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ORIGINAL ARTICLE

HDAC9 Polymorphism Alters Blood Gene Expression in Patients with Large Vessel Atherosclerotic Stroke

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Abstract

The histone deacetylase 9 (HDAC9) polymorphism rs2107595 is associated with an increased risk for large vessel atherosclerotic stroke (LVAS). In humans, there remains a need to better understand this HDAC9 polymorphism's contribution to large vessel stroke. In this pilot study, we evaluated whether the HDAC9 polymorphism rs2107595 is associated with differences in leukocyte gene expression in patients with LVAS. HDAC9 SNP rs2107595 was genotyped in 155 patients (43 LVAS and 112 vascular risk factor controls). RNA isolated from blood was processed on whole genome microarrays. Gene expression was compared between HDAC9 risk allele-positive and risk allele-negative LVAS patients and controls. Functional analysis identified canonical pathways and molecular functions associated with rs2107595 in LVAS. In HDAC9 SNP rs2107595 risk allele-positive LVAS patients, there were 155 genes differentially expressed compared to risk allele-negative patients (fold change > |1.2|, $p < 0.05$). The 155 genes separated the risk allele-positive and risk allele-negative LVAS patients on a principal component analysis. Pathways associated with HDAC9 risk allele-positive status involved IL-6 signaling, cholesterol efflux, and platelet aggregation. These preliminary data suggest an association with the HDAC9 rs2107595 risk allele and peripheral immune, lipid, and clotting systems in LVAS. Further study is required to evaluate whether these differences are related to large vessel atherosclerosis and stroke risk.

Keywords SNP · Polymorphism · Gene expression · Large vessel stroke · Ischemic stroke · Atherosclerosis

Introduction

Large vessel atherosclerosis is an important cause of ischemic stroke. Understanding genomic differences that place individuals at risk of large vessel stroke could aid in the development of novel stroke prevention strategies. Histone deacetylase 9 (HDAC9) is a class IIa histone deacetylase located at 7p21 [1, 2]. A GWAS study conducted by the International Stroke Genetics Consortium and the Wellcome Trust Case Control Consortium 2 demonstrated an association between HDAC9 and large vessel atherosclerotic stroke [3]. The A risk allele at HDAC9 single nucleotide polymorphism (SNP) rs2107595, which displays incomplete penetrance, increases odds for large vessel stroke [4–6]. However, the underlying functional relationship of rs2107595 to LVAS remains unclear.

HDAC9 regulates gene expression by removing acetyl groups from proteins that regulate chromatin state [7]. Hypo-acetylation at gene promoters typically leads to transcriptional repression by restricting accessibility of transcriptional machinery to chromatin [8, 9]. Balancing acetylation/deacetylation plays a key role in disease states such as atherosclerosis [1, 8, 9].

HDAC9 is upregulated in human carotid and aortic atherosclerotic plaques [4]. Cells that express HDAC9 include monocytes, macrophages, T lymphocytes, vascular endothelial cells, and smooth muscle cells [1]. Coronary artery disease patients exhibit significantly elevated expression of HDAC9 in plasma [6].

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HDAC9 deletion disrupts lipid homeostasis and genes involved in inflammation [10]. Loss of HDAC9 increases histone-3 acetylation at macrophage ABCA1, ABCG1, and PPAR-γ promoters, resulting in increased HDL-mediated cholesterol efflux and macrophage polarization toward the anti-inflammatory M2 phenotype [10]. HDAC9 deficiency also reduces atherosclerotic lesion size in ApoE$^{-/-}$ mice [1].

In humans, there remains a need to better understand how HDAC9 SNP rs2107595 contributes to LVAS. In this novel pilot study, we evaluated how HDAC9 SNP rs2107595 relates to blood cell gene expression in patients with LVAS and in vascular risk factor controls (VRFC).

