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ORIGINAL ARTICLE

Rs10230207 genotype confers changes in *HDAC9* and *TWIST1*, but not *FERD3L* in lymphoblasts from patients with intracranial aneurysm

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Abstract

Intracranial aneurysms (IA) are weakened outpouchings of the arterial wall in the cerebrovasculature. Rupture of an IA often leads to devastating consequences. The early identification of IA patients is crucial for management of their condition. A genetic variant at rs10230207, located nearby the *HDAC9*, *TWIST1*, and *FERD3L* genes, is associated with IA. HDAC9 is a class IIa histone deacetylase that mediates vascular smooth muscle cell dysfunction. TWIST1 is a mechanosensitive transcription factor and its expression is reduced in unstable carotid atherosclerotic plaques. In this study, the expression of the *HDAC9*, *TWIST1*, and *FERD3L* genes was characterized and associated with the presence of the rs10230207 genetic variant. Allelic discrimination and gene expression analysis were performed using lymphoblasts from 85 population controls and 109 IA patients. Subjects that were heterozygous (GT) within rs10230207 were 4.32 times more likely to have an IA than those that were homozygous for the reference allele (GG; 95%CI 1.23 to 14.16). Subjects that were homozygous (TT) were 8.27 times more likely to have an IA than those that were GG (95%CI 2.45 to 27.85). While the presence of the risk allele was not associated with changes in *FERD3L* gene expression, the risk allele was associated with increased *HDAC9* and decrease in *TWIST1* mRNA expression. The significant inverse correlation between *HDAC9* and *TWIST1* gene expression suggests that changes in the expression of both of genes may contribute to the formation of IAs.

Keywords Intracranial aneurysm · *HDAC9* · *TWIST1* · *FERD3L* · rs10230207

Introduction

Intracranial aneurysms (IA) are weakened outpouchings of the cerebral vascular wall and occur in 3–5% the general population [1, 2]. Subarachnoid hemorrhage (SAH) is a serious complication of IA and is associated with devastating outcomes. Over 40% of patients with SAH due to IA rupture die within 30 days [3] and those patients that do survive often experience significant disabilities and loss of independence [4, 5]. Therefore, early identification of patients with IA is crucial for improved patient management and the prevention of the devastating consequences of rupture.

While there are several common lifestyle-related risk factors for IA formation, there is evidence that supports a genetic contribution. Genetic contributions to IA formation are supported by the presence of familial disease [6–8]. While the general population has a 3–5% incidence rate of IA, first and second-degree relatives of an IA patient have an increased incidence rate of 8.8–13.9% [9, 10].

Genome-wide association studies (GWAS) in cases that were enriched for a family history of IA have identified several loci on chromosomes 1p34.3-p36.13, 7q11, 19q13.3, and Xp22 that may contain predisposing genes [11]. Recently, a GWAS study of IA patients (2617 cases and 2548 controls) of European ancestry identified a genetic variant on chromosome 7 that is associated with the increased risk of developing IA

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(rs10230207; $P = 4.14 \times 10^{-8}$) [12]. Rs10230207 (chr7.hg38.p12.19571684G > T) is not located within a protein coding region but is located nearby the *HDAC9*, *TWIST1*, and *FERD3L* genes (Fig. 1 [12]).

The proximity of rs10230207 to the *HDAC9*, *TWIST1*, and *FERD3L* genes is interesting because of their relationship with vascular integrity. Another genetic variant, rs2107595, is located between the *HDAC9*, *TWIST1*, and *FERD3L* genes. The rs2107595 variant selectively increases the expression of *HDAC9*[13], a class IIa histone deacetylase and is associated with ischemic stroke and large vessel ischemic disease [14–18]. Furthermore, increased *HDAC9* expression is associated with thoracic aortic aneurysm [19–21]. It is also notable that *TWIST1* is a mechanosensitive transcription factor that is expressed in regions of adult arteries where blood flow is disturbed and promotes atherosclerosis by inducing endothelial cell proliferation and inflammation [22, 23]. Importantly, decreased *TWIST1* expression is associated with decreased atherosclerotic plaque stability [24]. Based on these findings, we hypothesized that the presence of the IA risk allele within the rs10230207 locus would act as a proximal expression quantitative trait loci (eQTL) and effect the expression of the *HDAC9*, *TWIST1*, and *FERD3L* genes.

