP site in the target template sequence by measuring fluorescence signals and using two SNP variant specific probes. For SNP rs604273, the major allele is the nucleotide C at the SNP site, and the minor allele is the nucleotide T at the SNP site. Furthermore, the SNP probe for the nucleotide C is attached to a fluorescent FAM dye, and the SNP probe for the nucleotide T is attached to a fluorescent VIC dye. When a SNP probe matches with its complementary sequence, the fluorophore is cleaved, and the fluorescent signal is measured by the machine. Table 1

shows the association between the fluorescence signal levels and the corresponding genotype of SNP rs604723 in the 510 human gDNA samples.

Table 1: Determination of the sequence's genotype based on fluorescent signal levels

Fluorescence signal detected	Genotype
VIC dye only	Homozygous T/T
FAM dye only	Homozygous C/C
VIC and FAM dye both	Heterozygous C/T

The positive controls for this experiment were pGL3-DHS2T and pGL3-DHS2C, which were previously made by the lab. The positive control, pGL3-DHS2T, was made by isolating DHS2T from human DNA and putting it into the vector pGL3. DHS2T is the DHS where the SNP rs604723 is found with the minor allele T. The second positive control, pGL3-DHS2C, was made by performing site-directed mutagenesis on pGL3-DHS2T. The negative control was water.

The samples were prepared on 96-well plates, where each reaction well was prepared by following the protocol described in Table 2.

Table 2: Sample Preparation for Allelic Discrimination Assay

Reagent	Quantity
Taqman Genotyping MasterMix (20X)	5 μ L
SNP Probe (40X)	0.5 μ L
Wet DNA/control (10 ng/ μ L)	1 μ L
DEPC-H ₂ O	3.5 μ L

Minor Allele Frequency Calculation

After genotyping all the samples obtained from the Heart Healthy Lenoir Study, the data was combined with the genotype data from two other hypertensive studies: UNC Family Medicine Study, a longitudinal study directed by co-PI Dr. Anthony Viera, and CAD, Dr. George Stouffer and Dr. Craig Lee's study. The SNP genotyping assays for rs604723 were previously performed by the lab for these two studies, and I used the genotype data to calculate the Minor Allele Frequency (MAF) of hypertensive vs. non-hypertensive populations and populations of different races.

MAF, which is the frequency of the least common allele in a given population, is calculated as follows:

$$MAF = \frac{\# \text{ of samples with genotype } T/T + 0.5 * \# \text{ of samples with genotype } C/T}{\text{Total \# of samples}}$$

The statistical analysis on the results obtained was done by a post-doc student's t-test, and the p-values < 0.05 were considered statistically significant.

Results:

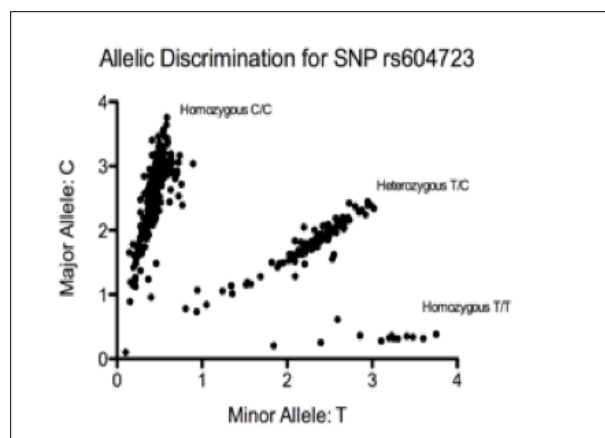


Figure 2: Allelic Discrimination Plot for SNP rs604723 for 510 human cDNA samples obtained from Lenoir County Database

To evaluate the presence of the SNP rs604723, 510 patient gDNA samples, obtained from the Heart Healthy Lenoir Study, were genotyped through an allelic discrimination assay. The allelic discrimination plot for SNP rs604723 of *GRAF3* gene shows that there were 13 patients with the genotype T/T, 112 with the

genotype T/C, and 385 patients with the genotype C/C (Figure 2).

Previously, the lab had performed an allelic discrimination assay for SNP rs604723 on the samples obtained from UNC Family Medicine Study and CAD Study. In UNC Family Medicine Study, the blood pressure data for each patient was collected during two visits, and the patients were not on any antihypertensive therapy before taking part in the study. The patients with systolic blood pressure (SBP) of 140 mm Hg and higher in both visits were considered hypertensive. The MAF for the hypertensive population was 22.08%, which is lower than the MAF for the non-hypertensive population, which was 26.09% (Table 3). However, this dataset does not reach statistical significance.

Furthermore, for the patients of UNC Family Medicine Study, the average SBP and diastolic blood pressure (DBP) was compared between patients with different genotypes. The patients homozygous for minor allele T had the lowest average SBP, 126.29 mm Hg versus 129.58 mm Hg in patients homozygous for the major allele C, but this difference is not statistically significant ($p = 0.199$). A more pronounced and statistically significant effect was observed when comparing DBP, which averaged 76.87 mm Hg in patients homozygous for the minor allele T and 81.79 mm Hg in patients homozygous for the major allele C ($p = 0.028$; Table 4).