Methods

Study Subjects

LVAS patients and VRFC were prospectively recruited from the University of California, Davis, from 2012 to 2014. The institutional review board approved the study protocol, and written informed consent was obtained from each patient. Stroke diagnosis criteria are available in the Supplementary Methods. VRFC had no clinical history of stroke or cardiovascular disease, and no recent infection. Controls were of similar age, sex, and vascular risk factor profile as stroke patients.

SNP Genotyping

Blood collection and RNA/DNA isolation methods are available in Supplementary Methods. The SNP rs2107595 was genotyped using the Single Tube TaqMan SNP Genotyping Assay per manufacturer protocol (Life Technologies Corporation, NY). PCR amplification was performed using 50 ng of gDNA with the Applied Biosystems-7900HT Fast Real-Time PCR System. TaqMan Genotyper Software was used to plot R_n values based on fluorescence signals from each sample and used to determine allele representation. Assays were performed in triplicate and included positive controls of two homozygotes and one heterozygote on each replicate. Positive control DNA was obtained from the Coriell Biorepository (Coriell Institute for Medical Research, Camden, NJ). Genotype distribution of rs2107595 did not significantly deviate from Hardy-Weinberg equilibrium in both LVAS and control groups ($p > 0.05$). Subjects exhibiting one or both of risk alleles were deemed rs2107595 risk allele positive. Subjects exhibiting two non-risk alleles were deemed rs2107595 risk allele negative.

Gene Expression Array Hybridization and Statistical Analysis

RNA samples underwent two-cycle target labeling, and cDNA was hybridized to Affymetrix Human Transcriptome 2.0 Arrays per the manufacturer's GeneChip protocol (Affymetrix, Santa Clara, CA). These arrays cover all known protein-coding genes (Affymetrix GeneChip HTA Data Sheet). To minimize batch effect and technical variation, each batch contained equal numbers of randomly allocated stroke and control patients.

Raw gene expression data was normalized using robust multichip averaging (RMA) and $\log_2$ transformed as previously described [11]. Statistical analysis and principal component analysis were performed using Partek Genomics Suite 6.6. After removing unannotated transcripts, 33,007 probe sets were used for analysis. Gene expression of risk allele-positive LVAS patients was compared to risk allele-negative LVAS patients. Similarly, gene expression of risk allele-positive VRFC patients was compared to risk allele-negative VRFC patients. Univariate analysis was performed to identify potential confounding variables including age, sex, race, and vascular risk factors. Variables with $p < 0.05$ were then included in the multivariate model. Probesets with a $p < 0.05$ and fold change $\geq |1.2|$ were considered significant, as previously described [12–15]. Functional pathways represented greater than expected by chance (Fisher's exact test) based on the differentially expressed genes were identified using Ingenuity Pathway Analysis (IPA; QIAGEN, Redwood City, CA, www.ingenuity.com) and literature review.

Results

Patient Characteristics

A total of 43 LVAS and 112 VRFC patients were included in this study. There were no statistically significant differences in patient demographics including age, sex, vascular risk factors, or smoking status between LVAS and VRFC groups (Table 1). Of 43 LVAS patients, 24 were rs2107595 risk allele positive and 19 risk allele negative. Of the 112 VRFC patients, 31 were rs2107595 risk allele positive and 81 risk allele negative. There were significantly more rs2107595 risk allele-positive LVAS patients (56%) than rs2107595 risk allele-positive VRFC patients (28%) ($p < 0.001$, chi-square). There were no significant differences between rs2107595 risk allele-positive and risk allele-negative LVAS groups (Supplementary Table 1), nor were there significant differences between VRFC risk allele-positive and risk allele-negative groups (Supplementary Table 2).