In the present study, the genetic variation at rs10230207 was identified in immortalized lymphoblasts obtained from IA cases and population controls from within the US population. The association between the presence of the risk allele and IA was replicated in our sample population. Allelic discrimination indicated that in the sample population, the risk allele is the major allele. Furthermore, the presence of the risk allele was associated with increased expression of *HDAC9* and decreased expression of *TWIST1* mRNA.

Materials and methods

Lymphoblast cell culture This study used immortalized lymphoblast samples from patients with unruptured IA and age matched population controls banked in the NINDS Repository as well as their clinical data. NINDS Repository sample numbers listed in the supplementary table correspond to the samples used and are located in the Coriell Biorepository catalog (https://www.coriell.org). Cells were passaged two times in RPMI-1640 supplemented with 10% fetal bovine serum so that the final concentration of cells was 1×10^6 cells/mL. While we were sent 111 cases and 94 population controls, only 109 of the cases and 85 population controls had viable cultures and were used for further genotyping and gene expression assays.

Allelic discrimination Allelic discrimination assays were used to determine the presence of the risk allele. Genomic DNA was extracted from lymphoblasts using DNAzol reagent (Invitrogen) and used as a template for custom TaqMan SNP genotyping assays directed toward rs10230207, rs11767221, and rs2107595 (Thermo). Subject genotype for all three SNPs is listed in the supplementary table.

Real-time PCR Total mRNA was extracted from lymphoblasts using Trizol reagent (Invitrogen) and used as a template for reverse transcriptase using a SuperScript VILO cDNA synthesis kit. Human TaqMan primer and probe sets were used to quantitate the expression of housekeeping gene, *TBP*, and the genes of interest: *HDAC9*, *TWIST1*, and *FERD3L* mRNA from each of the samples. The ΔΔCT method [25] was used to calculate the relative fold change from the reference samples and the fold change was rank normalized to determine if gene expression was associated with allele status.

Protein extraction and immunoblot analysis Cells were washed two times with PBS and nuclear extracts were prepared using the EpiQuik Nuclear Extraction kit (Epigentek). Protein was measured using a bicinchoninic acid assay (Sigma). For detection of nuclear proteins, 20 μg of nuclear extract was mixed with 1X Laemmli loading buffer containing 10% 2-mercaptoethanol and analyzed by SDS-PAGE/Western blot. Separated proteins were transferred onto a PVDF membrane using semi-dry transfer in Towbin transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3, and 20% methanol). Primary antibodies were purchased from Abcam and used at the following concentrations: HDAC9 (ab109446; 1:20000), TWIST1 (ab49254; 1:1000), and FERD3L (ab126381; 1:1000). Secondary antibody was purchased from Li-Cor Biosciences and used at a concentration of 1:10000. The amount of protein was expressed relative to the nuclear envelope protein Lamin B1 (ab65986; 1:5000).

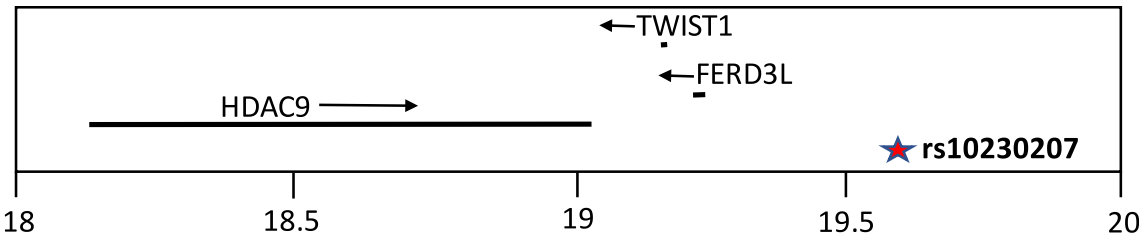

Fig. 1 Proximity of rs10230207 to *HDAC9*, *TWIST1*, and *FERD3L* genes

Statistics Numeric data are expressed as means ± SEM. Statistical differences in mRNA expression were analyzed using non-parametric Krusskal-Wallis tests and then evaluated using a post hoc Dunn's test to determine differences between groups. Multivariable logistic regression models were used to estimate crude and adjusted odds ratios (aORs) to determine the association between genotype and IA. Models were adjusted for age, race (white, other), sex, smoking history (never, former, current), and hypertension history (yes, no) which were identified a-priori as important covariates. Statistics were performed using Prism GraphPad software and STATA 15.1. *P* values that were less than 0.05 were considered statistically significant.

