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Copy Number Variation analysis in 98 individuals with PHACE syndrome

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Conflict of Interest:
The authors state no conflict of interest.

Web Resources:
Toronto Database of Genomic Variants: http://projects.tcag.ca/cgi-bin/variation/gbrowse/hg19/
Decipher: http://decipher.sanger.ac.uk/
OMIM: http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim
UCSC Genome Browser: http://genome.ucsc.edu/cgi-bin/hgGateway
PubMed: http://www.ncbi.nlm.nih.gov/pubmed/
Ingenuity: http://ingenuity.com/
dbVAR: http://www.ncbi.nlm.nih.gov/dbvar/studies/nstd54/

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Abstract

PHACE syndrome is the association of large segmental facial hemangiomas and congenital anomalies, such as posterior fossa malformations, cerebral arterial anomalies, coarctation of the aorta, eye anomalies and sternal defects. To date, the reported cases of PHACE syndrome have been sporadic suggesting that PHACE may have a complex pathogenesis. We report here genomic copy number variation (CNV) analysis in 98 individuals with PHACE syndrome as a first step in deciphering a potential genetic basis of PHACE syndrome. A total of 3,772 CNVs (2,507 duplications and 1,265 deletions) were detected in 98 individuals with PHACE syndrome. CNVs were then eliminated if they failed to meet established criteria for quality, spanned centromeres or did not contain genes. CNVs were defined as “rare” if not documented in the Database of Genomic Variants. Ten rare CNVs were discovered (size range: 134–406 kb), located at 1q32.1, 1q43, 3q26.32-3q26.33, 3p11.1, 7q33, 10q24.32, 12q24.13, 17q11.2, 18p11.31, and Xq28. There were no rare CNV events that occurred in greater than one subject. Therefore, further study is needed to determine the significance of these CNVs in the pathogenesis of PHACE syndrome.

Keywords

PHACE syndrome; PHACES syndrome; Pascual-Castroviejo II syndrome; aortic arch anomaly; hemangioma; copy number variation

Introduction

Children with large infantile hemangiomas (IH) on the face and scalp are at risk for associated birth defects. In 1978, Pascual-Castroviejo described a female patient with a facial hemangioma and Dandy-Walker syndrome with abnormal intracranial cerebral arteries(Pascual-Castroviejo, 1978b). He subsequently described seven additional female patients with hemangioma and neurovascular anomalies (Pascual-Castroviejo, 1978a). In 1985, the term sternal malformation/vascular dysplasia syndrome was used to describe a

child with sternal cleft and cutaneous, craniofacial hemangiomas as well as airway and liver hemangiomas (Hersh *et al.*, 1985). In 1996, Frieden et al proposed the acronym PHACE (*p*osterior fossa malformations, infantile *h*emangiomas, *a*rterial anomalies of the great cerebral vessels, *c*oarctation of the aorta, *e*ye anomalies and sternal defects) to describe this condition (OMIM: 606519) (Frieden *et al.*, 1996). Children with PHACE are at risk for cerebral vasculopathy that can lead to arterial ischemic stroke in a subset of individuals (Drolet *et al.*, 2006; Siegel *et al.*, 2012). Hemangiomas on the lower body can be associated with congenital anomalies in LUMBAR syndrome (*l*ower body hemangioma, *u*rogenital anomalies, *u*lceration, *m*yelopathy, *b*one deformities, *a*norectal or *a*rterial anomalies and *r*enal anomalies)(Iacobas *et al.*, 2010). Recently, identification of the genes for the overgrowth syndromes associated with vascular malformations have been rapidly unfolding (Kurek *et al.*, 2012; Riviere *et al.*, 2012), however the genes for the hemangioma syndromes have yet to be elucidated.

All reported cases of PHACE to date have been sporadic, yet the consistent phenotype and the characteristic brain and aortic anomalies provide evidence for a genetic etiology. PHACE has a significant female predominance suggesting that there may be a contribution from the X chromosome or hormonal influences (Haggstrom *et al.*, 2007). X-linked inheritance has been suggested, although males do not have increased severity (Levin and Kaler, 2007; Metry *et al.*, 2008). Preliminary X-inactivation studies in this PHACE cohort did not identify significant skewing in most affected females or their mothers. Although linkage studies have been performed on solitary IH identifying a locus at 5q, and subsequent studies have pointed to the involvement of VEGFR2 (Jinnin *et al.*, 2008; Walter *et al.*, 1999), there have been no linkage studies performed on PHACE specifically. There have been no significant prenatal environmental factors or toxins identified in association with PHACE (Metry *et al.*, 2006).

