

Short Communication

Integrative Pediatrics and Child Care

Decreased Phosphorylated ERK 1/2 in Individuals with Autism

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Received date: March 24, 2019; Accepted date: April 10, 2019; Published date: April 18, 2019

Abstract

Autism spectrum disorders (ASDs) are complex, highly heritable neurodevelopmental disorders affecting ~1 in 60-100 children. The extracellular signal-regulated kinases, ERK1 and ERK2, are central elements of one of the most prominent intracellular signaling cascades, the mitogen activated protein kinase (MAPK) pathway. They are genetically linked to ASDs and other syndromes typified by intellectual disability. In this study, we measured the concentration of phosphorylated (activated) ERK 1 and 2. We present evidence that ERK is decreased in individuals with autism, and that ERK levels are associated with decreased Epidermal Growth Factor Receptor (EGFR).

Keywords: ERK, Autism, EGFR, Kinases, Phosphorylated

Introduction

Growth factors, through receptor tyrosine kinases, recruit a large network of signaling proteins to execute their cellular programs. The first of these networks to be discovered was the Ras-Raf-ERK signal transduction cascade, defined by Extracellular Signal-regulated Kinase-1 (ERK1) and ERK2 [1]. During growth factor stimulation, the ERK phosphorylation cascade is linked to cell surface receptor tyrosine kinases (RTKs) (11). ERK1 and ERK2 regulate transcription indirectly by phosphorylating the 90 kDa Ribosomal Protein S6 Kinases (RSKs), a family of broadly expressed serine/threonine kinases activated in response to mitogenic stimuli, including growth factors [2].

Autism spectrum disorders (ASDs) are complex, highly heritable neurodevelopmental disorders affecting ~1 in 60-100 children. Copy number variations (CNVs) and other chromosomal rearrangements are associated with ~10-20% of ASDs. CNV of human chromosome *16p11.2* is one of the most common genetic linkages to autism and

deletion of this region accounts for ~1% of ASDs [3], demonstrated by the fact that individuals heterozygous for the *16p11.2* deletion exhibit a range of clinical symptoms including ASD, language impairment, intellectual disability (ID), anxiety, attention deficit hyperactivity disorder and epilepsy [4-6].

The extracellular signal-regulated kinases, ERK1 and ERK2, are central elements of one of the most prominent intracellular signaling cascades, the mitogen activated protein kinase (MAPK) pathway. The ERKs play critical roles in brain development and synaptic plasticity [7] and are activated in response to a broad range of stimuli including growth factors, neurotransmitters, morphogens and transient increases in synaptic calcium [8]. Importantly, they are genetically linked to ASDs and other syndromes typified by intellectual disability [9,10], and mutations in elements of the ERK/MAPK pathway alter the activity of the ERKs, resulting in a group of genetic disorders collectively known as "RASopathies". These syndromes are typified by intellectual disabilities, developmental and

language deficits, ASD, and psychiatric disease [11,12].

In this study, we measured phosphorylated ERK 1 and 2 in autistic individuals and neurotypical controls. We compared these concentrations with concentrations of other intracellular proteins and with the level of symptom severity in the same individuals.

This data suggests that decreased ERK levels are associated with autism and involvement of ERK may be related to EGF/EGFR signaling.

Materials and Methods

Subjects

Cellular phosphorylated ERK 1 and 2 concentrations was measured in 41 autistic children and 12 age and gender similar neurotypical, controls.

The diagnostic criteria used in this study were defined by DSM-IV criteria. In 2012, the separate diagnostic labels of Autistic Disorder, Asperger's Disorder, and Pervasive Developmental Disorder-not otherwise specified (PDD-NOS) were replaced by one umbrella termed "Autism Spectrum Disorder".

