Increased Epidermal Growth Factor Receptor (EGFR) Associated with Hepatocyte Growth Factor (HGF) and Symptom Severity in Children with Autism Spectrum Disorders (ASDs)

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ABSTRACT

BACKGROUND: One in 88 children in the US is thought to have one of the autism spectrum disorders (ASDs). ASDs are characterized by social impairments and communication problems. Growth factors and their receptors may play a role in the etiology of ASDs. Research has shown that epidermal growth factor receptor (EGFR) activation is associated with nerve cell development and repair. This study was designed to measure plasma levels of EGFR in autistic children and correlate these levels with its ligand, epidermal growth factor, other related putative biomarkers such as hepatocyte growth factor (HGF), the ligand for MET (MNNG HOS transforming gene) receptor, as well as the symptom severity of 19 different behavioral symptoms.

SUBJECTS AND METHODS: Plasma EGFR concentration was measured in 33 autistic children and 34 age- and gender-similar neurotypical controls, using an enzyme-linked immunosorbent assay. Plasma EGFR levels were compared to putative biomarkers known to be associated with EGFR and MET and severity levels of 19 autism-related symptoms.

RESULTS: We found plasma EGFR levels significantly higher in autistic children, when compared to neurotypical controls. EGFR levels correlated with HGF and high-mobility group protein B1 (HMGB1) levels, but not other tested putative biomarkers, and EGFR levels correlated significantly with severity of expressive language, conversational language, focus/attention, hyperactivity, eye contact, and sound sensitivity deficiencies.

CONCLUSIONS: These results suggest a relationship between increased plasma EGFR levels and designated symptom severity in autistic children. A strong correlation between plasma EGFR and HGF and HMGB1 suggests that increased EGFR levels may be associated with the HGF/Met signaling pathway, as well as inflammation.

KEYWORDS: EGFR, EGF, HGF, HMGB1, autism, symptom severity

Introduction

Autism spectrum disorders (ASDs) comprise a diverse group of conditions characterized by problems in social actions, communication deficits, and stereotypical repetitive behaviors. One in 88 children in the US is thought to have ASD.[1]

The epidermal growth factor receptor (EGFR; ErbB-1; HER1 in humans) is a member of the ErbB family of receptors, and a member of a subfamily of four closely related receptor tyrosine kinases (RTKs): EGFR (ErbB-1), HER2/c-neu (ErbB-2), Her 3 (ErbB-3), and Her 4 (ErbB-4). These cell-surface receptors are members of the epidermal growth factor (EGF) family of extracellular protein ligands.[2] EGFR is activated by binding to its specific ligands, including EGF and transforming growth factor α. Upon activation, EGFR changes to a homodimer,[3] which stimulates its intrinsic intracellular protein-tyrosine kinase activity.[4] This autophosphorylation

elicits downstream activation of several signal transduction cascades, principally the MAPK/ERK (mitogen activated kinases/extracellular signal regulated kinases), Akt and JNK pathways, leading to DNA synthesis and cell proliferation.[5]

EGFR is important in nerve cell development and repair. Activation of EGFR is a common, regulatory pathway that triggers quiescent astrocytes into reactive astrocytes in response to neural injuries in the optic nerve, and perhaps other parts of the CNS.[6] An EGFR inhibitor, erlotinib, has shown delayed disease progression in a mouse model of ALS.[7] Alzheimer's-like memory loss has been reversed in animal models by blocking EGFR signaling.[8] Inhibition of EGFR/MAPK suppresses microglia activation and associated cytokine production. This reduces neuroinflammation-associated secondary damage, thus providing neuroprotection to spinal cord injury rats.[9] Recently, research has demonstrated that EGFR is a preferred target for treating Amyloid-β–induced memory loss.[9]

Genetic variants of RTKs, such as MET, have been implicated in the etiology of autism.[10,11] Growth factors, hepatocyte growth factor (HGF) and EGF, which are the signaling ligands for MET and EGFR, respectively, are decreased in autism.[12,13]

Attachment of growth factors to their receptors regulates many aspects of neuronal growth and differentiation. Its signaling also is in part responsible for neuronal survival, migration, and synaptic signaling.[14] These growth factors also act as immune modulators.[15–18] Since there is immune dysfunction in the nervous system[19–22] in children with autism, it is plausible to think that growth factors are involved.