There is growing evidence that small structural chromosomal abnormalities contribute to a broad array of complex congenital malformations (Southard *et al.*, 2012). High-density arrays now provide a powerful method for evaluating genome-wide copy number variation (CNV) and have been used to advance knowledge of the genetic basis of numerous disorders including thoracic aortic aneurysms (Prakash *et al.*, 2010), autism spectrum disorders (Glessner *et al.*, 2009) and developmental brain disorders (Aldinger *et al.*, 2009; Pinto *et al.*, 2010). The objective of this study is to comprehensively evaluate the contribution of copy number variation in PHACE syndrome.

Results

Ninety-eight individuals diagnosed with PHACE were genotyped and analyzed for CNVs. Of these, 94 individuals with PHACE were genotyped using the Affymetrix Human Mapping GeneChip 6.0 arrays and analyzed with the Genotyping Console™ Software (Figure 1: Flowchart of CNV Analysis and Identification). A collaborating group also provided an additional cohort of 4 individuals with PHACE which were run and analyzed with Roche Nimblegen programs and these results were included in this analysis (see Supplemental methods). Eighty-two individuals with PHACE were female and 16 male. Additional demographic data are summarized in Table 1. A control group included 91

individuals without PHACE from an unrelated research cohort (see methods for a description of this cohort).

CNV in 98 PHACE syndrome cases

First, we looked at CNV burden and found a total of 3,696 CNVs (2,473 duplications and 1,223 deletions) in 94 individuals with PHACE using Genotyping Console, averaging 39 CNVs per individual (18–83 CNVs) with a mean length of 328 kb (range: 25 kb-23.8 MB). In the control cohort of 91 individuals, a total of 3,484 CNVs (2,506 duplications and 978 deletions) were identified, averaging 38 CNVs per individual (18–91 CNVs) with a mean length of 267 kb (25 kb-23.4 MB). The average number of CNVs per individual was not found to be statistically different between subjects and controls (p=0.91). In data provided by a collaborating group from an additional cohort of 4 individuals with PHACE a total of 76 CNVs (34 duplications and 42 deletions) with a mean length of 373 kb (range: 27 kb-3.9 Mb) were detected using the Roche NimbleGen array. Thus, a total of 3,772 CNVs (2,507 duplications and 1,265 deletions) were detected in 98 individuals with PHACE syndrome. CNVs were then eliminated from further analysis if they failed to meet established criteria for quality, spanned centromeres or did not contain genes. Of the CNVs meeting our criteria as described in Figure 1, only two CNVs were excluded based on identification in the control cohort (1q34 and 2p13.2). In these 98 individuals with PHACE, we identified rare CNVs greater than 100kb at 1q32.1, 1q43, 3q26-3q26.33, 3p11.1, 7q33, 10q24.32, 12q24.13, 17q11.2, 18p11.31, and Xq28. The genes contained in the reported CNVs are shown in Supplemental Table 1 and clinical phenotypes are detailed in Supplemental Table 2. There were no rare CNV events that occurred in greater than one individual with PHACE.

Validation

Taqman qPCR was used to validate 9 discovered rare CNV regions. The 7q33 region was validated with the Nimblegen chip. Taqman probes for selected genes in each genomic region were designed as follows: 1) *KLHL12*, *CYB5R1*, and *PPFIA4* (1q32.1), 2) *FH* and *KMO* (1q43), 3) *PIK3CA* and *MFN1* (3q26.32-3q26.33), 4) *EPHA3* (3q11.1), 5) *ARL3* (10q24.32), 6) *TPCN1* and *SLC24A6* (12q24.13), 7) *TNFAIP1* and *VTN* (17q11.2), 8) *EMILIN2* and *MYOM1* (18p11.31), and 9) *AFF2* (Xq28). Additional candidate regions (tested but not listed above) that failed to validate with Taqman qPCR were excluded from further analysis.