Table 1 Demographics for patients with large vessel atherosclerotic stroke (LVAS) and vascular risk factor controls (VRFC)

Variables	LVAS ($n = 43$)	VRFC ($n = 112$)	p value
Mean age (years)	65.49 ± 1.59	63.83 ± 1.15	0.43
Sex, male (%)	30 (69.8%)	59 (52.7%)	0.07
Hypertension (%)	35 (81.4%)	76 (67.8%)	0.38
Diabetes (%)	18 (41.2%)	26 (23.2%)	0.07
Hyperlipidemia (%)	28 (65.1%)	62 (55.3%)	0.58
Smoking status (%)	13 (30.2%)	18 (16.1%)	0.10

Differentially Expressed Genes

In LVAS, there were 155 differentially expressed genes (DEG) in rs2107595 risk allele-positive compared to risk allele-negative patients (Supplementary Table 3). Of these, 139 (89.1%) were upregulated and 16 (10.9%) were downregulated in risk allele-positive patients. A principal component analysis plot of these 155 genes demonstrates separation of rs2107595 risk allele-positive and risk allele-negative LVAS by gene expression (Fig. 1). In VRFC, there were 8 DEG in rs2107595 risk allele-positive compared to risk allele-negative patients (Supplementary Table 4). The CHML (choroideremia-like protein) gene was shared by comparisons of risk allele$^{+/-}$ LVAS to risk allele$^{+/-}$ VRFC.

Functional Analysis

Pathway analysis for the 155 genes associated with rs2107595 identified canonical pathways involving IL-6 signaling, ERK/MAPK signaling, LXR/RXR activation, and the role of macrophages, fibroblasts, and endothelial cells in rheumatoid arthritis (Table 2). SNP rs2107595 risk allele-positive LVAS was associated with leukocyte recruitment, chronic inflammation, immune response of macrophages, cholesterol efflux, and platelet aggregation (Table 2). GMFG (glial growth factor-γ; FC 1.24, $p = 0.02$) and LRP1 (low-density lipoprotein receptor-related protein 1; FC − 1.23, $p = 0.01$) are involved in endothelial cell function and angiogenesis [16, 17] and were differentially expressed in LVAS risk allele-positive patients.

Literature review indicates the following genes have previously been associated with atherosclerosis and were differentially expressed in a pro-atherogenic manner between risk allele-positive and risk allele-negative patients: CX3CR1, HCAR2, HMGB1, JUNB, LRP1, PDE3B, PTGS1, S100A8, THBS1, TUBB2A, and VCAN. Of note were HMGB1 (high mobility group box 1; FC 1.20, $p = 0.03$) and THBS1 (thrombospondin 1; FC − 1.25, $p = 0.04$).

Discussion

The HDAC9 risk allele at rs2107595 was associated with differences in blood cell gene expression in patients with LVAS. Risk allele-positive LVAS patients had increased gene expression involved in inflammation, lipid metabolism, and platelet aggregation. Mechanisms by which these changes occur require further study, but may include HDAC9 risk allele regulation of gene expression in leukocytes, or indirect