EGF helps in controlling cell division and differentiation of nervous tissue[23,24] and has been found to promote wound healing.[25]

EGF concentration and gene mutation has been linked to autism. Frequency of EGF single-nucleotide polymorphisms is in children with autism.[26] Plasma EGF levels in adults[27] and children with autism[11,28] were found to be significantly decreased. However, in one report of younger autistic children, EGF was increased.[29]

High-mobility group protein B1 (HMGB1) is a marker of inflammatory diseases. It acts by binding to lipopolysaccharides and IL-1 (Interleukin-1), to initiate Toll-like receptor 4–mediated inflammation[30] and then is released from activated macrophages. HMGB1 is dependent on various processes such as phosphorylation by calcium-dependent protein kinase C,[31] acetylation, and methylation[32] and was found to be associated with the generation and recurrence of seizures in experimental animals.[33,34] This inflammatory marker is increased in autistic children,[35] and in neural tissue and malignant cells, and receptor activation by HMGB1 leads to MAPK activation with increased cell growth.[36]

Because several studies have associated abnormal levels of EGF and HGF with autism, and the EGF/EGFR signaling pathway plays a role in regulating neural growth, proliferation, differentiation, and migration, it is important to continue studying the elements of this pathway to determine if abnormal levels are associated with abnormal neurodevelopment and symptoms in individuals with autism.

This study was designed to determine plasma levels of EGFR in children with autism and evaluate the correlation of these EGFR levels with inflammatory and regulatory biomarkers, as well as severity of 19 different symptoms related to autism.

Materials and Methods

Enzyme-linked immunosorbent assays (ELISAs) were used to measure plasma EGFR and other biomarkers (ELISA kits, R and D Systems, Minneapolis, MN, and USCN Life Sciences, Wuhan, China). The ELISA and serum protocols have been previously reported and are discussed below.[37]

All reagents and specimens were equilibrated to room temperature before each assay was performed. A 1:51 dilution of the patient samples was prepared by mixing 10 µL of the patient's plasma with 0.5 mL of plasma diluent. One hundred microliters of calibrators (20–200 Eu/mL antibodies), positive and negative control plasma, plasma diluent alone, and diluted patient samples were added to the appropriate microwells of a microculture plate (each well contained affinity-purified polyclonal IgG to the appropriate marker). Wells were incubated for 60 minutes (±5 minutes) at room temperature, and then washed 4× with wash buffer. One hundred microliters of prediluter anti-human IgG conjugated with HRP (horseradish peroxidase) was added to all microwells, incubated for 30 minutes (±5 minutes) at room temperature, and then washed 4× with wash buffer. One hundred microliters of enzyme substrate was added to each microwell. After approximately 30 minutes at room temperature, the reaction was stopped by adding 50 µL of 1 M sulfuric acid, and then the wells were read at 405 nm with an ELISA reader (BioRad Laboratories, Inc., Hercules, CA, USA).

Serums. All serums, experimental and control serums, were treated in an identical manner, frozen at −70 °C immediately after collection and cell/serum separation, and then stored at −70 °C until thawing for use in ELISAs.

Subjects. Plasma and diagnostic criteria (below) have been previously reported.[37] Plasma EGFR concentration was measured in 33 autistic children and 34 age- and gender-similar neurotypical controls.

The diagnostic criteria used in this study were defined by DSM-IV (Diagnostic and Statistical Manual of Mental Disorders) criteria. In 2012, the separate diagnostic labels of autistic disorder, Asperger's disorder, and pervasive developmental disorder-not otherwise specified were replaced by one umbrella termed "Autism Spectrum Disorder."

Plasma from consecutive individuals with diagnosed autism (n = 33; 28 male; mean age 10.3 years) and controls (n = 34; 25 male; mean age 9.6 years) was obtained from patients presenting at the Health Research Institute (HRI, which is a comprehensive treatment and research center, specializing in the care of persons with neurological disorders,

including autism) over a 2-year period. All autistic individuals who presented to HRI were asked to participate. 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, which is a repository of biomaterials and phenotypic and genotypic data to aid research on ASDs) 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 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.

Severity of disease. The Pfeifer questionnaire, severity criteria, and statistical methodology have been previously reported.[37]

An autism symptom severity questionnaire was used to evaluate symptoms. The questionnaire (Pfeiffer 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.

Statistics. Inferential statistics were derived from unpaired t-test and odds ratios with 95% confidence intervals. Pearson moment correlation test was used to establish the degree of correlation between the groups ($r > 0.3$ or < -0.3; $P < 0.1$).

Results

We found that plasma levels of EGFR in children with autism ($m = 13{,}200 \pm 804$ pg/mL) was significantly higher when compared to neurotypical controls ($m = 11{,}312 \pm 369$ pg/mL) ($P = 0.03$) (Fig. 1).

EGFR levels correlated with HGF levels ($r = 0.55$; $P = 0.007$) (Fig. 2) and HMGB1 ($r = 0.42$; $P = 0.003$) (Fig. 3), and increased EGFR plasma levels correlated with severity of symptoms related to expressive language, conversational language, focus/attention, hyperactivity, eye contact, and sound sensitivity (Table 1).