De novo versus Inherited CNVs

The *de novo* versus inherited status was determined by testing the mother and father for the identified CNV using the Taqman qPCR analysis. Parental DNA for either one or both parents was not available for 5 cases. Inherited CNVs included 1q32.1, 3p11.1, 10q24.32, 17q11.2, and 18p11.31 (Supplemental Table 1). *De novo* status for all other validated CNVs was indeterminate as one or both parental DNA samples were unavailable for analysis

Functional network pathway analysis

We screened the CNV regions for known genes important in vasculogenesis such as *TGFBR1*, *TGFBR2*, *VEGF*, *NOTCH1*, and *NOTCH2*; however, CNVs containing these

specific genes were not identified in this cohort. As a next step in the analysis, validated CNVs were analyzed with the Ingenuity program to identify gene networks and enrich for common pathways (see methods). The analysis identified direct and indirect interactions between the genes in the CNV and the AKT/PIK3/VEGF pathways (Figure 2). A duplication containing the gene *PIKC3A* is of interest because PIKC3A has indirect interactions with mTOR, NOS2, EGF and HIF-1α and a direct interaction with ERK and AKT1. MYOG, CYP17A1, and CHI3L1 also showed interactions with these pathways.

Discussion

As a first step in determining the genetic influence in the development of PHACE, we utilized genome wide CNV analysis in a discovery cohort of 98 individuals with PHACE to evaluate CNV regions. This is the largest series to date analyzing the contribution of copy number variation in PHACE. We report here several rare CNVs (see methods for selection criteria) which were not found in the Database of Genomic Variants. We have further reviewed the CNVs in the Database of Genomic Structural Variation (dbVar) containing a dataset with copy number variants from 15,767 children with developmental delay. There was no overlap between the CNVs in individuals with PHACE and the CNVs reported as pathogenic in the publication from Cooper et al(Cooper *et al.*, 2011). Overlap was noted with the CNVs recorded in dbVar from the Cooper cohort, however the majority of the reported overlapping CNVs were much larger, encompassing several additional genes. In the dbVar database, there was no reported clinical interpretation, validation or testing of inheritance for these CNVs. As these variants have not been previously linked with a specific disease phenotype other than developmental delay, and in rare cases craniofacial dysmorphism or unspecified abnormalities of the cardiovascular system, they should be considered 'variants of unknown significance' until larger datasets for PHACE are investigated or functional studies are performed. In this study we did not find any large, rare CNVs occurring in more than one individual with PHACE.

The CNVs reported in this paper contain genes that interact with the HIF-1α, VEGF, AKT and mTOR pathways (Figure 2) which have been implicated in angiogenesis and hemangioma pathogenesis (Boscolo *et al.*, 2011; Chen *et al.*, 2005; Karar and Maity, 2011). The CNV gain at 3q26.32 contains the *PIK3CA* gene. PIK3CA (phosphoinositide 3-kinase catalytic subunit α) interacts with AKT1, VEGF and HIF-1α (Figure 2). PIK3CA, specifically, has been demonstrated to promote angiogenesis via VEGF with a positive correlation between PIK3CA and VEGF expression in ovarian cancer cells (Zhang *et al.*, 2003). The mutations in the *PIK3CA* gene have recently been implicated in a condition characterized by megalencephaly and growth dysregulation with variable asymmetry, skin capillary malformations, distal limb malformations, and variable cortical malformation (MCAP) (Mirzaa *et al.*, 2012; Riviere *et al.*, 2012).

In addition, *PIK3CA* gene mutations have been identified in CLOVES syndrome (*c*ongenital *l*ipomatous *o*vergrowth with *v*ascular, *e*pidermal, and *s*keletal anomalies)(Kurek *et al.*, 2012). The 1q32.1 region also contains the liprin-α4 gene promoter (*PPFIA4*) which is directly activated by binding of the hypoxia-inducible factor 1α (*HIF-1α*) (Mattauch *et al.*, 2010). *HIF-1α* functions as a transcriptional regulator of the adaptive response to hypoxia

and plays a role in embryonic vascularization. A portion of the *MYOM1* gene was present in both a 26 kb loss and a 247 kb gain, in two unrelated individuals with PHACE. The significance of this finding is uncertain. The myomesin genes play an important structural and functional role in striated muscle and myomesin mutations have been identified in a family with hypertrophic cardiomyopathy (Siegert *et al.*, 2011).