Results

Immortalized lymphoblasts from IA patients have differences in HDAC9, TWIST1, and FERD3L gene expression

A risk allele associated with IA, located in an intergenic region on chromosome 7 near the *HDAC9*, *TWIST1*, and *FERD3L* genes, was recently detected in a GWAS (Fig. 1**)** [12]. Although this region is located outside of the coding regions of these genes, SNPs associated with rs10230207 may cause changes in gene expression that may also be associated with IA susceptibility. To determine if the expression of these genes was altered in patients with IA, immortalized lymphoblasts were obtained from patients with unruptured IA ($n = 111$) and population controls ($n = 94$). Table 1 lists the clinical characteristics of the samples included in this study.

The mRNA expression of the *HDAC9*, *TWIST1*, and *FERD3L* in the IA cases was compared with the population controls. *HDAC9* gene expression was upregulated in the IA subject lymphoblasts compared with the population controls (Fig. 2a; 1 ± 0.104 vs 1.304 ± 0.1477; $P = 0.0313$). Conversely, *TWIST1* mRNA expression was decreased in the IA subject lymphoblasts compared with the population controls (Fig. 2b; 1 ± 0.184 vs 0.648 ± 0.078; $P = 0.0311$). There was no significant change in *FERD3L* mRNA expression in the IA subject lymphoblasts compared with the population controls (Fig. 2c; 1 ± 0.185 vs 0.800 ± 0.127; $P = 0.1085$).

To determine if the changes in the expression of each gene could be associated with each other, the correlation between the ΔΔCT values of each gene was analyzed (Fig. 3). *TWIST1* and *FERD3L* expression had very weak but significant correlation with HDAC9 expression ($R = 0.168$ and 0.254, respectively; $P = 0.03$ and 0.001, respectively). Although the expression of *FERD3L* was very low and there appeared to be no significant difference in the expression between cases and controls, *FERD3L* expression was strongly correlated with *TWIST1* expression (Fig. 3c; $R = 0.6032$; $P < 0.0001$).

The risk allele within the rs10230207, but not the rs2107595 locus is associated with IA incidence in the NINDS sample population

Previous GWAS studies indicated that rs10230207 is associated with IA in Dutch and Finnish populations [12]. To determine if rs10230207 is also associated with IA in the NINDS cohort, immortalized lymphoblasts from IA cases and population controls were genotyped for the SNP status of rs10230207 (Fig.4). To confirm rs10230207 status, the population was also genotyped for the SNP status of rs11767221 which is in high linkage disequilibrium with rs10230207 (Supplemental). Based on the SNP genotyping data, subjects were considered positive for harboring the risk allele if they were heterozygous or homozygous for the alternative allele (GT or TT) in both genotyping assays. Table 2 lists the genetic relative risk for subjects that are heterozygous or homozygous for the risk allele. Crude logistic models indicate that subjects heterozygous for the risk allele (TG) had a significant increase of 4.32 times the odds of having IA compared with subjects with the reference genotype (GG; OR, 4.32, 95% CI, 1.23 to 14.16). Homozygous (TT) subjects were also at a significant risk of IA compared with the reference genotype (GG; OR, 8.27, 95% CI, 2.45 to 27.85). The net effect of adjustments

Table 1 Subject characteristics

Characteristic	IA cases	Population controls
Total number	111	94
Sex (females/males)	53/58	46/48
Age (years)	50.6 (11.9)	50.5 (17.8)
Race (Caucasian/African American/Other)	97/5/9	57/34/3
With hypertension (%)	61.6%	17.0%
With diabetes (%)	8.0%	4.3%
With family history of cerebrovascular disease	81.3%	7.4%
Smokers (%)	63.4%	28.7%

