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Association of EGF +61A/G (rs4444903) Polymorphism and Serum EGF Levels with Autism Spectrum Disorder: A Case–Control Study

Farhad Mashayekhi *, Masoumeh Khalili, Zivar Salehi

Department of Biology, Faculty of Sciences, University of Guilan, Rasht, Iran; E-Mails: mashayekhi@guilan.ac.ir; umistbiology20@gmail.com; sinless.kh@gmail.com; salehiz@guilan.ac.ir

* **Correspondence:** Farhad Mashayekhi; E-Mails: mashayekhi@guilan.ac.ir; umistbiology20@gmail.com

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Abstract

Research has demonstrated the significant role of epidermal growth factor (EGF) in synaptic plasticity and the pathogenesis of Autism spectrum disorder (ASD). This study aimed to assess the correlation between EGF +61A/G (rs4444903) polymorphism and its serum level with ASD. Blood samples were collected from 200 ASD and 200 controls for DNA extraction, and genotyping was carried out using Restriction Fragment Length Polymorphism (RFLP). Additionally, EGF serum concentration was determined using enzyme-linked immunosorbent assay (ELISA). In ASD, the frequencies of GG, AG, and AA genotypes were 15%, 41%, and 44%, respectively, while in controls, they were 5%, 30%, and 65%. The genotypes of rs4444903 demonstrated an influential contribution to the susceptibility to ASD under co-dominant (GG versus AA), recessive (GG versus AA+AG), and dominant (AG+GG versus AA) models. Additionally, the frequencies of A and G alleles were 68.8% and 31.2% in ASD, and 79.8% and 20.2% in controls, respectively ($P < 0.0001$). Furthermore, the G allele was found to be associated with an increased risk of ASD ($P = 0.0001$). Notably, the EGF concentration in the serum samples of the ASD group was lower than in controls (792.65 ± 178.19 and 1265 ± 213.32 pg/ml, respectively; $P = 0.0001$). In ASD patients, the GG genotype is connected to lower serum EGF levels. The serum concentrations for carriers of GG, AG, and AA were measured at 515 ± 109.63, 716.22 ± 102.26, and 886.11 ± 119.69 pg/ml, respectively. The

results of this project suggest that there may be a relationship between the EGF +61A/G (rs4444903) polymorphism and its serum levels with the risk of ASD. Furthermore, the GG genotype seems to be linked to decreased EGF expression in individuals with ASD.

Keywords
Autism spectrum disorder; EGF +61A/G; restriction fragment length polymorphism; enzyme-linked immunosorbent assay

1. Introduction

Autism Spectrum Disorder (ASD) is defined as a neurodevelopmental disorder with the presence of impaired social communication [1]. The incidence of ASD has steadily increased in recent decades. It has been suggested that the increase in ASD occurrence may be partially attributable to diagnostic criteria that evolved before the publication of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), increased public awareness, and mandatory treatment. Additionally, previously unrecognized ASDs are being diagnosed in high-functioning school-aged children [2, 3].

The pathophysiology of ASD is not fully understood. However, genetics and environmental factors are involved in the pathogenesis of ASD [4]. Genetic contributions to ASD arise from a diverse set of mutational mechanisms along many biological pathways [5]. Environmental factors are also related to the risk of ASD. Prenatal risks include the age of parents, and maternal metabolic diseases also play a vital role in ASD. In utero risks include valproic acid (Depacon), maternal infections, and pesticide exposure, which are important in ASD [6].