Genes involved with embryonic development

The candidate gene *VTN* in region 17q11.2 interacts with a biological network involved in embryonic cranial mesenchyme and vascular tissue development, as well as endothelial-mesenchymal interactions in development (Arciniegas *et al.*, 2006). Such interactions with the cranial mesenchyme have been shown to be important in normal cerebellar development and disruption can lead to Dandy-Walker malformation (Aldinger *et al.*, 2009). This CNV in this female proband was inherited from the mother; therefore we consider this to be a variant of unknown significance. A gain of the ephrin receptor tyrosine kinase (*EPHA3*) gene at chromosome 3p11.1 was identified in an individual with a sternal cleft and supraumbilical raphe. *EPHA3* is involved in the guidance and coordination of the migration of motor and sensory axons during development and in cardiac valve development (Bevins *et al.*, 2011; Jayasena *et al.*, 2005; Oates *et al.*, 1999; Stephen *et al.*, 2007). A gain of the *ADORA*1 gene was found in a patient with arterial anomalies and coarctation of the aorta. The adenosine A1 receptor (*ADORA*1) gene has been shown to participate in axon guidance for the retino-tectal patterning through the engrailed homeoprotein transcription factors and ephrin A5 (Stettler *et al.*, 2012). It is notable that a gain in the *EMILIN2* gene at 18p11.31 was identified in a patient with a posterior fossa malformation and both carotid and cerebral arterial anomalies. *EMILIN2* protein expression has been demonstrated in the mouse heart and aorta (Doliana *et al.*, 2001; Sa and Hoover-Plow, 2011). Fibrosis and decreased elastic fibers seen in aorta tissue after coarctation repair has been reported, suggesting that abnormalities in elastic fibers could potentially play a role in the pathogenesis of PHACE (Prada *et al.*, 2010).

Summary

PHACE is a complex condition which is likely to have multifactorial risk factors. Although several rare CNVs were identified in this cohort, none were found in more than one patient. Five discovered CNVs, 1q32.1, 3p11.1, 10q24.32, 17q11.2, and 18p11.31 were inherited, although there was no family history of either PHACE or IH in any of these individuals. It is interesting to note the chromosomal regions, 1q32.1, 3p11.1, 10q24.32, 17q11.2, and 18p11.31 were enriched for genes involved in the HIF-1α, VEGF and mTOR signaling pathways. Larger datasets will be necessary to determine the significance of the CNVs reported in this manuscript. These findings are consistent with our current understanding of PHACE as a complex condition and more sophisticated approaches, including next-generation sequencing of both germline and tissue DNA, will likely be necessary to determine if there are genetic factors in the pathogenesis of this disorder.

Limitations

The greatest challenges with CNV analysis are discriminating the effects of common and rare CNV polymorphisms and the disease relevance of structural chromosomal abnormalities. We used the existing databases of published CNVs including the University of California Santa Cruz (UCSC) Genome Browser, the Database of Genomic Variants (DGV) and others to filter variants that appear to be common in the general population. We recognize a limitation of this study is that we may have excluded some of the CNVs with a potential influence in PHACE based on a previously reported association with a different disorder. When available, we used a family-based approach to determine the *de novo* status. Array CNV analysis is limited by its resolution to detect events. It is also possible that the coronary artery disease control group may have shared some common CNV with the PHACE cohort, wherein the causative CNV could have been masked. We recognize this as another limitation of this study.

Materials and Methods

For additional methods for the samples run on Nimblegen array, see the supplemental methods.

Study Populations

The study has ongoing approval from the Institutional Review Board (IRB) at Children's Hospital of Wisconsin (CHW) and was approved previously by the IRBs at Oregon Health and Science University (OHSU), the University of Utah, and the Committee for Human Research (CHR) at the University of California at San Francisco (UCSF). Written informed consent was obtained from all study subjects upon enrollment into the PHACE International Clinical Registry and Genetic Repository and includes information about DNA sharing and Database of Genotypes and Phenotypes and adherence to the Helinski Guidelines. Ninety-four individuals with PHACE were genotyped on the Affymetrix Human Mapping GeneChip 6.0 arrays and analyzed with the Genotyping Console™ Software-Affymetrix program. The diagnosis of definite or possible syndrome was made at participating institutions using previously described criteria (Metry *et al.*, 2009) and confirmed by central review of magnetic resonance imaging (MRI), magnetic resonance angiography (MRA), or echocardiogram reports. Internal controls for the study included 91 subjects with coronary artery disease who did not carry a diagnosis of PHACE. Individuals in the control cohort were consented under an unrelated research study which allows DNA information sharing for research related purposes. Parent and control cohorts were genotyped on the same platform and subjected to equivalent QC measures and CNV analysis used for affected cases.