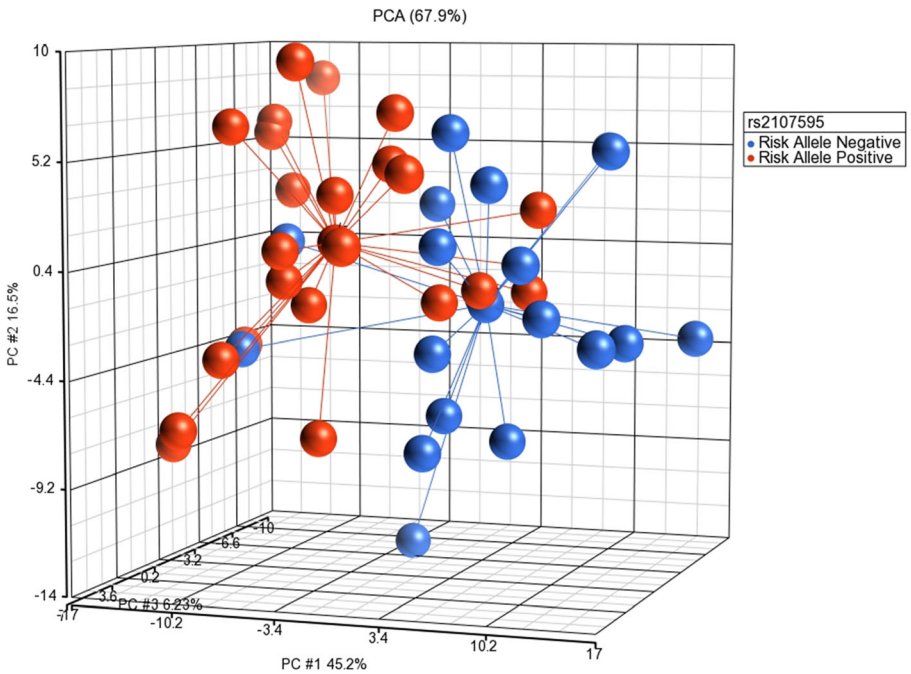

Fig. 1 Principal component analysis showing separation of rs2107595 risk allele-positive and risk allele-negative LVAS patients based on the expression of 155 DEG

Table 2 Functional analysis of the 155 genes differentially expressed in HDAC9 SNP rs2107595 risk allele-positive compared to risk allele-negative LVAS patients

	rs2107595 RAP vs RAN	Genes	*p* value
Canonical pathways	IL-6 signaling	CEBPB, CXCL8, IL1R2, TNFAIP6	9.25E−03
	Role of macrophages, fibroblasts, and endothelial cells in RA	CEBPB, CXCL8, IL1R2, LRP1, LTB, PLCG2	1.51E−02
	ERK/MAPK signaling	DUSP1, PLCG2, PRKAR1A, TLN1	4.00E−02
	LXR/RXR activation	IL1R2, LY96, S100A8	4.35E−02
Molecular functions	Leukocytes recruitment	CLEC4E, CX3CR1, CXCL8, HCAR2, HMGB1, LRP1, LY96, S100A8, THBS1, TPT1	1.77E−04
	Chronic inflammation	CCDC59, CEBPB, CLEC4E, CX3CR1, CXCL8, CYP4F3, DUSP1, HMGB1, IGF1R, IL1R2, LTB, PANK2, PDE3B, PTGS1, RPS13, S100A8, THBS1, TNFAIP6, TP53INP1, USP15	2.38E−04
	Macrophage immune response	ABCA7, CEBPB, HMGB1, LY96, mir-142, THBS1	4.44E−04
	Cholesterol efflux	ABCA7, HCAR2, LRP1, S100A8	5.98E−03
	Platelet aggregation	DAB2, PLCG2, RAB27B, THBS1, TLN1	1.26E−02

regulation through leukocyte interactions with endothelial cells, or smooth muscle cells affected by the HDAC9 risk allele.

In this study, the risk allele does not seem to alter HDAC9 expression levels. However, a previous study showed a significant increase of HDAC9 expression in both heterozygous and homozygous patients with the risk allele [1]. Explanations for why the two studies differ include the following: the 2015 study was performed on peripheral blood mononuclear cells (monocytes, lymphocytes), whereas the current study was performed on whole blood; and the current study utilized controls matched for vascular risk factors (hypertension, hyperlipidemia, diabetes, and statin therapy) compared to the LVAS group, as opposed to healthy patients. The different findings do raise the possibility that HDAC9 expression is regulated differently in different leukocyte subsets. Thus, the risk allele, whether through changes in HDAC9 expression or not, may affect deacetylase activity differently than the non-risk allele, leading to changes in gene expression. Indeed, both HDAC5 and HDAC7 alter gene expression through deacetylase actions on gene promoters [18, 19]. The risk allele at rs2107595 may affect HDAC9's deacetylase activity and alter gene expression in leukocytes. Alternatively, HDAC9 could affect endothelial cells [20, 21] and/or vascular smooth muscle cells, which could alter leukocyte interactions with these cells and produce the differential blood cell gene expression found in this study.

