PPM1A Methylation Is Associated With Vascular Recurrence in Aspirin-Treated Patients

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Background and Purpose—Despite great efforts by pharmacogenetic studies, the causes of aspirin failure to prevent the recurrence of ischemic events remain unclear. Our aim was to study whether epigenetics could be associated with the risk of vascular recurrence in aspirin-treated stroke patients.

Methods—We performed an epigenetic joint analysis study in 327 patients treated with aspirin. In the discovery stage, we performed a nested case–control study in 38 matched ischemic stroke patients in whom 450 000 methylation sites were analyzed. Nineteen patients presented vascular recurrence after stroke, and 19 matched patients did not present vascular recurrence during the first year of follow-up. In a second stage, 289 new patients were analyzed by EpiTYPER.

Results—The following 3 differentially methylated candidate CpG sites, were identified in the discovery stage and analyzed in the second stage: cg26039762 (P=9.69×10^{-06}, *RAF1*), cg04985020 (P=3.47×10^{-03}, *PPM1A*), and cg08419850 (P=3.47×10^{-03}, *KCNQ1*). Joint analysis identified an epigenome-wide association for cg04985020 (*PPM1A*; P=1.78×10^{-07}), with vascular recurrence in patients treated with aspirin.

Conclusions—The pattern of differential methylation in *PPM1A* is associated with vascular recurrence in aspirin-treated stroke patients. **(*Stroke*. 2016;47:1926-1929. DOI: 10.1161/STROKEAHA.116.013340.)**

Key Words: aspirin ■ methylation ■ phenotype ■ platelet aggregation ■ stroke

Ischemic stroke patients are at high risk of having new cardiovascular events. The cumulative risk of vascular recurrence after a first stroke reaches up to 48% at 10 years.[1] Acetylsalicylic acid (aspirin) and clopidogrel are the most widely used antiplatelet agents in secondary prevention of stroke. Aspirin has been associated with a relative risk reduction of 25% in secondary cardiovascular events and 13% to 15% in secondary prevention of stroke.[2] A high platelet reactivity phenotype can be present in ≤60% of aspirin-treated patients and has been associated with an increased risk of recurrent ischemic events, and although it is a substantially heritable phenotype, very few genetic determinant have been identified. Furthermore, the causes of aspirin failure goes beyond high platelet reactivity and are still largely unknown.[3]

Only polymorphisms in *PEAR1* have been confirmed as genetic risk factors of aspirin failure.[4] The rs12041331 polymorphism is associated with platelet aggregation, increased platelet reactivity,[5] and vascular recurrence in aspirin-treated patients,[4] although these polymorphisms do not explain the whole variability observed in aspirin resistance or in vascular responsiveness. Other genomic regulations, such as epigenetics modifications could be associated with the risk of vascular recurrence in patients treated with aspirin.

We aimed to analyze the whole epigenome of stroke patients treated with aspirin, to find altered methylation sites associated with vascular recurrence.

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Materials and Methods

Study Patients

Thirty-eight subjects from a cohort of 1900 ischemic stroke patients recruited prospectively at Vall d'Hebron Hospital (Spain), who started aspirin treatment after a first ischemic stroke, were analyzed. Nineteen participants presented a new vascular event (ischemic stroke, myocardial infarction, peripheral vascular disease, or cardiovascular death) during the first year of follow-up and were matched one-to-one (age, sex, TOAST [Trial of Org 10172 in Acute Stroke Treatment]) with 19 participants without a vascular event. Second-stage analysis was performed in 289 prospective enrolled participants from 4 independent cohorts with ischemic stroke or ischemic atherothrombotic disease; 29 presented a vascular recurrence within 1 year of follow-up (Table I in the online-only Data Supplement).

Discovery: HumanMethylation450 Assay

DNA was extracted from blood samples using standard methods. Samples were obtained during the first 24 hours after stroke onset, before initial aspirin administration.

