Intracranial Aneurysm–Associated Single-Nucleotide Polymorphisms Alter Regulatory DNA in the Human Circle of Willis

Melanie D. Laarman, MSc; Marit W. Vermunt, PhD; Rachel Kleinloog, PhD; Jelkje J. de Boer-Bergsma, BSc; Netherlands Brain Bank; Gabriël J.E. Rinkel, PhD; Menno P. Creyghton, PhD; Michal Mokry, PhD; Jeroen Bakkers, PhD*; Ynte M. Ruigrok, PhD*

Background and Purpose—Genome-wide association studies significantly link intracranial aneurysm (IA) to single-nucleotide polymorphisms (SNPs) in 6 genomic loci. To gain insight into the relevance of these IA-associated SNPs, we aimed to identify regulatory regions and analyze overall gene expression in the human circle of Willis (CoW), on which these aneurysms develop.

Methods—We performed chromatin immunoprecipitation and sequencing for histone modifications H3K4me1 and H3K27ac to identify regulatory regions, including distal enhancers and active promoters, in postmortem specimens of the human CoW. These experiments were complemented with RNA sequencing on the same specimens. We determined whether these regulatory regions overlap with IA-associated SNPs, using computational methods. By combining our results with publicly available data, we investigated the effect of IA-associated SNPs on the newly identified regulatory regions and linked them to potential target genes.

Results—We find that IA-associated SNPs are significantly enriched in CoW regulatory regions. Some of the IA-associated SNPs that overlap with a regulatory region are likely to alter transcription factor binding, and in proximity to these regulatory regions are 102 genes that are expressed in the CoW. In addition, gene expression in the CoW is enriched for genes related to cell adhesion and the extracellular matrix.

Conclusions—CoW regulatory regions link IA-associated SNPs to genes with a potential role in the development of IAs. Our data refine previous predictions on SNPs associated with IA and provide a substantial resource from which candidates for follow-up studies can be prioritized. **(*Stroke*. 2018;49:447-453. DOI: 10.1161/STROKEAHA.117.018557.)**

Key Words: circle of Willis ■ computational biology ■ epigenomics ■ gene expression ■ intracranial aneurysm ■ polymorphism, single nucleotide ■ subarachnoid hemorrhage

Intracranial aneurysms (IAs) are saccular shaped outpouchings of the circle of Willis (CoW), a system of arteries located at the base of the brain. Rupture of an IA results in aneurysmal subarachnoid hemorrhage (aSAH). The consequences of aSAH are enormous because of the relatively young age at which it occurs (mean of 50 years) and the high case fatality and morbidity.[1]

First-degree relatives of patients with aSAH have an increased risk of developing an IA and subsequent aSAH,[2,3] which suggests the presence of genetic risk factors for IA and aSAH. Genome-wide association studies (GWASs) have indeed identified single-nucleotide polymorphisms (SNPs) that significantly associate with IA.[4–6] These SNPs are not necessarily the causal variants but may rather be in linkage disequilibrium with the causal variants.

The majority of the SNPs identified in the IA GWASs are situated in noncoding regions[4–6] as has been described for >90% of SNPs from other GWASs.[7] These SNPs often overlap with genomic sites with putative regulatory activity, such as distal enhancers and promoters.[7] Transcription factors (TFs) can bind to both distal enhancers and promoters and thereby regulate gene expression. TF-binding sites may be disrupted by disease- and trait-associated SNPs, which could lead to alterations in gene expression.[7] Indeed, recent studies show that disease-associated SNPs often alter the activity of noncoding regulatory regions.[8,9]

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From the Department of Neurology and Neurosurgery (M.D.L., R.K., G.J.E., Y.M.R.), Hubrecht Institute-KNAW, and Division of Heart and Lungs, Department of Medical Physiology (J.B.), University Medical Center Utrecht, the Netherlands (M.D.L., M.W.V., M.P.C., J.B.); Department of Genetics, University Medical Center Groningen, University of Groningen, the Netherlands (J.J.d.B.-B.); Netherlands Institute for Neuroscience, Amsterdam (N.B.B.); and Division of Pediatrics, Wilhelmina Children's Hospital, Utrecht, the Netherlands (M.M.).

*Drs Bakkers and Ruigrok contributed equally.