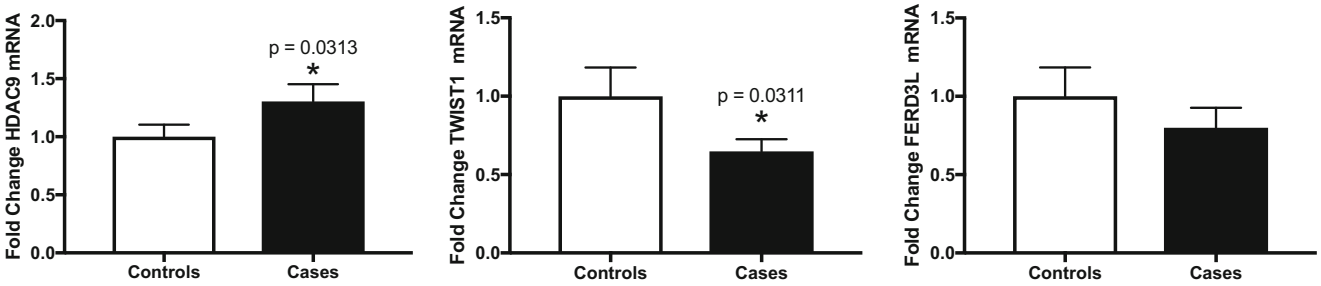

Fig. 2 Expression of *HDAC9*, *TWIST1*, and *FERD3L* mRNA in lymphoblasts obtained from IA cases compared with population controls. a The expression of *HDAC9* was increased in cases compared with controls (Student's *t* test; $*P = 0.0313$). **b** The expression of *TWIST1* was decreased in cases compared with controls (Student's *t* test; $*P = 0.0311$). **c** The expression of *FERD3L* was not significantly decreased compared with population controls. Bars represent the mean ΔΔCT values, error bars are + 1 SEM, controls $n = 76$, cases $n = 91$

(age, race, sex, smoking, and hypertension history) was marginal as heterozygous subjects (aOR, 5.32 95%CI, 2.14 to 13.21) and homozygous subjects (aOR, 8.37, 95%CI, 3.37 to 20.74) remained at a statistically significant increased risk for IA.

Another genetic variant, rs2107595, is reported to selectively increase the expression of *HDAC9*[13] and is associated with ischemic stroke and large vessel ischemic disease. However, this variant is not in linkage disequilibrium with rs10230207. To determine if rs2107595 is also associated with IA, lymphoblasts were genotyped for the SNP status of rs2107595. Table 2 lists the genetic relative risk for subjects that are heterozygous or homozygous for the alternate allele at rs2107595. Crude logistic models indicate that subjects that are heterozygous (AG) or homozygous (GG) for the alternate allele were not at increased risk of IA. Thus, consistent with the lack of linkage disequilibrium with the IA SNP, the large vessel ischemic stroke SNP did not contribute to the risk of IA.

Rs10230207, but not rs2107595 changes HDAC9, TWIST1, and FERD3L gene expression

Rs10230207 is located nearby the *HDAC9*, *TWIST1*, and *FERD3L* genes and may act as an eQTL, affecting the expression of these genes. *HDAC9* gene expression was analyzed in lymphoblasts from IA cases and population controls and stratified according to the genotype. The risk allele was associated with increases in *HDAC9* expression (Table 3; Fig. 5a). Although *FERD3L* gene expression was very low, a similar trend was observed for *FERD3L*; however, the value did not reach statistical significance (Table 3; Fig. 5c). The mRNA expression of *TWIST1* was negatively affected by the presence of the risk allele (Table 3; Fig. 5 b). HDAC9, TWIST1, and FERD3L gene expression were also analyzed as a function of rs2107595 allele status. In our test population, there was no significant difference in the expression of any of these genes (Table 3).