Plasma and white blood cells from consecutive individuals with diagnosed autism ($n=41$; 27 male; mean age 10.5 years) and controls ($n=16$; 12 male; mean age 9.3 years) were obtained from patients presenting at the Health Research Institute (HRI)* over a two year period. All autistic individuals who presented to HRI were asked to participate, and patients who participated in this study were randomly chosen from all patients who volunteered. Neurotypical control plasma was obtained from HRI and the Autism Genetic Resource Exchange (AGRE)** and randomly chosen from a selection of about 200 samples. The autistic individuals in this study met the DSM-IV criteria and many were diagnosed using The Autism Diagnostic Interview-Revised - ADI-R before presenting to the HRI.

Patient consent was obtained from all patients involved in this study and this study was approved by the IRB of the HRI.

Cellular phosphorylated ERK 1 and 2 concentrations was measured using an ELISA.

ELISA

To measure cellular phosphorylated ERK 1 and 2

(eBiosciences, San Diego, CA)

1. 50 μ L/well of 1X Cell Lysis Mix (negative control) and 50 μ L/well Positive Control Cell Lysate (positive control) to separate assay wells for controls.
2. 40 μ l of lysis buffer (contains a combination of detergents, phosphatase inhibitors, salts and buffers) was added to each of the control and experimental wells.
3. 10 μ l of buffy coat cells (experimental and controls) were added to appropriate wells and mixed gently.
4. 50 μ L/well of Antibody Cocktail mix (detection antibody and HRP conjugated antibody) was added to all the assay test wells. The plate was incubated for 1 hr at room temperature on a microplate shaker (~300 rpm).
5. Wells were washed with 300 μ L/well 1X Wash Buffer 4 times.
6. 100 μ L of Detection Reagent (TMB) was added to each well and the wells were incubated for 10-30 minutes.
7. After colour development, 100 μ L of Stop Solution was added to each well.
8. Absorbance was measured using a colorimetric (spectrophotometric) plate reader (BioRad) set at 450 nm.

To ensure reproducibility of results, samples were run in duplicate and reported concentrations were the result of the average of at least two separate assays.

Buffy coat white blood cells

All experimental and control cells were separated from whole blood using centrifugation and were treated in an identical fashion-refrigerated (4 C) immediately after collection and cell/serum separation, then used within 4 hours for inductively-coupled plasma-mass spectrometry for zinc concentration determination. Frozen buffy coat was placed at -70C and used for ELISAs within 6 months of retrieval.

Statistics

Inferential statistics were derived from t-test with 95% confidence intervals.

Severity of disease

An autism symptom severity questionnaire was used to evaluate symptoms. The questionnaire (HRI Questionnaire) asked parents or caregivers to assess the severity of the following symptoms: Awareness, Expressive

Language, Receptive Language, (Conversational) Pragmatic Language, Focus, Attention, Hyperactivity, Impulsivity, Perseveration, Fine Motor Skills, Gross Motor Skills, Hypotonia (low muscle tone), Tip Toeing, Rocking/Pacing, Stimming, Obsessions/Fixations, Eye Contact, Sound Sensitivity, Light Sensitivity, and Tactile Sensitivity. The symptoms were rated by parents/guardians on a scale of 0-5 (5 being the highest severity) for each of these behaviors.

**The Health Research Center (HRI) is a comprehensive treatment and research center, specializing in the care of with neurological disorders, including Depression.*

Results

We used an ELISA to measure ERK 1 and 2 in individuals with autism (N=41) and age and gender similar neurotypical controls (N=16). We found ERK levels to be significantly decreased in these individuals compared to controls ($p=0.007$) (Figure 1).

