Integrative Genomic Analysis in Autism Brain Tissue Reveals Pro-Inflammatory Signaling

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Introduction

Autism spectrum disorders (ASD) constitute a variety of neurodevelopmental disorders defined clinically by difficulties in socialize and restricted or repetitive behaviors during childhood onset. The cause of ASD is complex and involves both genetic and environmental risk factors. Disruption of epigenetic mechanisms regulating brain developmental processes has been proposed as a potential mechanism linking ASD environmental and genetic risk (Risch, et al., 2014).

Recent studies mostly focused on genetic approaches that dissect the molecular basis of autism. Neural development involves innumerable biological events simultaneously, i.e., neurogenesis, gliogenesis, cellular migration, cell differentiation, synapse formation, etc. Several growth factors play indispensable roles to shape the postnatal brain and further neurobiological process. Vascular endothelial growth factor (VEGF) plays a role in neurodevelopment as a neurotrophin and potent growth factor secreted by neurons involved in the regulation of vasculogenesis and vascular function (Takahashi and Shibuya, 2005). VEGF also acts as a signaling protein in the developed central nervous system, functioning in neuroprotection, neuronal survival, axonal outgrowth, long-term potentiation, and learning. Altered VEGF levels are apparent in multiple psychiatric disorders. With respect to autism, serum levels of VEGF and its receptors were found to be altered in patients with severe autism.

Here, we performed an analysis that integrates genetic variation, RNA expression, and protein expression measured from the frontopolar cortex (Brodmann Area 10) of individuals with ASD and matched controls. Overall, we found converging evidence for increased VEGF expression in the frontopolar cortex of individuals with ASD.

Samples & Methods

Samples

Brain tissues were obtained through the Autism Tissue Program. They were dissected postmortem from the frontopolar cortex (BA10) of individuals with ASD (*n*=16) and controls (*n*=14). Subjects were predominately male (26M:4F), white (27 white, 3 African-American), and ranged in age from 2-57 years old. RNA integrity was measured prior to RNA-Seq analysis and all samples had RIN > 6.

RNA-Seq Analysis

RNA-seq libraries were constructed from poly-A selected RNA isolated from frontopolar cortex of ASD samples and controls and sequenced on the Ion Proton System at the Cedars Sinai Genomics Core. We performed pseudo-alignment with kallisto to obtain normalized gene-level expression values and tested for differential expression with sleuth.

Cytokine & Chemokine Protein Expression in Brain

Brain tissues were homogenized and cytokine levels were measured from filtered supernatants using the Milliplex MAP Human Cytokine/Chemokine Magnetic Bead Panel, which includes 41 different cytokines and chemokines. Homogenates were thawed at room temperature and run diluted with PBS at 1:5. Manufacturer recommended assay protocol was followed, with tissue homogenates incubated in the plate for 12 h at 4 °C. Tissue analyte concentrations were normalized to total protein concentrations of homogenate supernatant for each sample, and presented as pg/ml protein homogenate. We excluded all analytes below the level of detection, according to their respective standard curves.

Quantiative PCR

Total RNA was extracted using Trizol and purified using a Qiagen Rneasy Mini Kit, with on-column DNase treatment. cDNA was synthesized from 500ng of total RNA with SuperScript IV, using random hexamers and oligo-dT. Quantiative PCR (qPCR) was carried out using the QuantStudio 3 System, with either custom primers and PowerSYBR or TaqMan probes and NEB Luna Universal Probe qPCR Master Mix. Reactions were run in triplicate in three independent experiments. The geometric mean of cycle threshold values (C_T) for the housekeeping gene *ACTB* was used as an internal control to normalize the variability, and expression values are reported as C_T differences from *ACTB* (Δ*ACTB*).

Weighted Gene Co-expression Network Analysis (WGCNA)

We performed WGCNA using the top 5000 differentially expressed genes from the RNA-seq experiment, with a minimum module size of 30. We correlated module eigengenes with demographic factors and cytokine/chemokine levels, to identify co-expression modules associated with cytokine/chemokine expression.