Discussion

A GWAS identified a novel region on chromosome 7 that is associated with the increased risk of developing IA (rs10230207; $P = 4.14 \times 10^{-8}$) [12]. These SNPs are located in a noncoding region nearby the *HDAC9*, *TWIST1*, and *FERD3L* genes. We have confirmed the association between this SNP and IA, in a non-enriched human sample. We have also shown for the first time that the variant at rs10230207 is associated with an increase in *HDAC9* and a decrease in *TWIST1* expression in lymphoblasts from patients with IA.

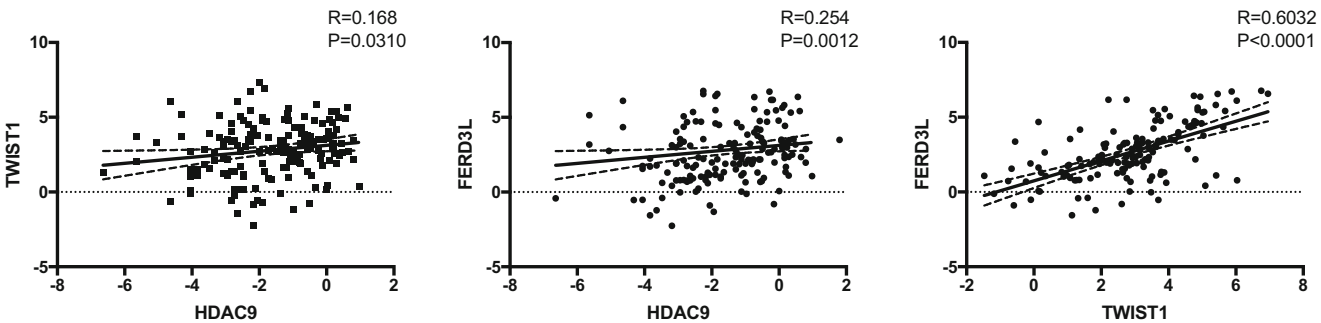

Fig. 3 Correlation of gene expression in all samples analyzed. a Correlation between *HDAC9* and *TWIST1* gene expression. Pearson correlation coefficient (*R*) was 0.168 ($P = 0.031$). **b** Correlation between *HDAC9* and *FERD3L* gene expression ($R = 0.254$, $P = 0.0012$). **c** Correlation between *TWIST1* and *FERD3L* ($R = 0.6032$, $P < 0.0001$)

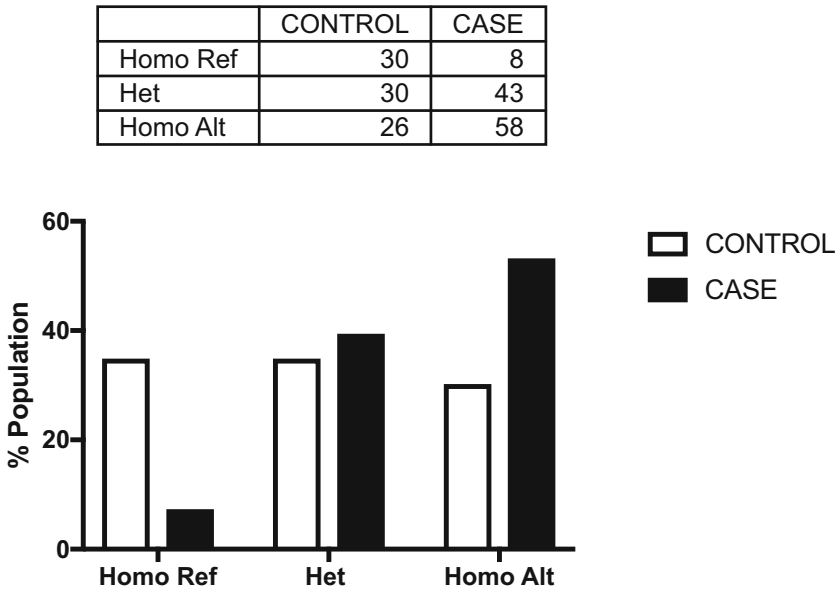

Fig. 4 rs10230207 SNP genotypes for cases and controls. Case and control lymphoblasts were genotyped for the allele status of the rs10230207 variant and categorized as homozygous for the reference allele (Homo Ref; G/G), heterozygous (Het; T/G), or homozygous for the risk allele (Homo Alt; T/T)