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Correspondence to Ynte M. Ruigrok, PhD, Department of Neurology and Neurosurgery, University Medical Center Utrecht, Heidelberglaan 100, 3584CX Utrecht, the Netherlands. E-mail ij.m.ruigrok@umcutrecht.nl

To gain insight into the relevance of the IA-associated SNPs, we set out to identify regulatory regions and assess the overall gene expression in postmortem specimens of the CoW. Using these data, we aimed to (1) analyze the biological meaning of putative distal enhancers identified in the CoW; (2) determine whether the identified putative CoW regulatory regions overlap with IA-associated SNPs; (3) investigate the possible effect of the IA-associated SNPs on these regulatory regions with the use of available datasets and computational methods; and (4) identify potential target genes of the CoW regulatory regions that overlap with IA-associated SNPs.

Materials and Methods

The data sets discussed in this publication have been made publicly available in the NCBI Gene Expression Omnibus database and can be accessed through Gene Expression Omnibus Series accession number GSE107196 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE107196).

Postmortem CoW Specimens

Four CoW specimens (Figure 1A) were obtained from the Netherlands Brain Bank, Netherlands Institute for Neuroscience, Amsterdam. All material was collected from donors whose written informed consent for brain autopsy and the use of the material and clinical information for research purposes had been obtained by the Netherlands Brain Bank (http://www.brainbank.nl). Donor characteristics can be found in Table I in the online-only Data Supplement. The CoW specimens were flash frozen in liquid nitrogen and stored at −80°C until further use.

Experimental Procedures

Regulatory regions in the human CoW were identified with chromatin immunoprecipitation followed by sequencing (ChIP-seq) as previously described (Figure 1A).[10] A short version of the methods are available in the online-only Data Supplement, and Table II in the online-only Data Supplement contains ChIP-seq sample and sequencing characteristics.

Gene expression in the human CoW was investigated by RNA sequencing (RNA-seq) as previously described (Figure 1A).[11] Expanded methods are available in the online-only Data Supplement, and Table III in the online-only Data Supplement contains RNA sample and sequencing characteristics.

Biological Meaning of Distal Enhancers Through Gene Ontology

We obtained distal enhancers from our ChIP-seq data by removing all H3K27ac-enriched regions that overlap with a known promoter (defined as 2 kb around the transcription start site of a gene) from the total set of H3K27ac-enriched regions. We studied the biological meaning of these distal enhancers using GREAT (Genomic Regions Enrichment of Annotations Tool)[12] (http://bejerano.stanford.edu/great/public/html/). Expanded methods are available in the online-only Data Supplement.

Enrichment of IA-Associated SNPs in CoW Regulatory Regions

To determine whether IA-associated SNPs are enriched in CoW regulatory regions compared with matched control SNPs, we calculated how many IA-associated SNPs overlap with a CoW regulatory region (Figure 2A), as previously described.[7,8] We performed this analysis for the H3K4me1- and H3K27ac-enriched regions, both separately and together, and for both the 19 SNPs from the IA GWAS and the 332 SNPs, including the SNPs in strong linkage disequilibrium. Expanded methods are available in the online-only Data Supplement.

Predicted Effects of the IA-Associated SNPs on the Overlapping Regulatory Regions

We used the RegulomeDB database (www.regulomedb.org)[13] to investigate possible effects of the IA-associated SNPs on the overlapping regulatory regions, such as perturbations of TF-binding sites. The RegulomeDB output is based on ChIP-seq, footprinting, and position weight matrix data from a variety of cell types. Subsequently, we used the online tool DAVID[14,15] to analyze gene ontology enrichment for all TFs that can bind to the regulatory regions that overlap with an IA-associated SNP, according to RegulomeDB.

Potential Target Genes of CoW Regulatory Regions That Contain an IA-Associated SNP

To get insight in potential target genes of the CoW regulatory regions that overlap with an IA-associated SNP, we used publicly available data sets[16] that describe topologically associated domains (TADs). Regulatory regions and their target genes are thought to interact within such TADs.[17] We selected those TADs that contain ≥1 regulatory regions that in turn overlap with an IA-associated SNP. The genes for which the transcription start site lies within the genomic region of these TADs were filtered for gene expression based on our RNA-seq data to obtain only the genes that are expressed in the CoW. Expanded methods are available in the online-only Data Supplement.