Ingenuity Pathway Analysis (IPA)

We performed IPA on co-expression networks identified by WGCNA, primarily focusing on networks that are significantly correlated with cytokine/chemokine protein expression.

Cytokine & Chemokine Quantitative Trait Loci (QTLs)

Genome-wide genotyping was performed on the brain tissues, using the Omni5Exome BeadChip array. We extracted genotypes from genes (±100kb) whose expression was significantly associated with VEGF, according to our WGCNA analysis. We then performed QTL analysis using plink, to identify polymorphisms that influence VEGF expression.

Results

RNA-Seq reveals multiple differentially expressed genes. qPCR validation supports greater expression of *VEGFA* and *ADGRG1* in ASD.

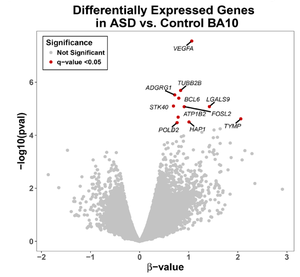

Fig 1: Eleven genes are significantly upregulated in frontopolar cortex (BA10) brain tissues from individuals on the autism spectrum, according to RNA-seq analysis. Note: A 1-point change in β-value is approximately a 2-fold difference in gene expression across diagnosis.

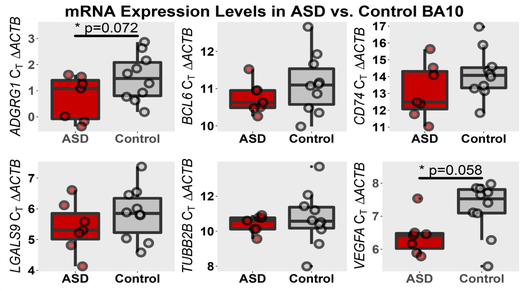

Fig 2: ADGRG1 and VEGFA gene expression in the frontopolar cortex (BA10), as measured by qPCR, is consistent with RNA-seq. ASD samples express approximately 2-fold more VEGFA and 1.5-fold more ADGRG1 than control samples and the comparisons for both genes across diagnosis show a trend towards significance. The remaining genes, while consistent with the changes in expression observed via RNA-seq, were not significant when compared across diagnosis. Note: Gene expression is normalized to β-actin (ACTB), with lower numbers indicative of higher expression. Abbrv: ASD = Autism Spectrum Disorder, BA10 = Brodmann Area 10, C_T = cycle threshold.

VEGF protein levels are higher in ASD frontopolar cortex *versus* controls. WGCNA finds co-expression modules associated with VEGF

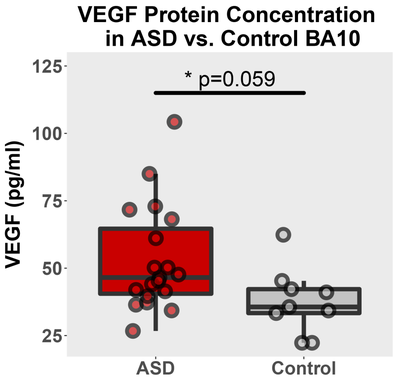

Fig 3: VEGF protein, as measured by the Milliplex MAP Human Cytokine/Chemokine panel, shows a trend towards higher expression in the frontopolor cortex of ASD samples versus Controls (p=0.059). This is consistent with the differences in VEGFA RNA expression, as measured by RNA-seq and qPCR.

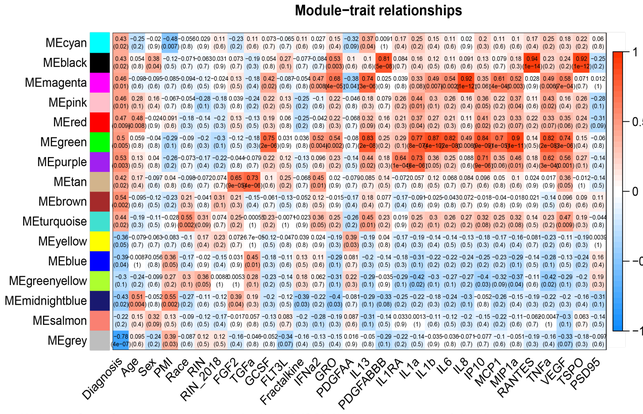

Fig 4: Weighted Gene Co-expression Network Analysis (WGCNA) shows that many of the chemokine and cytokine analyte levels are significantly positively associated with the "green" co-expression module. Three of the cytokines not association with the green module, including PDGF-AA, RANTES, and TSPO, are associated with the "black" co-expression module. Note: Values inside of the boxes represent correlation between the module eigengene values and the respective traits, with red representing positive correlations and blue representing negative correlations.