SNPs located within the *HDAC9* gene have been associated with ischemic stroke caused by atherosclerosis and large vessel disease [14–18]. HDAC9 is a class IIa histone deacetylase that mediates transcriptional dysregulation in vascular smooth muscle cells and its upregulation results in the formation of the HDAC9-MALAT1-BRG1 complex, transcriptional dysregulation, and loss of contractile phenotype in vascular smooth muscle cells [19]. Disruption of the HDAC9-MALAT1-BRG1 complex restores contractile protein expression and inhibits experimental aortic aneurysm growth [19]. TWIST1 is associated with vascular remodeling and has also been indirectly linked to aneurysm pathogenesis [22]. TWIST1 is a mechanosensitive transcription factor that is expressed in regions of adult arteries where blood flow is disturbed [22, 23], the same locations that IAs tend to form [26]. AKT ser42 phosphorylation of TWIST1 is essential for the survival of vascular endothelial cells [23, 27, 28] and this event has been implicated in transient endothelial phenotypic switching via TGF-β-SMAD signaling [22]. Based on the association of

Table 2 Genotype distribution and allele frequencies of the rs10230207 variant in IA patients and population controls

	Cases	Population controls	OR (95% CI)
rs10230207	(n=109)	(n=85)	
GG (%)	8 (7)	30 (35)	1.0
TG (%)	43 (39)	30 (35)	4.32 (1.23 to 14.16)
TT (%)	58 (53)	25 (29)	8.27 (2.45 to 27.85)
T (%)	159 (73)	90 (53)	
G (%)	59 (27)	80 (47)	
*rs2107595	(n=109)	(n=85)	
AA (%)	2 (2)	3 (4)	
AG (%)	26 (24)	21 (25)	1.0
GG (%)	50 (46)	57 (67)	0.38 (0.03 to 2.64)
A (%)	42 (19)	28 (17)	1.20 (0.57 to 2.52)
G (%)	176 (81)	142 (83)	

*No rs2107595 genotype was detected for 23 cases and 3 population controls

Table 3 Association of the rs10230207 and rs2107595 variants and mRNA expression of *HDAC9*, *TWIST1*, and *FERD3L*

	Mean rank difference	*P*-value
rs10230207		
HDAC9		
GG vs. TG	-0.4372	0.0316
GG vs. TT	-0.3151	0.1128
TWIST1		
GG vs. TG	0.1282	0.5493
GG vs. TT	0.4482	0.0351
FERD3L		
GG vs. TG	-0.2117	0.3442
GG vs. TT	-0.4538	0.4538
rs2107595		
HDAC9		
AA vs. AG	-0.2404	0.7458
AA vs. GG	0.2494	0.7458
TWIST1		
AA vs. AG	-0.5782	0.4995
AA vs. GG	-0.2624	0.6234
FERD3L		
AA vs. AG	-0.6156	0.3144
AA vs. GG	-0.1169	0.7904