Statistics

Statistical approaches for analysis of ChIP-seq and RNA-seq data were performed as previously described.[10,11] Likewise, the statistical analysis for enrichment of IA-associated SNPs in CoW regulatory regions was performed as previously described.[8] Additional statistical methods can be found in the online-only Data Supplement.

Results

Identification of CoW Regulatory Regions

Using ChIP-seq to search for regulatory regions in the CoW, we identified a total of 48 717 unique H3K4me1-enriched regions (both poised and active enhancers) and 40 696 unique H3K27ac-enriched regions (active enhancers and active promoters; Table IV in the online-only Data Supplement). There is a substantial overlap between the 4 samples; 50.9% of all H3K4me1-enriched regions and 64.5% of all H3K27ac-enriched regions are present in at least 2 samples (Figure IIIA and IIIB in the online-only Data Supplement). Representative ChIP-seq tracks for H3K4me1 and H3K27ac enrichment of all 4 CoW samples are shown in Figure 1B.

Gene Expression in the CoW

Using RNA-seq, we identified a total of 13 481 genes that are expressed in at least 2 samples. Figure 1B shows an example of the RNA-seq data from all 4 CoW samples. RNA expression is strongly correlated between the samples, with an average Pearson correlation coefficient between each of the samples of 0.95 (Figure IIB–IIG in the online-only Data Supplement) and 74.2% of the genes expressed in all 4 samples (Figure IIIC in the online-only Data Supplement). Gene ontology analysis with GOrilla[18,19] revealed a strong enrichment of genes linked to cell adhesion and the extracellular matrix (Figure IIC in the online-only Data Supplement).

Biological Meaning of CoW Distal Enhancers Through Gene Ontology

We identified 28 052 putative distal enhancers after removing the H3K27ac-enriched regions that overlap with a known