Genome-wide DNA methylation (DNAm) was assessed using the HumanMethylation450 (Illumina) assay. All samples (n=38) were processed in a single working batch. Quality Control and differentially methylated CpG (DMC) sites analysis were performed as described elsewhere,[6] using the R computing environment (3.1.3 version; Table II in the online-only Data Supplement).

Rs120411331 polymorphism (*PEAR1*) was checked to discover whether it could be a confounding factor in the EWAS (Epigenome-Wide Association Study) analysis.

CpGs Selection

One DMC with epigenome-wide significance[7] (*RAF1*; $P<10^{-06}$) was selected for further replication. Candidate DMCs for second-stage analysis were defined as: top 100 significant DMCs from the univariate analysis, which also appear in the top 100 DMCs after multivariable analysis. Multivariable analyses were adjusted for principal components and DNAm potential covariates (age, sex, and current smoking). From a total of 36 candidate CpGs, those mapped on genes previously associated with cardiovascular disease were considered for further analysis. A PubMed search (http://www.ncbi.nlm.nih.gov/pubmed) was undertaken using the following key words: aspirin, atherosclerosis, cardiovascular, stroke, and vascular. Finally, from 4 candidate DMCs, only 2 CpGs (*PPM1A* and *KCNQ1*) were located in regions suitable for EpiTYPER analysis (Figure IV in the online-only Data Supplement).