DNA and Tissue Repository

The DNA and tissue repository for the PHACE syndrome International Clinical Registry and Genetic Repository has been maintained in the Children's Research Institute at MCW. DNA was extracted from saliva obtained from buccal swabs and blood collected from each subject (4 ml in an EDTA tube). The quantity and quality of the DNA was measured with the Nanodrop *(Thermo Scientific)*.

Genotyping and CNV Data Analysis and Novelty Assessment

Two hundred eighteen samples (118 PHACE subjects, and 100 control samples) were hybridized to the Affymetrix 6.0 GeneChips. Genotyping was performed with the Affymetrix Human Mapping GeneChip 6.0 arrays to assay a minimum of roughly 1.9M probes for analysis per standard protocol (see Supplemental methods). CEL files were exported to Genotyping Console (Affymetrix) for analysis per standard protocols. Stringent quality parameters were applied. Samples with greater than 92 CNV segments, out-of-bound, and duplicate patient samples were excluded from further analysis, resulting in a total of 94 individuals with PHACE and 91 controls for the study. Regions of CNV in our participants with PHACE were compared to CNVs previously reported in public CN databases (Toronto Database of Genomic Variants (DGV)) to determine regions that were novel to individuals with PHACE. An additional search for CNV regions in Decipher was conducted to look for any associated phenotypes and in OMIM for known diseases. Candidate genes within discovered genomic regions were determined for its potential role in disease pathogenesis based on information gathered from sources in PubMed, UCSC Genome Browser, and Ingenuity. We used the following criteria for CNV detection: 1) Size greater than 100 kb, 2) less than 5% overlap with reported CNVs in the DGV or our control cohort, and 3) we only included regions containing genes. CNVs with a less than 100 kb copy loss or gain were noted if found within a larger CNV region discovered in another individual. An average distance between markers of ≤ 2 kb was required to determine adequate probe density. Once rare CNVs were discovered in our patient cohort, validation was performed with the Applied Biosystems TaqMan quantitative polymerase chain reaction (qPCR) and *de novo* status was ascertained when parental genotypes were available. Finally, the validated CNV were cross-referenced against the published neurodevelopmental CNVs at http://www.ncbi.nlm.nih.gov/dbvar/studies/nstd54/ (Cooper *et al.*, 2011).

Quantitative Polymerase Chain Reaction Validation

Findings from our CNV analysis with genotyping console were validated with the Applied Biosystems TaqMan qPCR. Primers and probes used to detect CNVs with qPCR were ordered from Applied Biosystems (pre-mixed assay) (see Supplemental methods).

Functional Analysis: Ingenuity Network Pathways

Candidate genes within discovered genomic regions from CNV analysis with Genotyping Console were imported into the Ingenuity Pathways Analysis (IPA) Web server for functional analysis (maximum 35 genes). The IPA software utilizes the IngenuityR Knowledge Base (http://ingenuity.com, accessed Jan 2012) which contains information on millions of biological interactions and functional annotations derived from published data. This referenced information is used to model relationships between genes, proteins, complexes, cells, cellular components, and diseases amongst other molecules. The Path Explorer function of the IPA program searches for interactions between genes and was utilized to create the shortest interconnected pathway for genes contained in detected CNV regions. Shared pathways were subsequently noted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

CNV	copy number variation
PHACE	an acronym for Posterior fossa brain malformation, large facial Hemangiomas, Arterial anomalies, Cardiac anomalies and aortic coarctation, and Eye abnormalities
DGV	Database of Genomic Variants
dbVar	Database of Genomic Structural Variation