Discussion

The MET and EGFR RTKs when signaled by their ligands, HGF and EGF, respectively, through a cascade of signaling reactions, modulate the ERK and PI3K intracellular regulatory pathways. ERK and PI3K activate mammalian targets, which, through other kinases, increase messenger RNA translation to influence developmental functions as diverse as the cell cycle, cell survival, differentiation, and motility. The
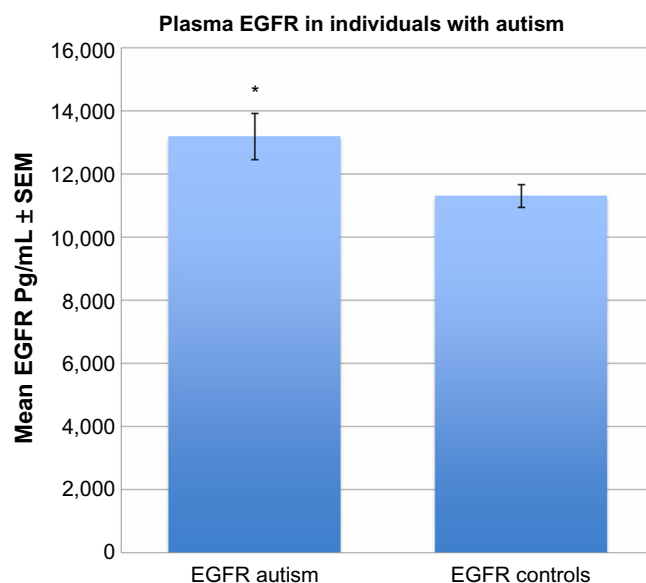
Figure 1. EGFR plasma levels in autistic children (*) are significantly higher than controls ($P = 0.03$).

key genes involved in met proto-oncogene–RTK have been implicated in ASD risk.[10]

Different genetic routes to altered RTKs, such as MET and EGFR, function by way of modulation of ERK/PI3K signaling pathways. It has been proposed that, combined with environmental factors, such as biochemical stressors, they may modulate the degree of dysfunction of the clinical features of autism.[10]
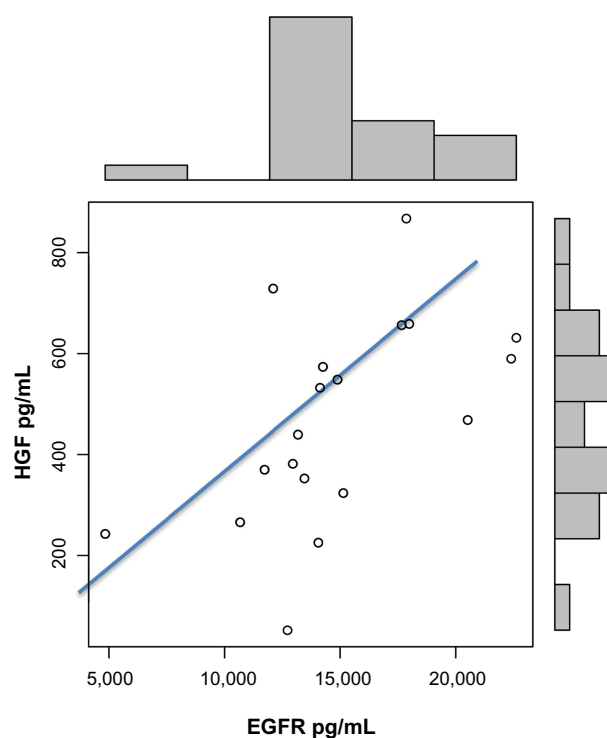
Figure 2. Correlation between EGFR and HGF in autistic children ($r = 0.55$; $P = 0.007$).
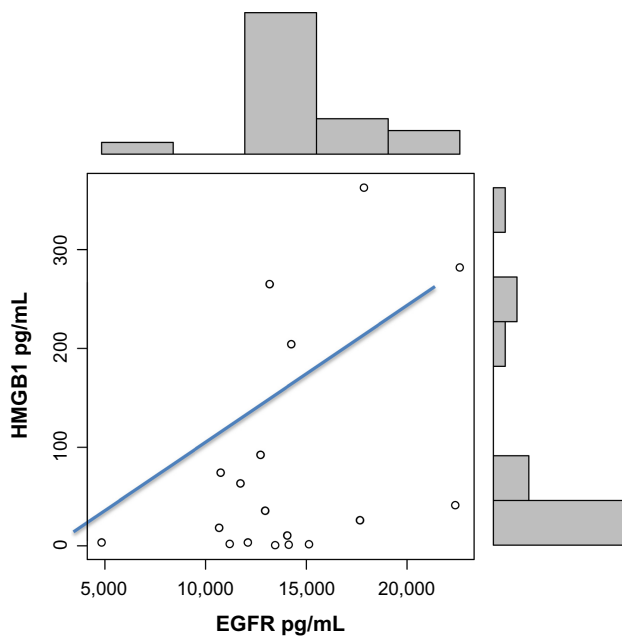
Figure 3. Correlation between EGFR and HMGB1 in autistic children ($r = 0.42$; $P = 0.03$).