The WGCNA "green" co-expression network correlated with VEGF is consistent with VEGFA pathway activation. A genetic variant upstream of *METRNL* is associated with higher VEGF protein concentrations in the frontopolar cortex.

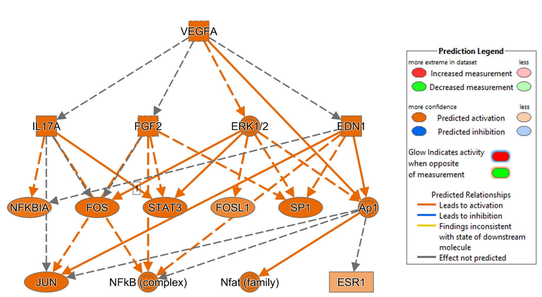

Fig 5: Ingenuity Pathway Analysis (IPA) using RNA-seq expression values of genes in the "green" WGCNA module, which was associated with ASD diagnosis, identifies an expression network where VEGF is a significant upstream regulator ($p=9.22x10^{-11}$). All VEGF pathway genes are activated in a manner consistent with their predicted activation status according to IPA.

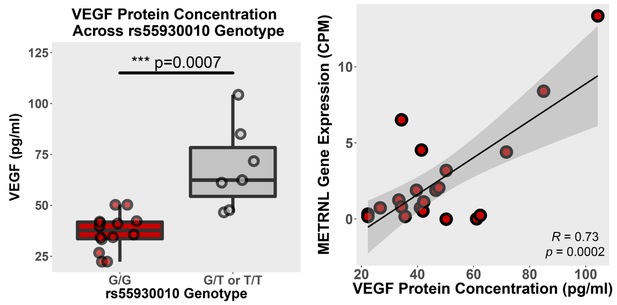

Fig 6: A. VEGF protein expression in the frontopolar cortex (BA10) significantly differ across rs55930010 genotype. Minor allele carriers of rs55930010 (G/T or T/T genotype) express significantly more VEGF protein (p=0.0007), relative to major allele homozygotes (G/G). B. Expression of the METRNL gene, according to RNA-Seq, is significantly correlated with VEGF protein levels, consistent with WGCNA results.

Discussion

VEGF expression, measured by RNA-seq, qPCR, and immunoassay is increased in ASD frontopolar cortex relative to controls. Furthermore, gene co-expression and pathway analysis support the upregulation of a network of genes consistent with VEGF activation and neuroinflammation, more generally. The role of VEGF in neurodevelopment, specifically as it relates to neurogenesis and neuronal survival, is significant in the context of ASD. Neuroanatomical studies reveal accelerated growth of the frontal cortex and overall frontal lobe volume early in development, with a greater number of neurons in these brain regions, relative to typically-developed controls. Neuroanatomical studies in other psychiatric disorders reveal an inverse relationship between serum VEGF and brain volume, and previous studies in ASD found decreases VEGF levels in the serum. However, the relationship between supernatant brain VEGF levels, peripheral VEGF levels, and neuroanatomical features is still emerging. We found a possible relationship between VEGF protein levels and a SNP upstream of *METRNL*. *METRNL* is structurally related to meteorin, a molecule that regulates glial cell development and has roles in neuroinflammation, angiogenesis, and pre-eclampsia.

Conclusions

- **Frontopolar cortex from individuals with ASD exhibit increased VEGF levels, relative to typically developed controls**
- **Gene expression profiles are consistent with increased VEGF signaling and neuroinflammation.**
- **A polymorphism upstream of *METRNL* could affect VEGF levels in the frontopolar cortex.**

References

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