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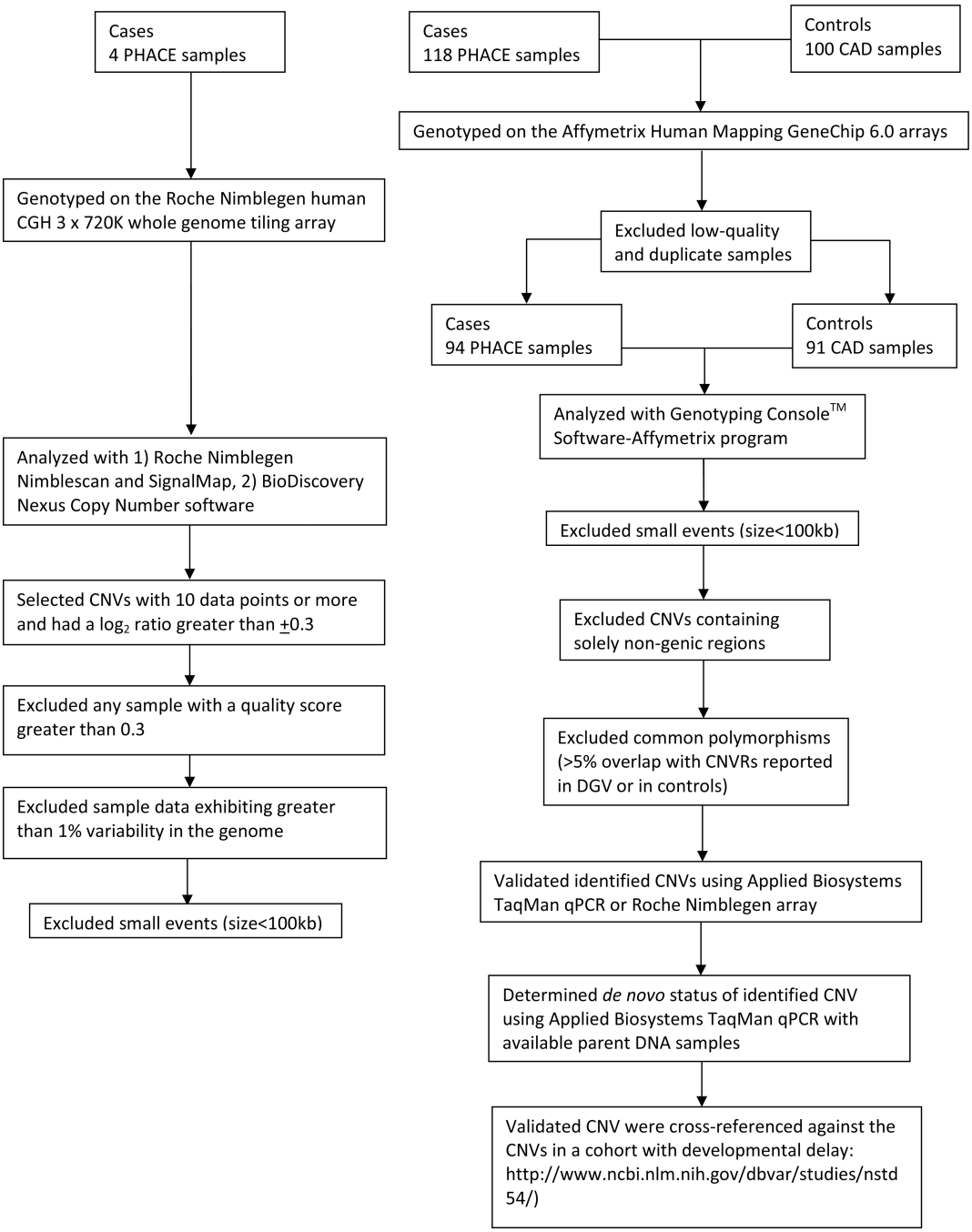

Figure 1. Flowchart of CNV Analysis and Identification

Algorithm used to identify large and rare CNVs in 98 individuals with PHACE syndrome.