Table 1. Correlation between EGFR levels and sympton severity in autistic children.

SYMPTON	SYMPTON SEVERITY CORRELATION WITH EGFR
Expressive language	$r = 0.41$; $P = 0.01$
Conversational language	$r = 0.31$; $P = 0.05$
Focus/Attention	$r = 0.3$; $P = 0.06$
Hyperactivity	$r = 0.32$; $P = 0.04$
Eye contact	$r = 0.4$; $P = 0.02$
Sound sensitivity	$r = 0.41$; $P = 0.01$

Our laboratory has demonstrated that plasma HGF and EGF (the ligands for MET and EGFR, respectively) are significantly decreased in autistic children,[10,11] and in the data reported here, we have found that EGFR is increased. We also found in this study and previous studies that EGF and EGFR levels, but not HGF levels, correlate with symptom severity in autistic children.[10,11]

It is not surprising that RTKs are associated with autism, because other neurobehavioral and mood disorders such as bipolar disorder and schizophrenia are linked to altered markers associated with these pathways.[38–40]

It is plausible that higher EGFR levels are the result of altered RTK suppressor proteins such as mitogen-inducible gene protein (MIG-6). MIG-6 can be induced by HGF and functions as a negative feedback regulator of the MET pathway by inhibiting HGF-induced cell migration and proliferation.[41] Overall, this RTK cascade, initiated by EGFR signaling, functions in cellular proliferation, differentiation, and survival, and its inappropriate activation is a common occurrence in human cancers. Specifically, as an example of the effect of a decreased suppressor protein, low Mig-6 expression is associated with high levels of EGFR and altered ERK phosphorylation in certain cancers.[42]

EGF has been found to play an integral role in nerve cell development,[24,43] as well as intestinal mucosa development.[44–46] The inflammatory marker HMGB1 is increased in autistic children.[35] Our laboratory has previously reported a relationship between decreased EGF and increased HMGB1, as well as hyperactivity-related symptom severity in a group of autistic children.[13]

We also reported significantly decreased HGF in autistic children (mean age of approx. 10 years) with severe GI disease,[12] which did not correlate with symptom severity. Since we previously found that EGF correlated with hyperactivity-related symptoms, it is reasonable to suggest that EGF is more important than HGF as a putative biomarker.

In this study, we found that plasma EGFR was significantly higher in autistic children. EGFR levels correlated with HGF and HMGB1 (as we had found with EGF), although the clustering of these data suggests that there may be more than one population distribution represented, and therefore may require a larger sample size to confirm. We also found that EGFR correlated with high symptom severity in six autism-related behaviors, including hyperactivity.

Our observations of decreased EGF and increased EGFR in autistic children suggest that decreased EGF may be the result of increased ligand binding to its receptor, resulting in decreased plasma/serum EGF. Decreased EGF has been associated with inflammatory conditions such as colitis.[44,45] We did not find a correlation between decreased EGF and EGFR in this patient group. However, both EGF and EGFR correlate with HMGB1, suggesting that their abnormal levels may be associated with inflammation and generally increased neuroimmune activity. Our data suggest that decreased EGF and increased EGFR are associated with higher symptom severity, suggesting that their levels are associated with abnormal behavior and, as a result, their association with the etiology of autism.

In summary, these data support the notion that the EGFR/ERK pathway, associated with cell growth, differentiation, and division, may be associated with the etiology of autism. High EGFR levels are associated with many cancers. These increased levels are, in turn, associated with unregulated cell division. The data reported in this study demonstrate a correlation between increased EGFR and increased severity of autistic symptoms. We also found a correlation between EGFR and HGF and the inflammatory marker, HMGB1. This supports evidence that this (EGFR) RTK pathway, possibly associated with decreased HGF levels and inflammation, is associated with the etiology of autism.

Acknowledgments

Author Contributions

Conceived and designed the experiments: AJR. Analyzed the data: AJR. Wrote the first draft of the manuscript: AJR. Made critical revisions: AJR. The author reviewed and approved of the final manuscript.

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