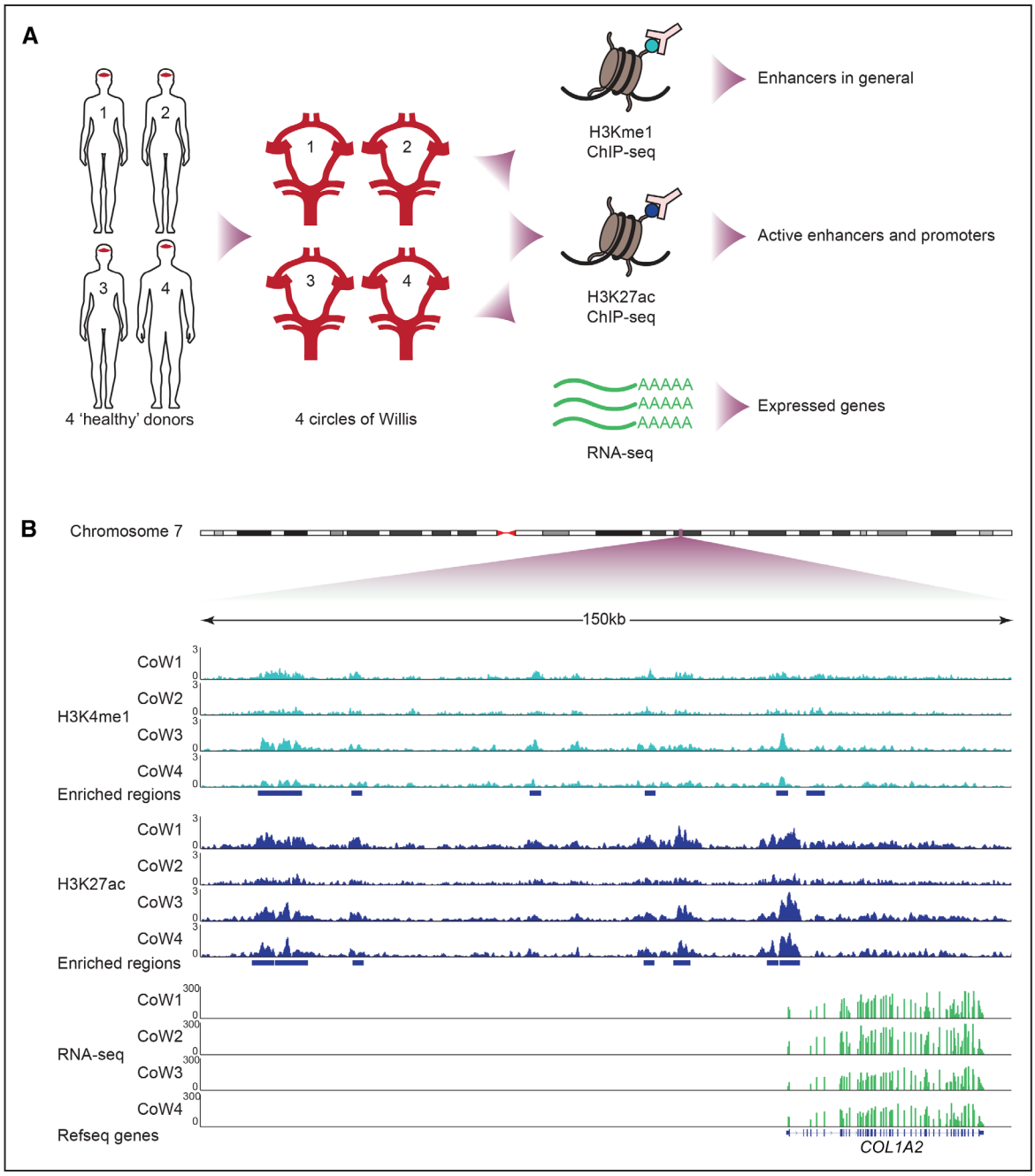

Figure 1. Chromatin immunoprecipitation followed by sequencing (ChIP-seq) and RNA sequencing (RNA-seq) on 4 human circles of Willis (CoWs). **A**, Graphical representation of the experimental setup. The CoWs from 4 donors were used for (1) ChIP-seq with antibodies against H3K4me1 to identify enhancers in general and antibodies against H3K27ac to identify active enhancers and promoters and (2) RNA-seq to identify expressed genes. **B**, Genome browser view of a 150-kb genomic region around the *COL1A2* gene, a gene that has been linked to intracranial aneurysm in previous studies. For all 4 CoW samples, tracks are presented for H3K4me1 and H3K27 enrichment (including dark blue bars depicting identified enriched regions) and RNA-seq.

promoter. When we investigated the biological meaning of these distal enhancers using GREAT,[12] the term IA was found to be significantly enriched in the disease ontology category (Table 1). This was based on 82 enhancers located nearby 15 genes that have been implicated in IA disease pathogenesis in previous studies, such as genome-wide linkage, candidate gene SNP association, and differential gene expression studies (Table VII in the online-only Data Supplement). In addition to this, biological processes related to vasculature development, the extracellular matrix, and cell adhesion were enriched (Table 1), which is in line with the observed terms for the expressed genes (Figure IIC in the online-only Data Supplement). These results suggest a potential role for regulatory regions in the pathology of IA.

Enrichment of IA-Associated SNPs in CoW Regulatory Regions

We find that IA-associated SNPs are strongly enriched in (active) CoW regulatory regions (both promoters and distal enhancers) compared with random-matched control SNPs.