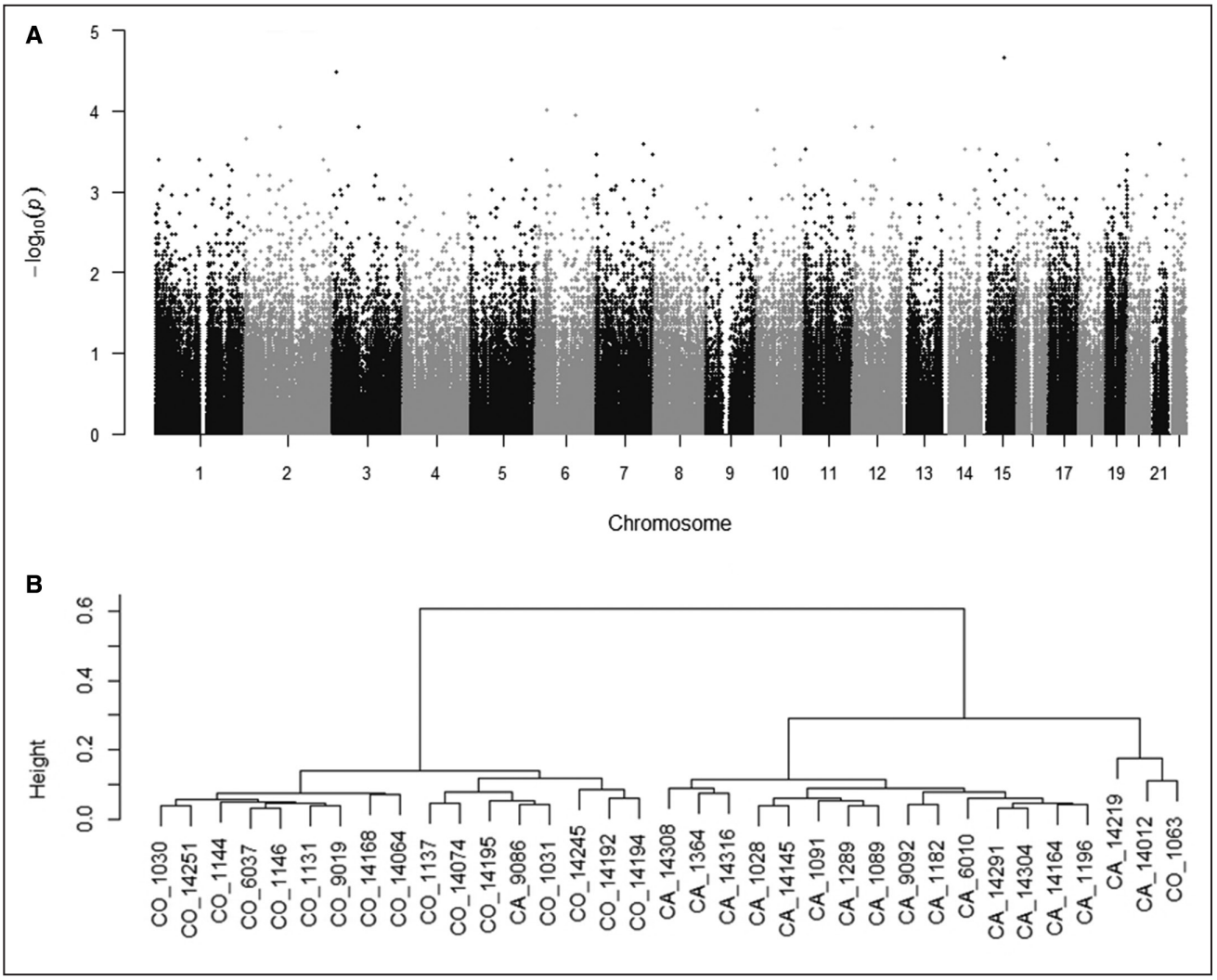

Figure. A, Manhattan plot for the EWAS (Epigenome-Wide Association Study) of aspirin-treated stroke patients. *x* axis: chromosome position; *y* axis: $-\log_{10}$ of *P* values. **B**, Hierarchical cluster analysis of most significant differentially methylated CpG associated (Table) with vascular recurrence. CA indicates recurrence patients; and CO, nonrecurrence patients.

Second-Stage EpiTYPER Assay

An averaged 400-bp region was sequenced using EpiTYPER (Sequenom) around each CpG selected from the discovery stage in 289 new patients.

Statistical Analysis

DMC sites were calculated using the Mann–Whitney *U* test and multivariable generalized linear analysis. Sample size calculation indicated that 19 subjects per condition were needed to achieve a 10^{-05} significance level and 80% statistical power. A joint analysis strategy using the DerSimonian–Laid test was performed to increase the statistical power in the 2-stage study as previously suggested.[8]

Complete methodology is available in the Materials and Methods section in the online-only Data Supplement.

Results

Discovery

DNAm levels were obtained for 485 577 CpGs sites (Figure [A]). After data preprocessing and quality control analysis one DMC was associated with vascular recurrence at epigenomewide level ($P<10^{-06}$), covariant adjustment analysis was also performed (Table; Table IV in the online-only Data Supplement). Table IV in the online-only Data Supplement shows the 36 candidate CpG sites which were best ranked in both univariate and covariate analyses, including cg04985020 (*PPM1A*) and cg08419850 (*KCNQ1*). Figure (B) shows a hierarchical cluster analysis, which was able to distinguish vascular recurrence patients from nonvascular patients, with only 2 samples misclassified.

Second-Stage Analysis

Second-stage analysis was performed for the epigenome-wide significant CpG, cg26039762 (*RAF1*; $P=9.69\times10^{-06}$), and 2 additional candidate CpG sites, cg04985020 (*PPM1A*; $P=3.46\times10^{-04}$) and cg08419850 (*KCNQ1*; $P=3.46\times10^{-04}$). All 3 CpGs map genes involved in atherosclerosis and vascular process. The cg04985020 (*PPM1A)* was associated with vascular recurrence ($P=0.027$). The same cg04985020 methylation pattern was observed in each of the 4 validation-stage cohorts and when nonstroke and cardioembolic patients were excluded from the analyses (Materials and Methods section in the online-only Data Supplement). Additionally, the following 3 CpG sites surrounding cg26039762 (*RAF1*) were de novo associated with vascular recurrence: RAR1_10 ($P=0.0015$), RAF1_6 ($P=0.0043$), and RAF1_3 ($P=0.01$; Table VI in the online-only Data Supplement). However, cg26039762 (*RAF1*) and cg08419850 (*KCNQ1*) were not associated in this second stage.