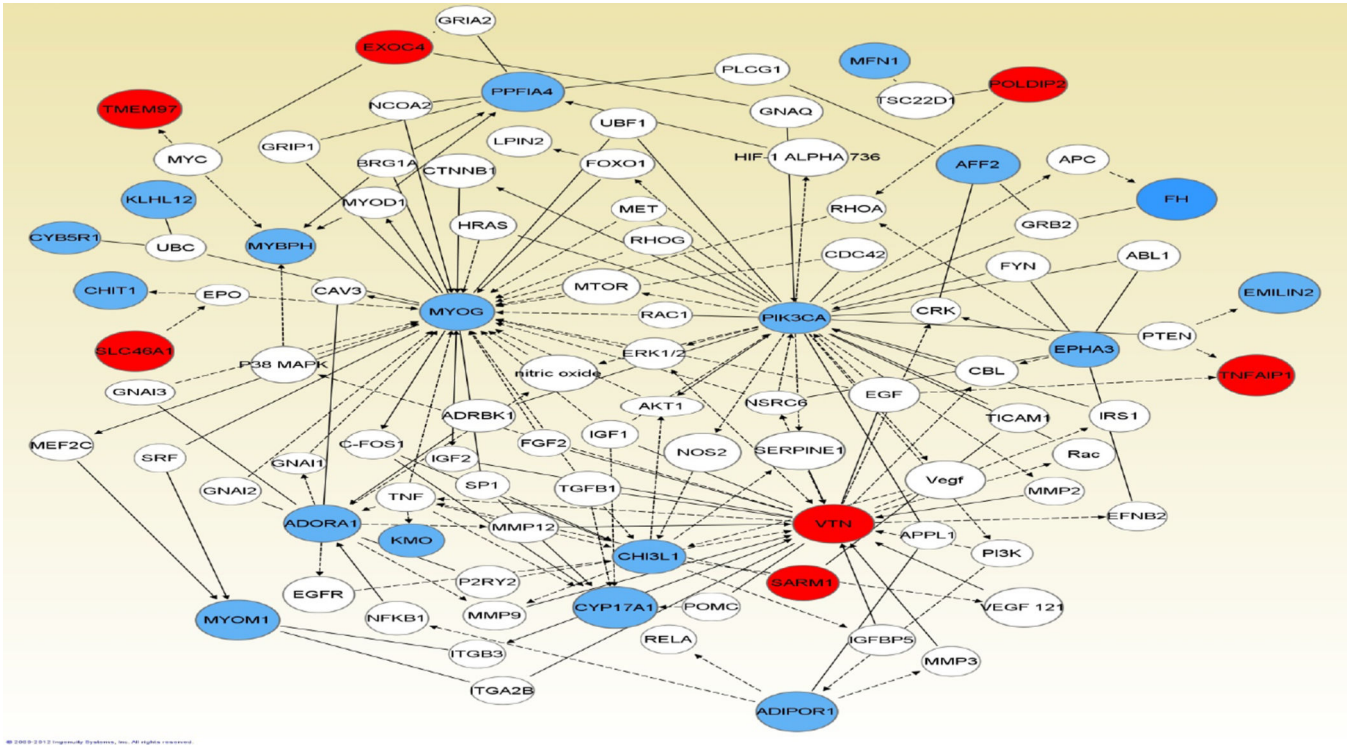

Figure 2. Network of interacting PHACE syndrome CNV genes

Genes within the PHACE-associated CNVs were analyzed using the Ingenuity Software Program (http://ingenuity.com). Genes with CNV gains are shown in blue and CNV losses are shown in red. All CNVs in this network are rare, not seen in the database of genomic variants or 91 controls from the study. Arrows indicate the action of one molecule on another. Lines between two molecules indicate binding only. Solid lines indicate direct interactions and dashed lines indicate indirect interactions.

Table 1

Clinical Characteristics of 98 individuals with PHACE Syndrome

Category	Number of Individuals with PHACE (Total: 98)
Gender	
Female	82
Male	16
Female: Male Ratio	5:1
Age at enrollment[1]	
Mean	35.3 months
Range	1 month-30 years
Gestational age (weeks)[2]	
Mean	38.7
Range	32–42
Ethnicity	
Hispanic or Latino	23
Non-Hispanic or Non-Latino	60
Other/Unknown/NA	15
Race	
American Indian/Alaska Native	1
Asian	3
Native Hawaiian or Other Pacific Islander	0
Black or African American	2
White	86
Other/Unknown/NA	6
Family History of Infantile Hemangioma	
Yes	8
No	28
Unknown/NA	62
PHACE phenotype	
Brain Malformations	33/84 (39%)
Infantile Hemangioma	90/90 (100%)
Arterial Anomalies	68/79 (86%)
Aortic Anomalies	39/87 (45%)
Congenital Eye Abnormalities	27/84 (32%)
Sternal malformations	22/83 (27%)
Maternal Factors	Number of Mothers (Total: 98)
Age at time of delivery[3]	
Mean	30 years
Range	20–44 years

Category	**Number of Individuals with PHACE (Total: 98)**
Pre-eclampsia	
Yes	10
No	60
Unknown/NA	28
Hypertension	
Yes	14
No	58
Unknown/NA	26
Number of Previous Miscarriages[4]	
Mean	0.43
Range	0–5

[1] Information for age at enrollment was available for 88 of 98 individuals with PHACE syndrome

[2] Information for gestational age was available for 80 of 98 individuals with PHACE syndrome

[3] Information for maternal age at delivery was available for 76 mothers

[4] Information for number of previous miscarriages was available for 65 mothers

NA=Information not available