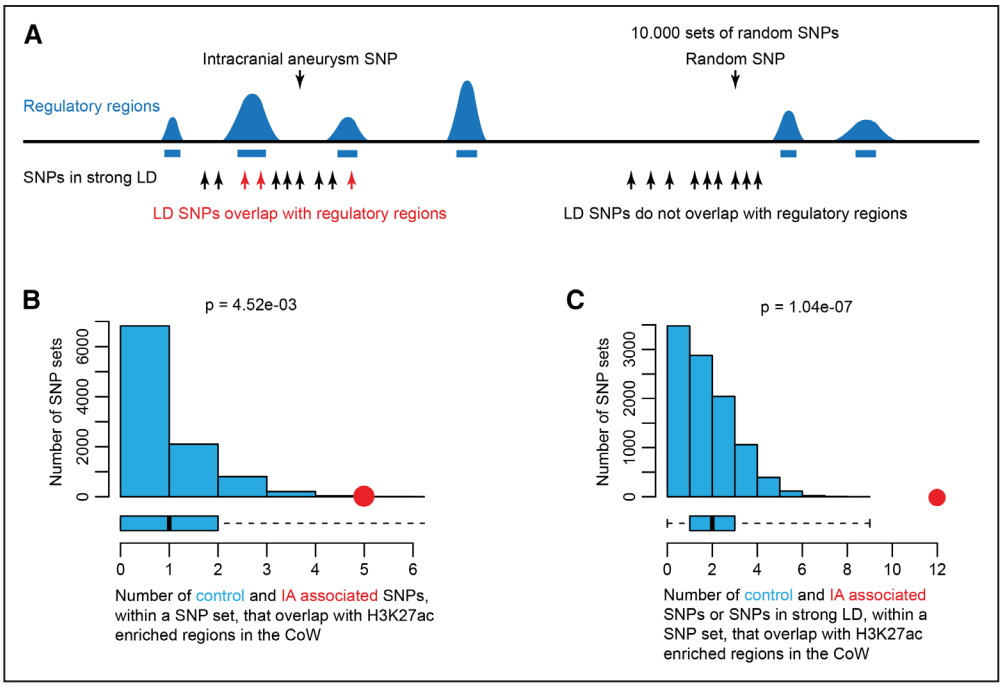

Figure 2. Intracranial aneurysm (IA)–associated single-nucleotide polymorphisms (SNPs) are enriched in circle of Willis (CoW) regulatory regions. **A**, Graphical representation of the method used to determine the overlap between IA-associated SNPs and CoW regulatory regions. Within the set of IA-associated SNPs, we calculated how many lie within the genomic coordinates of a CoW regulatory region. To control for overlap based on chance, the same analysis was done with 10.000 sets of random matched control SNPs (shown in **B**). This analysis was also performed for all SNPs in strong linkage disequilibrium (LD; r^2>0.8) with the IA-associated SNPs. SNPs in strong LD were also included for the 10.000 control SNP sets (shown in **C**). **B** and **C**, Number of SNPs in the set of IA-associated SNPs (red dot) that overlap with H3K27ac-enriched regulatory regions, compared with 10.000 sets of control SNPs (blue bars). Number of overlapping SNPs is depicted on the *x* axis. The box-and-whisker plot between the bar graph and the *x* axis shows the spread in the number of overlapping SNPs in the control SNP sets. IA-associated SNPs are enriched in CoW regulatory regions compared with the 10.000 sets of control SNPs. Analyzing only the original SNPs, shown in (**B**), we find a significant enrichment with a *P* value of 4.52e-03 (5 IA-associated SNPs compared with a median of 1 control SNP). When using also the SNPs in strong LD, shown in (**C**), we find a significant enrichment with a *P* value of 1.04e-07 (12 IA-associated SNPs compared with a median of 2 control SNPs).

When only the 19 SNPs from the IA GWAS were overlapped with H3K27ac-enriched regions, 5 IA-associated SNPs overlapped (*P*=4.52e-03). When also the SNPs in strong linkage disequilibrium were used, 12 of the IA-associated SNPs (or ≥1 SNPs in linkage disequilibrium; 31 in total) overlapped (*P*=1.04e-7; Figure 2B and 2C and additional overlap graphs in Figure V in the online-only Data Supplement). In total, 19 CoW (active) regulatory regions contain ≥1 IA-associated SNPs. This enrichment suggests that there is a link between IA-associated SNPs and regulatory regions in the CoW and that these CoW regulatory regions could be involved in the development of the disease.

Predicted Effects of the IA-Associated SNPs on the Overlapping Regulatory Regions

According to ChIP-seq, footprinting, and position weight matrix data from multiple cell types in RegulomeDB, a wide variety of TFs can bind to 15 of the 19 regulatory regions that overlap with an IA-associated SNP (Table VIII in the online-only Data Supplement). Gene ontology term analysis for these TFs shows that an enrichment of TFs involved in blood vessel–related processes, apoptotic processes, the MAP kinase signaling pathway, infection-related pathways, megakaryocyte development and platelet production, and obviously many transcription-related processes (Table IX in the online-only Data Supplement for the full gene ontology term lists). Some of the TFs are able to bind to multiple CoW regulatory regions, suggesting that these TFs may have an important role in IA (Table X in the online-only Data Supplement). The RegulomeDB analysis also indicated that 10 of the 31 IA-associated SNPs that overlap with a CoW regulatory region are likely to influence TF binding (Table 2).

Potential Target Genes of CoW Regulatory Regions That Overlap With an IA-Associated SNP

We identified 158 genes of which the transcription start site lies within the same TAD as an identified regulatory region that overlaps with an IA-associated SNP. Of those 158 genes, 102 genes are expressed in the CoW (Table XI in the online-only Data Supplement). The IA-causing genes are likely among these genes.