Lastly, the genotyping data obtained for the patients of Heart Healthy Lenoir Study was combined with the data from UNC Family Medicine Study and CAD Study in order to see if MAF of the SNP rs604723 varies between different races. The proportion of homozygous subjects in each race group (Caucasian, African American, and Other) is significantly different ($p < 0.001$). The Other race group included patients who identified themselves as non-Caucasian and non-African American, and a good majority

of these patients were of Hispanic descent. Notably, there is a significant decrease in the percentage of African Americans that contain the minor T allele (Table 5).

Table 3: Minor Allele Frequency (MAF) for SNP rs604723 is higher in hypertensive vs. non-hypertensive patients in the UNC Family Medicine Study. gDNA was isolated from patient's blood and then genotyped using a Taqman SNP genotyping assay for rs604723 (n=346). MAF between hypertensive and non-hypertensive patients was calculated. Patients were considered hypertensive if the systolic BP was greater than 140 for 2 clinic visits. T corresponds to the minor allele and C corresponds to the major allele. No statistical significance reached.

	Genotype frequency			Sum	Minor Allele Frequency
	T/T	T/C	C/C		
Hypertensive	12	78	141	231	22.08%
Non-hypertensive	8	44	63	115	26.09%

Table 4: There is correlation between blood pressure and genotype of GRAF3 SNP rs604723 in patients in the UNC Family Medicine Study. gDNA was isolated from patient's blood and then genotyped using a Taqman SNP genotyping assay for rs604723 (n = 348 patients). The average SBP and DBP for each of the three genotypes were calculated. T corresponds to the minor allele and C corresponds to the major allele. The difference in the average SBP between T/T patients and C/C patients is not statistically significant (p=0.199), but the difference in the average DBP between T/T patients and C/C patients is statistically significant (p=0.028).

	Genotype Frequency		
	T/T	T/C	C/C
Average Systolic Blood Pressure (mm Hg)	126.29	129.03	129.58
Average Diastolic Blood Pressure (mm Hg)	76.87	81.14	81.79
Number of Patients	19	123	206

Table 5: Minor Allele Frequency (MAF) is greater in Caucasian patients than African American patients in the Heart Healthy Lenoir Study, UNC Family Medicine Study, and CAD Study. gDNA was isolated from patient's blood and then genotyped using a Taqman SNP genotyping assay for rs604723 (n=505 in the Heart Healthy Lenoir Study, n=350 in the UNC Family Medicine Study, n=369 in the CAD Study). MAF between patients of different races was calculated. Other race group consists of Hispanic patients, and non-Caucasian, non-African American patients. T corresponds to the minor allele and C corresponds to the major allele. The proportion of homozygous subjects in each race group is significantly different ($p < 0.001$).

Race	Genotype Frequency			Sum	Minor Allele Frequency
	T/T	T/C	C/C		
Caucasian	47	302	392	741	26.72%
African American	0	40	390	430	4.65%
Other	6	21	26	53	31.13%

Discussion:

The original goal was to combine the genotyping data obtained for the Heart Healthy Lenoir Study with data from UNC Family Medicine Study and CAD Study, compare the MAF of the SNP rs604723 in hypertensive vs. non-hypertensive population, and conclude whether this SNP is more frequent in non-hypertensive population. However, as nearly all of the patients in the Heart Healthy Lenoir Study were treated with anti-hypertensive medicines prior to our assessment, we are not able to use Heart Healthy Lenoir Study's data to draw conclusions regarding the association between the SNP rs604723 and hypertension.

For the dataset of UNC Family Medicine Study in which all patients were untreated at the start of the study, the MAF was higher in non-hypertensive population by 4.01%, but this dataset does not reach statistical significance (Table 3). This correlation when combined with the lab's studies in mice suggests that the SNP rs604723 may help

the non-hypertensive population maintain their BP by protecting *GRAF3* gene's function. Furthermore, patients homozygous for the minor allele T had the lowest average SBP and DBP among the three genotypic subgroups (Table 4). A statistically significant difference in the DBP between patients homozygous for the T allele and patients homozygous for the C allele ($p=0.028$) provided evidence for the hypothetical protective role of SNP rs604723 in BP regulation. Next, the prevalence of SNP rs604723 in different race populations was analyzed. One significant difference observed in the data collected from all the studies – Heart Healthy Lenoir Study, UNC Family Medicine Study, and CAD Study – was that the SNP rs604723 is more frequent in the Caucasian population than in the African American population (Table 5). If the SNP rs604723 does in fact has a protective role of reducing blood pressure by increasing more expression of *GRAF3*, it can help explain why the African American population is more prone to have salt sensitivity and HTN ¹⁸.