Joint analysis of discovery and replication stage revealed an epigenome-wide statistically significant meta *P* value[8] for *PPM1A* (meta $P=1.78\times10^{-07}$) but not for *RAF1*. *PPM1A* was independently associated with vascular recurrence when analyzed possible DNAm confounding factors (age, sex, and current smoking; $P=5.74\times10^{-03}$) and also *PEAR1* polymorphism (rs12041331; $P=9.82\times10^{-03}$). Additionally, *PPM1A* methylation levels were not influenced by cell-type proportions.

Table. DMC Sites Associated With Vascular Recurrence ($P<10^{-05}$)

DMCs Associated With Vascular Recurrence. Univariate Analysis.		
CpG ID	Gene	*P* Value
cg26039762	RAF1	9.69×10^{-6}
cg00094487	SPG21	1.20×10^{-5}
cg26568880	RDBP; SKIV2L	1.47×10^{-5}
cg20702204	CHFR	2.19×10^{-5}
cg22352818	…	2.19×10^{-5}
cg01112035	…	2.19×10^{-5}
cg16966962	LMOD1	3.23×10^{-5}
cg09747456	PANK1	3.90×10^{-5}
cg07580707	XPC; LSM3	4.69×10^{-5}
cg02533998	…	5.62×10^{-5}
cg00932677	…	6.72×10^{-5}
cg08859247	…	6.72×10^{-5}
cg00608860	MTHFSD; FLJ30679	6.72×10^{-5}
cg16933664	LOC100130987	8.02×10^{-5}
cg08808677	…	8.02×10^{-5}
cg26577201	CRYBA2	8.02×10^{-5}

DMC indicates differentially methylated CpG.

Discussion

Patients with vascular recurrence presented higher methylation levels of cg04985020 CpG (*PPM1A*) compared with nonrecurrent patients. Despite cg04985020 not being significant on the epigenome-wide level, in the discovery stage ($P=3.46\times10^{-04}$), the joint analysis revealed a meta *P* value of 1.78×10^{-07} that was significant on the epigenome-wide level.[7] These results suggest an association between DNAm levels of *PPM1A* and vascular recurrence during on-aspirin treatment, regardless of the disease (cardiovascular or stroke). The association was not confounded by common DNAm confounding factors or *PEAR1* polymorphism. The cg26039762 (*RAF1*) was not replicated, but 3 surrounding CpGs were associated de novo with vascular recurrence. Patients with vascular recurrence presented lower methylation levels of these 3 surrounding CpGs compared with nonvascular recurrence patients, similar to cg26039762 (*RAF1*) in the discovery stage. Additionally, hierarchical cluster analysis showed a potential role of DMCs as a model to classify patients within highest or lowest risk of vascular recurrence.

Protein phosphatase magnesium dependent 1A (PPM1A) is involved in the regulation of transforming growth factor (TGF)-β1 signaling and plasminogen activator inhibitor-1 transcription.[9] Plasminogen activator inhibitor-1 levels are associated with increased risk of cardiovascular events, particularly in the context of elevated tissue TGF-β1.[10] Raf1 proto-oncogene, serine/threonine kinase (*RAF1*), has been described in many vascular diseases, such as vascular dementia, angiogenesis processes, or cardiovascular events.[11] *RAF1* has also been described as taking part in signal transduction in the TGF-β/Smad pathway.[12] Furthermore, aspirin administration has been associated with decreasing TGF-β1 serum levels in hypercholesterolemic rats.[13] We hypothesize that PPM1A- and *RAF1*-altered methylation could be associated with vascular

recurrence because of the regulation of the TGF-β pathway; however, further studies are needed to confirm this hypothesis.

Pharmacoepigenomics is a growing field that could be used in the future to assist in personalized antiplatelet therapy or to find potential new drug targets. Our study suggests a remarkable role for epigenetics in the modulation of aspirin response.

Limitations

The small sample size in the discovery stage impeded the achievement of more significant values; however, joint analysis was useful to improve the results of our study. mRNA studies are needed to confirm the biological significance of our results.