Discussion

We have identified a genome-wide set of regulatory regions, including both poised and active distal enhancers and active promoters, and genome-wide expression profiles in the human CoW. Distal enhancers from the CoW are enriched in proximity to genes previously implicated in IA and in proximity to genes involved in vascular development, the extracellular matrix, and cell adhesion, which suggests a role for CoW

Table 1. Enriched Gene Ontology Terms Based on the Enrichment of Enhancers Close to Genes Associated With These Terms

Type of Annotation	Term Name	*P* Value
Osborne Annotated Disease Ontology	Intracranial aneurysm	3.77E-10
MGI Phenotype	Abnormal cell adhesion	1.40E-61
	Abnormal heart right ventricle outflow tract morphology	7.32E-42
	Abnormal pulmonary valve morphology	8.63E-41
	Abnormal circulating tumor necrosis factor level	3.53E-40
	Abnormal vascular branching morphogenesis	1.07E-38
	Overriding aortic valve	5.23E-38
	Aneurysm	8.86E-38
	Abnormal aortic valve morphology	9.34E-38
	Abnormal thymus development	8.50E-37
	Increased circulating tumor necrosis factor level	1.25E-35
Human Phenotype Ontology	Aortic dissection	8.36E-26
	Atrophic scars	2.43E-14
GO Molecular Function	Collagen binding	1.56E-47
	Calcium-release channel activity	6.56E-11
GO Biological Process	Cell-substrate junction assembly	2.98E-38
	Regulation of RNA stability	4.22E-37
	Regulation of mRNA stability	3.23E-31
	Collagen fibril organization	2.98E-30
	Platelet-derived growth factor receptor signaling pathway	1.56E-27
	Kidney vasculature development	1.15E-22
	Glomerulus vasculature development	3.26E-22
	Substrate adhesion-dependent cell spreading	1.31E-19
	Platelet formation	2.18E-18
MGI Expression Detected	TS23_arterial system	1.88E-69
	TS23_aorta	2.41E-65
	TS20_blood vessel	1.96E-41
	TS14_outflow tract	9.50E-28
	TS23_renal cortex arterial system	2.81E-25
	TS25_blood vessel	1.00E-22
	TS23_trachea cartilaginous ring	1.61E-20
	TS24_sclera	1.71E-20
	TS24_rib	4.10E-19
	TS9_parietal endoderm	1.76E-18
	TS28_brain blood vessel	5.90E-17

Derived from GREAT.[12] Gene ontology terms are sorted by type of ontology first and by *P* value second. GO indicates gene ontology; and MGI, Mouse Genome Informatics.

distal enhancers in these processes. Furthermore, we find that there is a significant enrichment of IA-associated SNPs in CoW regulatory regions, suggesting that these regions are involved in the pathogenesis of IA. Some of the SNPs that overlap with a CoW regulatory region are likely to alter TF binding and could therefore alter the expression of a target gene, providing a link between IA-associated SNPs and potential gene expression changes. Previous studies have suggested that the effects of SNPs that alter TF-binding sites are likely to be small,[7] which could explain the small cumulative effect of IA-associated SNPs. Other studies have suggested that the effects of the SNP on the TF-binding site may be context specific,[20] which might explain why IAs form mainly in certain locations in the CoW, where stimuli might be different. The identified regulatory regions that overlap with IA-associated SNPs are located in the same TADs as 102 genes that are expressed in the CoW. These 102 candidate target genes are an important focus for future studies.

This is to our knowledge the first study in which ChIP-seq and RNA-seq were performed on the human CoW. The resulting ChIP-seq and RNA-seq data sets provide a unique resource to investigate gene expression and the genomic location of regulatory regions of the CoW. Using CoW tissue for these techniques provides both advantages and disadvantages. Because it is not yet clear which cell type(s) contribute(s) to IA, using CoW tissue ensured that all cell types present in the CoW were subjected to our experiments. However, because of the presence of multiple cell types, the interpretation of the data is more challenging. Any result coming from only one of the cell types will be diluted by the presence of the other cell types and is therefore less easy to identify. We think that our data sets provide a good overall assessment of the CoW and a good starting point for future studies that could look further into specific cell types present in the CoW. Within the limits of this study, we aimed to get some insight into the target genes of the identified regulatory regions that overlap an IA-associated SNP, by using online available data sets from 2 different cell types: human embryonic stem cells and IMR90 cells (fetal lung cells).[16] These are cell types that are not present in the CoW, but because TADs are thought to be relatively consistent between cell types,[17] these data sets can still provide indicative information on potential target genes. However, these are potential interactions in other cell types and are therefore not conclusive for the CoW.