The risk allele at rs210759 is not causative for LVAS. Many people that exhibit the risk allele do not have stroke. It is possible that other factors interact with the HDAC9 polymorphism to contribute to stroke risk. The HDAC9 polymorphism displays incomplete penetrance which may be influenced by polymorphic alleles at other loci, epigenetic regulation, or environmental modifiers. This has been described in other diseases associated with a genetic risk such as BRCA1 and BRCA2 in breast cancer [22, 23]. This may explain why in our study risk allele-positive LVAS patients had differential gene expression involved in lipid metabolism and inflammation while controls did not. Risk allele-positive VRFC may have compensatory mechanisms or a different genetic and environmental background. Indeed, environmental factors such as diabetes and body mass index are known to influence risk of coronary artery disease in patients with the rs2107595 risk allele [6], and microbial environment can influence HDAC9 deacetylase activity [24]. Specifically, statins have been reported to affect histone acetylation [25], though there were no differences of statin usage in the risk allele-positive and risk allele-negative LVAS patients (Supplementary Table 1), and no differences in statin usage in the risk allele-positive and risk allele-negative VRFC patients (Supplementary Table 2). In addition, it is known that valproate is an HDAC inhibitor [26, 27]. In our study, no patients were treated with valproate.

Inflammation

HDAC9 SNP rs2107595 may increase risk of LVAS via its effect on leukocyte T_{reg} function. HDAC9 deacetylates and downregulates FOXP3 (forkhead box P3). This impairs T regulatory cell immunosuppression, resulting in increased inflammation which has been implicated in atherosclerosis [28]. Downregulation of FOXP3 is associated with increased HMGB1 and IL-6 (interleukin 6) expression, which we

observed in rs2107595 risk allele-positive LVAS patients (Table 1). This immune activation may contribute to atherosclerosis and stroke.

FOXP3 also decreases thrombospondin (THBS1) which was downregulated in rs2107595 risk allele-positive patients. THBS1 plays a protective role in atherosclerosis, impeding plaque maturation and rupture and suppressing the inflammatory response [29, 30]. THBS1 is downregulated in patients with atherosclerosis, promoting plaque inflammation and maturation [29, 31]. Thus, the rs2107595 risk allele may contribute to LVAS via its effect on T_{reg} function and inflammation.

Lipid Metabolism

ABCA7 and HCAR2 were both upregulated in LVAS HDAC9 risk allele-positive patients. ABCA7 is implicated in macrophage lipid efflux and clearance of apoptotic cells. It also plays a role in T cell proliferation [32] and is an Alzheimer's disease risk gene [33]. HCAR2 (hydroxylcarboxylic receptor 2) is a niacin receptor gene that mediates niacin decrease of lipoprotein(a) [34]. Nicotinic acid action on HCAR2 causes atherosclerosis to regress in humans and mice.

LRP1 is a signaling receptor that was downregulated in LVAS risk allele-positive patients. When expressed on adipocytes, it protects against diet-induced atherosclerosis and modulates atherosclerosis by regulating inflammation [35]. LRP1 regulates cholesterol accumulation in macrophages. This is important as excessive accumulation leads to foam cell formation which are precursors of atherosclerosis [36]. LRP1 also regulates plasma levels of blood coagulation factor VIII and other factors affecting coagulation [37] which could predispose to stroke.

Calgranulin S100A8 is a pro-inflammatory mediator of atherosclerosis and was upregulated in LVAS risk allele-positive patients. It is a major component of neutrophils, is upregulated in macrophages and foam cells, and influences leukocyte recruitment and inflammation by binding TLR4 and/or the receptor for advanced glycation end products (RAGE) [38]. Increased plasma levels of S100A8 predict cardiovascular events in humans, and deletion of this gene partly protects $Apoe^{-/-}$ mice from atherosclerosis [39]. Stroke-associated carotid plaques have high levels of S100A8.