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Disclosures

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References

1. van Wijk I, Kappelle LJ, van Gijn J, Koudstaal PJ, Franke CL, Vermeulen M, et al; LiLAC study group. Long-term survival and vascular event risk after transient ischaemic attack or minor ischaemic stroke: a cohort study. *Lancet*. 2005;365:2098–2104. doi: 10.1016/S0140-6736(05)66734-7.
2. Greer DM. Aspirin and antiplatelet agent resistance: implications for prevention of secondary stroke. *CNS Drugs*. 2010;24:1027–1040. doi: 10.2165/11539160-000000000-00000.
3. Pettersen AA, Arnesen H, Seljeflot I. A brief review on high on-aspirin residual platelet reactivity. *Vascul Pharmacol*. 2015;67-69:6–9. doi: 10.1016/j.vph.2015.03.018.
4. Lewis JP, Ryan K, O'Connell JR, Horenstein RB, Damcott CM, Gibson Q, et al. Genetic variation in PEAR1 is associated with platelet aggregation and cardiovascular outcomes. *Circ Cardiovasc Genet*. 2013;6:184–192. doi: 10.1161/CIRCGENETICS.111.964627.
5. Würtz M, Nissen PH, Grove EL, Kristensen SD, Hvas AM. Genetic determinants of on-aspirin platelet reactivity: focus on the influence of PEAR1. *PLoS One*. 2014;9:e111816. doi: 10.1371/journal.pone.0111816.
6. Gallego-Fabrega C, Carrera C, Reny JL, Fontana P, Slowik A, Pera J, et al. TRAF3 epigenetic regulation is associated with vascular recurrence in patients with ischemic stroke. *Stroke*. 2016;47:1180–1186. doi: 10.1161/STROKEAHA.115.012237.
7. Rakyan VK, Down TA, Balding DJ, Beck S. Epigenome-wide association studies for common human diseases. *Nat Rev Genet*. 2011;12:529–541. doi: 10.1038/nrg3000.
8. Skol AD, Scott LJ, Abecasis GR, Boehnke M. Joint analysis is more efficient than replication-based analysis for two-stage genome-wide association studies. *Nat Genet*. 2006;38:209–213. doi: 10.1038/ng1706.
9. Samarakoon R, Chitnis SS, Higgins SP, Higgins CE, Krepinsky JC, Higgins PJ. Redox-induced Src kinase and caveolin-1 signaling in TGF-β1-initiated SMAD2/3 activation and PAI-1 expression. *PLoS One*. 2011;6:e22896. doi: 10.1371/journal.pone.0022896.
10. Otsuka G, Agah R, Frutkin AD, Wight TN, Dichek DA. Transforming growth factor beta 1 induces neointima formation through plasminogen activator inhibitor-1-dependent pathways. *Arterioscler Thromb Vasc Biol*. 2006;26:737–743. doi: 10.1161/01.ATV.0000201087.23877.e1.
11. Liu Z, Liu Y, Li L, Xu Z, Bi B, Wang Y, et al. MiR-7-5p is frequently downregulated in glioblastoma microvasculature and inhibits vascular endothelial cell proliferation by targeting RAF1. *Tumour Biol*. 2014;35:10177–10184. doi: 10.1007/s13277-014-2318-x.
12. Watanabe-Takano H, Takano K, Hatano M, Tokuhisa T, Endo T. DA-Raf-mediated suppression of the Ras–ERK pathway is essential for TGF-β1-induced epithelial-mesenchymal transition in alveolar epithelial type 2 cells. *PLoS One*. 2015;10:e0127888. doi: 10.1371/journal.pone.0127888.
13. Mohamed AR, El-Hadidy WF, Mannaa HF. Assessment of the prophylactic role of aspirin and/or clopidogrel on experimentally induced acute myocardial infarction in hypercholesterolemic rats. *Drugs R D*. 2014;14:233–239. doi: 10.1007/s40268-014-0059-3.