Another interesting aspect is that the MAF was highest in the Other race group (Table 5). This race group included patients who identified themselves as non-Caucasian non-African American, and these patients were primarily of Hispanic descent. Thus, further analysis is needed to determine which specific race has the highest MAF. Also, a bigger sample size, with more detailed race breakdown information for the Other race group, is required to get accurate MAF information. Therefore, from the data analysis performed so far, trends have been found that indicate that SNP rs604723 plays a protective role in BP regulation by increasing *GRAF3* gene expression. However, a bigger sample size and further analysis of more

hypertensive studies that have patients who are un-treated at the time of data collection are needed before making a conclusion.

Given that BP is a highly variable trait, it is possible that there are numerous SNPs along the RhoA pathway that correlate with different levels of hypertensive risk and drug responses. Although screening for a single SNP such as rs604723 indicated very slight reduction in BP, it is likely that screening for multiple SNPs in the RhoA pathway could lead to a more accurate indication of the combination of SNPs along the RhoA pathway that may alter BP. Future experiments include: (1) analyzing the presence of a combination of SNPs occurring in the genes that regulate RhoA pathway in human populations (2) analyzing whether a combination of SNPs affect BP.

Since blood pressure is regulated by multiple organ systems, a universal anti-hypertensive treatment suitable for all patients is almost impossible to synthesize. The goal of our lab is to find out which SNPs in *GRAF3* and other molecules involved in the RhoA pathway alter the regulatory function of the pathway, how regulation is achieved, and which population subgroups are susceptible to having elevated BP due to elevated RhoA activity. Consequently, each population subgroup can be treated in a more personalized fashion with an anti-hypertensive treatment that targets RhoA signaling in that sub-population, instead of using a more general treatment that might not be very effective for every patient.

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References:

- 1) Wall HK, Hannan JA, Wright JS. Patients With Undiagnosed Hypertension: Hiding in Plain Sight. *JAMA*. 2014;312(19):1973-1974.
- 2) Cowley, Jr A. W. The genetic dissection of essential hypertension. *Nat. Rev. Genet.* 7, 829–840 (2006).
- 3) Davis, M. J. et al. Integrins and mechanotransduction of the vascular myogenic response. *Am. J. Physiol. Heart. Circ. Physiol.* 280, H1427–H1433 (2001).
- 4) Davis, M. J. & Hill, M. A. Signaling mechanisms underlying the vascular myogenic response. *Physiol. Rev.* 79, 387–423 (1999).
- 5) Hall, J. E. The kidney, hypertension, and obesity. *Hypertension* 41, 625–633 (2003).
- 6) Lifton, R. P., Gharavi, A. G. & Geller, D. S. Molecular mechanisms of human hypertension. *Cell* 104, 545–556 (2001)
- 7) Etienne-Manneville, S. & Hall, A. Rho GTPases in cell biology. *Nature* 420, 629–635 (2002).
- 8) Budzyn, K., Marley, P. D. & Sobey, C. G. Targeting Rho and Rho-kinase in the treatment of cardiovascular disease. *Trends Pharmacol. Sci.* 27, 97–104 (2006).
- 9) Loirand, G. & Pacaud, P. The role of Rho protein signaling in hypertension. *Nat. Rev. Cardiol.* 7, 637–647 (2010).
- 10) Amano, M. et al. Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *J. Biol. Chem.* 271, 20246–20249 (1996).
- 11) Kimura, K. et al. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho- kinase). *Science* 273, 245–248 (1996).
- 12) Mueller, B. K., Mack, H. & Teusch, N. Rho kinase, a promising drug target for neurological disorders. *Nat. Rev. Drug Discov.* 4, 387–398 (2005).
- 13) Wirth, A. et al. G12-G13-LARG-mediated signaling in vascular smooth muscle is required for salt-induced hypertension. *Nat. Med.* 14, 64–68 (2008).
- 14) Masumoto, A. et al. Possible involvement of Rho-kinase in the pathogenesis of hypertension in humans. *Hypertension* 38, 1307–1310 (2001).
- 15) Bai X, Lenhart KC, Bird KE, Suen AA, Rojas M, Kakoki M, Li F, Smithies, O, Mack CP, Taylor JM. The smooth muscle-selective RhoGAP GRAF3 is a critical regulator

of vascular tone and hypertension. *Nat Commun.* 2013;4:2910.

- 16) Wain, L. V. et al. Genome-wide association study identifies six new loci influencing pulse pressure and mean arterial pressure. *Nat. Genet.* 43, 1005–1011 (2011)
- 17) International Consortium for Blood Pressure Genome Wide Association Studies. Genetic variants in novel pathways influence blood pressure and cardiovascular disease risk. *Nature* 478, 103–109 (2011).
- 18) Peters, R. et al. Salt Sensitivity and Hypertension in African Americans: Implications for Cardiovascular Disease. *Prog Cardiovasc Nurs.* 2000; 15(4).