Platelet Aggregation

Disabled-2 (DAB2) is an adapter protein that is upregulated during megakaryocytic differentiation of hematopoietic cells. It is abundantly expressed in platelets where it is a key regulator of platelet signaling [40]. DAB2 is released from platelet granules and controls the extent of clotting reaction, platelet-fibrinogen interactions, and outside-in signaling [40]. DAB2 is required for platelet aggregation, fibrinogen uptake, and integrin αIIbβ3 activation stimulated by low concentrations of thrombin. As a result, bleeding time is prolonged and thrombus formation is impaired in DAB2-deficient mice [40]. Moreover, the combination of Arh and DAB2 is responsible for the majority of adaptor function in LDLR endocytosis and LDLR-mediated cholesterol homeostasis [41]. Thus, DAB2 could play a role in clotting and atherosclerosis associated with large vessel stroke.

PLCG2 (PLCgamma2) is tightly regulated to ensure efficient but limited platelet activation at sites of vascular injury [42]. Gain of function mutations in PLCG2 causes platelet hyperactivity and a pro-thrombotic state [42]. Platelet activation and thrombin generation are crucial steps in primary and secondary hemostasis. Thrombospondin-1 (TSP1) levels in human blood correlated with monocyte-platelet aggregates and thrombin generation indicating a pivotal role in regulating thrombosis [43]. Indeed, platelet-derived TSP1 modulates arterial thrombosis in vivo. Young patients with high cardiovascular risk and atherosclerosis have high levels of thrombospondin-1 platelet microparticles. Thrombospondin-1 levels correlate with carotid atherosclerotic plaque size and irregularity [44], and thrombospondin-1 levels immediately after stroke predict 6-month mortality and morbidity. Finally, TLN1 (talin 1) plays a role in αIIbβ3 inactivation in procoagulant platelets [45] and thus also plays a role in coagulation that could be crucial for outcomes in ischemic large vessel stroke.

Limitations

This is a novel pilot study of human stroke showing a relationship between an HDAC9 polymorphism and whole genome gene expression differences. Sample size was small; thus, evaluation in larger cohorts is warranted to confirm findings. There was no significant difference in phenotype between the few LVAS patients who were homozygous in comparison to LVAS patients who were heterozygous for the risk allele. Similarly, there was no significant difference in phenotype between the few VRFC who were homozygous in comparison to VRFC who were heterozygous for the risk allele. Due to the limited number of homozygous subjects ($n = 4$), we were unable to analyze gene expression differences between homozygous and heterozygous subjects in the LVAS and VRFC groups. Future studies are needed to assess the possibility of an allelic dosage effect on gene expression. Previous studies have shown that the risk allele at rs2107595 associates only with LVAS and not with any other stroke subtypes. However, future studies could utilize stroke patients without LVAS as controls in order to specifically assess the risk allele's

pro-atherogenic effects in comparison to the risk allele's pro-stroke effects.

Additional studies are needed to assess the relationship between leukocytes, endothelial cells, and smooth muscle cells with the risk allele. We were unable to determine whether observed changes in leukocyte gene expression relate to HDAC9 risk allele regulation in leukocytes, or an indirect effect of leukocyte interaction with endothelium or vascular smooth muscle cells affected by HDAC9 risk allele. We unfortunately do not have data sufficiently detailed to evaluate whether infarction of the insula or other particular brain location may affect gene expression. This will be important to consider in future studies given potential sympathetic effects on the spleen and peripheral cells.

While HDAC9 risk allele positivity is associated with LVAS, additional genetic or environmental factors may be required to result in stroke. While not every gene has the same pattern of expression across LVAS patients, certain pathways are consistently over-represented in these patients. Thus, RAP patients consistently over-express genes involved in IL-6 signaling, leukocyte recruitment, chronic inflammation, cholesterol efflux, and platelet aggregation. However, whether the identified genes and pathways directly increase stroke risk remains unclear. Experimental rodent stroke data support the importance of HDACs in stroke [46–48] and the need for further studies to evaluate the role of HDAC9 in stroke.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All patients or their surrogates provided informed consent for this study.

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