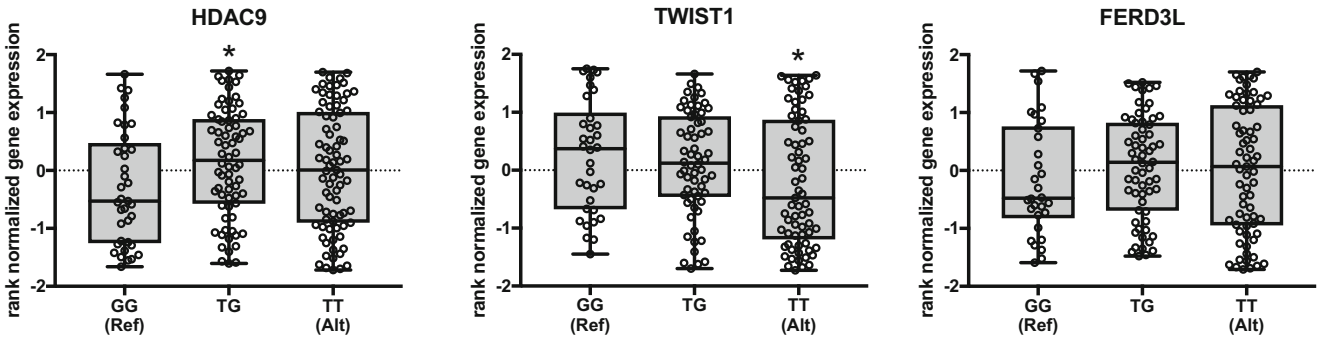

Fig. 5 rs10230207 SNP genotype and associated changes in *TWIST1* and *FERD3L*, *HDAC9*, expression. a Case and control lymphoblasts were genotyped and categorized as homozygous for the reference allele (GG), heterozygous (TG), or homozygous for the risk allele (TT) and mRNA expression of **a** *HDAC9*, **b** *TWIST1*, and **c** *FERD3L* was measured by qPCR. Gene expression was ranked and normalized and then compared using a Kruskal-Wallis test. The asterisk symbol indicates a significant difference from the homozygous reference allele (*HDAC9*, $P = 0.0316$, *TWIST1*, $P = 0.0351$)

the *HDAC9* and *TWIST1* genes with vascular integrity, we asked if the *HDAC9*, *TWIST1*, and *FERD3L* genes were differentially expressed in IA patients compared with population controls.

When lymphoblasts from patients with unruptured IA were compared with lymphoblasts from population controls, we found that *HDAC9* expression was upregulated and *TWIST1* expression was downregulated compared with the population controls. Although *FERD3L* expression was very low, gene expression of *FERD3L* was highly correlated with *TWIST1* expression which suggests these two genes may be co-repressed under disease or risk allele conditions. Optimally, IA associated gene expression should be measured in IA relevant cell types such as cerebrovascular smooth muscle and endothelial cells. Unfortunately, cerebrovascular tissue is not readily available for analysis. Therefore, gene expression analysis was limited to banked patient immortalized lymphoblasts. Because of this, we cannot exclude the possibility of differential regulation of these genes in IA relevant cell types. However, the benefit of measuring gene expression in patient and control white blood cells is the identification of a detectable marker for those at risk for IA from an easily obtainable sample of whole blood.

The presence of the risk variant at the rs10230207 locus may act as a proximal eQTL and affect the expression of the *HDAC9*, *TWIST1*, and *FERD3L* genes. By stratifying gene expression by allele status, we were able to determine if the *HDAC9*, *TWIST1*, and *FERD3L* genes were differentially expressed under the condition of the risk allele (T) compared with the reference allele (G) at rs10230207. Analysis of gene expression indicated that the presence of the risk allele resulted in upregulation of *HDAC9* and downregulation of *TWIST1* gene expression. Although HDAC9 mRNA expression as a function of the TT rs10230207 genotype trended toward an increase in expression, the change was not statistically significant ($P = 0.1128$). The lack of significance is likely a consequence of the low number of samples available for this study. Currently, it is unknown how the rs10230207 genetic variant changes the expression of the *HDAC9* and *TWIST1* genes. Possible mechanisms might include altering transcription factor binding [29], or sequence variation effects on DNA methylation [30].

While the risk allele at rs10230207 alters the expression of *HDAC9* and *TWIST1*, there were no statistically significant changes in *HDAC9*, *TWIST1*, or *FERD3L* expression as a function of rs2107595 allele status. A previous study by Azghandi et al.[13] detected statistically significant changes in *HDAC9* expression as a function of rs2107595 status using peripheral blood mononuclear cells from 77 healthy patients. However, in both their study and ours, there was a very low number of samples that were homozygous for the rs2107595 reference allele, which likely under-powered the analysis (Azghandi study AA $n = 4$ out of 77 total samples, this study AA $n = 5$ out of 194 samples).

How might dysregulated *HDAC9* and *TWIST1* expression put the carrier of the risk allele at increased risk for IA formation? The correlation among the expression of *HDAC9*, *TWIST1*, and *FERD3L* and their associated changes that occur in the presence of the risk allele suggests that these proteins, under healthy conditions may work in a concerted fashion to protect vascular integrity. Further studies are underway to determine the effects of HDAC9 upregulation and/or TWIST1 downregulation on vascular cell phenotypic switching.

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