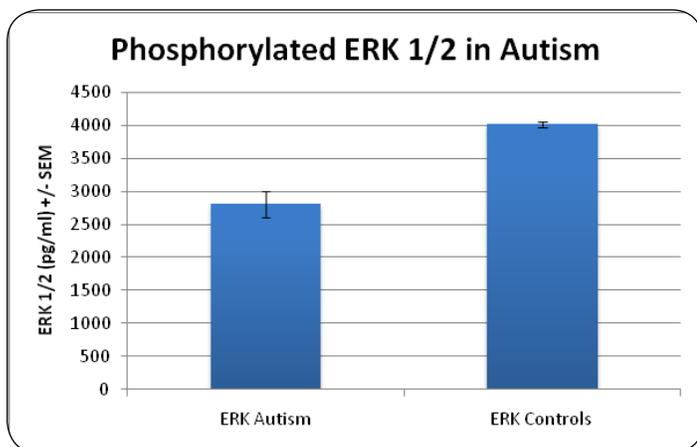


Figure 1: Phosphorylated ERK levels are significantly lower in individuals with autism (2806 +/- 197 pg/ μ l) compared to neurotypical controls (4012 +/- 46 pg/ μ l)

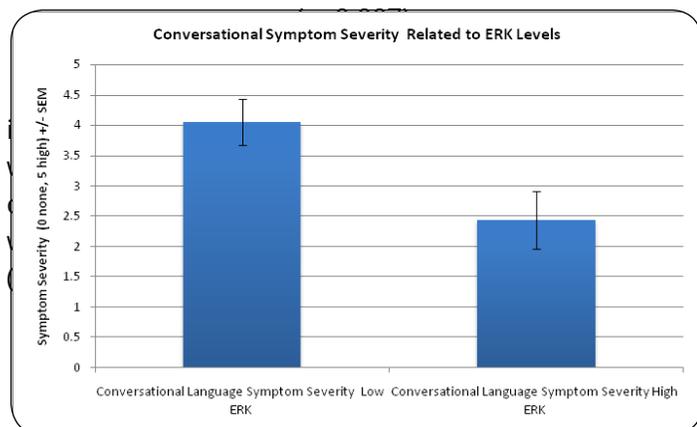


Figure 2: Individuals with autism with Low ERK (less than 2000 pg/ μ l) had a mean conversational symptom

severity of 4.06 +/- 0.38, compared to those with high ERK (greater than 2000 pg/ μ l) (mean symptom severity of 2.433 +/- 0.48) ($p=0.027$).

We also compared the ERK concentrations of individuals with autism with levels of other intracellular biomarkers. We found that there was a negative correlation between the phosphorylated ERK 1 and 2 levels and Epidermal Growth Factor Receptor (EGFR) ($r=-0.45$; $p=0.04$).

Discussion

ERK MAP kinases are important for normal cortical development and function and are genetically linked to intellectual disabilities, including disorders of cognition and CNVs [13,14].

Microdeletion of a region in chromosome 16p11.2, increases susceptibility to autism spectrum disorder (ASD) and accounts for up to 1% of the ASD population [15]. Patients carrying this microdeletion exhibit cognitive disability, language delay, ASD, and seizures [16]. Deletion of the entire 16p11.2 region, containing the ERK genes, in mice leads to diverse phenotypes, including hyperactivity, deficits in contextual conditioning and novel object recognition [17].

Loss of the 16p11.2 loci results in intellectual disability, enlarged head, obesity and, often, autism. This region spans 27 genes - including the ERK1 gene. Studies have shown a paradoxical increase in ERK signaling in mid-neurogenesis in 16p11.2 del mice, which is coincident with the development of aberrant cortical cyto-architecture. The 16p11.2 del mice exhibit anxiety-like behaviors and impaired memory [6,18]. We suggest that ERK levels become decreased in the adults because of altered EGFR signaling.

ERK2 plays a critical role in regulating social behaviors as well as cognitive and emotional behaviors. Our results demonstrate that ERK levels are lower in our autistic group and that these decreased levels are associated with conversational symptoms. We also found a reverse correlation of ERK with EGFR in the autistic group. We suggest that therapies designed to raise ERK levels, and/or lower EGFR levels in autistic individuals may improve these behavioral symptoms.

Funding

This work was funded, in part, by the Autism Research Institute.

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