In the present study, the focus was on noncoding IA-associated SNPs because the majority of the SNPs identified in the IA GWASs are situated in noncoding regions. IA-associated SNPs residing in coding regions of the genome could be further characterized, for example, by generating knockout animal models of the genes containing the specific SNPs.

In the future, chromatin conformation capture techniques, such as 4C, should be used to experimentally identify the target genes in the CoW.[17,21] Furthermore, the activity of the regulatory regions should be tested in a reporter system, in which the expression of a fluorescent protein is controlled by the activity of the regulatory region. This also enables testing the effect of the disease-associated allele of the IA-associated SNPs on regulatory region activity and thereby their functionality.[8,22]

Table 2. CoW Regulatory Regions That Overlap an IA-Associated SNP

Genomic Location of Regulatory Region	Overlapping SNPs	Predicted SNP Effect (RegulomeDB[2])
chr1:6860146-6864028	rs7536222	Likely to affect binding
	rs17029864	Minimal
chr1:154988813-154991491	rs905938	Minimal
chr2:198157412-198159793	rs1429417	Likely to affect binding and linked to expression of a gene target
	rs1429418	Minimal
chr2:198170139-198173607	rs13033821	Likely to affect binding and linked to expression of a gene target
chr2:198362711-198366749	rs2605039	Likely to affect binding and linked to expression of a gene target
chr4:148401140-148405145	rs6841581	Likely to affect binding
chr5:122458512-122460512	rs2287696	Minimal
chr9:22102161-22104379	rs1333042	Minimal
	rs7859727	Minimal
	rs1537373	Minimal
chr9:22106440-22108440	rs1333043	Minimal
chr10:104613043-104615229	rs3824754	Minimal
chr10:104676976-104681326	rs12221064	Likely to affect binding
	rs17115213	Likely to affect binding and linked to expression of a gene target
chr10:104825148-104827148	rs3781285	Likely to affect binding and linked to expression of a gene target
chr10:104912111-104914635	rs11191582	Likely to affect binding and linked to expression of a gene target
chr11:102138109-102140905	rs2124216	Likely to affect binding and linked to expression of a gene target
chr12:95509507-95513683	rs6538596	No prediction
	rs7977572	Minimal
	rs6538597	No prediction
chr13:33726495-33730203	rs17764067	Minimal
chr18:20183852-20187886	rs8096784	Minimal
	rs8098265	Minimal
chr18:20290257-20292257	rs1530716	Minimal
chr20:17591386-17597126	rs13734	Minimal
	rs12480846	Minimal
	rs6136149	Minimal
	rs12479820	Minimal
	rs1132274	Minimal

Minimal refers to minimal binding evidence for transcription factors. CoW indicates circle of Willis; IA, intracranial aneurysm; and SNP, single-nucleotide polymorphism.

These analyses may identify a causal relationship between the IA-associated SNPs and altered gene expression. Once the target genes are established, their function should be studied in a model animal to gain more insight in the largely unknown pathogenesis of the disease. In addition, studying the cell type–specific expression of these target genes in tissue sections of the human CoW could finally elucidate which cell type(s) are involved in the pathogenesis of the disease.

Summary

Our data demonstrate that IA-associated SNPs are enriched in CoW regulatory regions and that 19 of these regulatory regions contain IA-associated SNPs, which implicates the involvement of these regulatory regions in IA pathogenesis. Within the same TADs as these regulatory regions are 102 genes that are expressed in the CoW. Among these genes are likely genes that are involved in IA. The data presented in this study provide new angles to study the functional relevance of IA-associated SNPs. Chromatin conformation capture techniques can be used to experimentally identify the target genes of the regulatory regions that overlap with an IA-associated SNP, and the activity of these regulatory regions can be tested in a reporter assay in which the expression of a fluorescent protein is controlled by the activity of the regulatory region. These experiments will help to further elucidate the pathogenesis of IA.

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Disclosures

None.

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