Single-nucleotide polymorphisms (SNPs) help predict an individual's response to certain drugs, susceptibility to environmental factors such as toxins, and risk of developing diseases [7]. Genetic polymorphisms in many genes, including Neuropilin-2 (NRP2), SH3 and multiple ankyrin repeat domains 3 (SHANK3), Glutathione S-Transferase (GST), matrix metalloproteinase-9 (MMP-9), Contactin-associated protein-like 2 (CNTNAP2) and Insulin-like growth factor 1 (IGF-1), were suggested to be linked with ASD [8-11]. Growth factors, including epidermal growth factor (EGF), have been shown to be involved in mechanisms underlying neuronal dysfunction in ASD [12]. EGF plays a key role in brain development and function and is widely expressed in neurons, astrocytes and oligodendrocytes, where it supports cognitive functions [13]. It was suggested that neural cell survival might depend on EGF signaling [14]. EGF binds to its receptor (EGFR), initiating its action [15]. EGF plays an important role in nervous system development by regulating key functions such as cell differentiation, axonal growth, myelination, and synaptic plasticity. Synaptic plasticity is a key feature of the central nervous system (CNS). It is suggested to be important in physiological processes such as learning and memory, as well as in complex behaviors, including goal-directed behavior [16]. EGF signaling was suggested to be important in brain development [13]. Decreased EGF expression is important in the pathophysiology of ASD [17].

Epidermal growth factor (EGF), through binding to its receptor EGFR, activates intracellular signaling pathways that regulate neural network formation, maintenance, and synaptic plasticity—processes essential for learning, memory, and adaptive behavior. Disruption of synaptic plasticity has been strongly implicated in neurodevelopmental disorders, including ASD [18]. Emerging

evidence indicates that abnormal EGF signaling may contribute to ASD pathogenesis. Several studies have reported altered plasma EGF levels in children with autism, and reduced EGF availability may impair neuronal connectivity, synapse formation, and cortical maturation during critical developmental periods [19]. In addition, dysregulated EGFR signaling may affect glial cell function, myelination, and neuroinflammatory responses, thereby further disturbing communication between brain regions involved in social interaction and cognition [20].

Genetic investigations have also examined polymorphisms in the EGF gene and related signaling pathways as potential ASD risk factors [21], and EGF dysregulation has been associated with greater symptom severity, particularly in language, attention, and social domains [22]. Notably, EGFR is expressed in astrocytes and microglia and contributes to the regulation of inflammatory processes; excessive EGFR-mediated inflammation may therefore exacerbate neurodevelopmental abnormalities. Moreover, altered EGF expression has been reported in other neuropsychiatric disorders characterized by impaired neuroplasticity, such as schizophrenia, bipolar disorder, and major depressive disorder [23]. Collectively, these findings support the hypothesis that dysregulation of EGF-mediated neurodevelopmental mechanisms plays a role in ASD etiology and may represent a potential therapeutic target.

Gene variation has been reported to be important in disease susceptibility. As EGF is critical in synapse formation and pathogenesis of Autism [24], and the EGF +61A/G (rs4444903) variant is important in regulating the EGF expression [25], this project aimed to study the association of the EGF +61A/G (rs4444903) genetic variant and its blood levels with ASD.

2. Methods

Two hundred ASD patients (171 boys and 29 girls) 2-7 years of age (6.23 ± 2.91 years) and 200 control subjects (169 boys and 31 girls) 2-7 years age (5.92 ± 2.98 years) (P = 0.98) was included in this study. Normal subjects were also examined to exclude neurological disorders. DSM-5 criteria for ASD were used to diagnose patients with ASD [26]. Children who attended the Sharivar Hospital in Rasht, Iran, for routine check-ups and had no history or diagnosis of ASD were also requested by their parents or guardians to contribute to the project by donating blood. These children were regarded as the control group. Informed consent was obtained from the parents or guardians after approval of the experimental protocol by the Graduate Education Council of the Faculty of Sciences of the University of Guilan under number 41424. The procedures followed were in accordance with the responsible ethical standards of the institutional or regional committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 1983.

Inclusion criteria were: 1. children aged 2-7 years; 2. meeting DSM-5 criteria for ASD; 3. no medication or psychotropic drug use for at least 6 months; 4. Psychiatric disorders, neurological disorders, metabolic disorders or infectious diseases, malignancies, hepatic or renal dysfunction, and routine medications with no history or current diagnosis. Genetic or other known causes of autism were also ruled out. The control group comprised 200 children aged 2-7 years who were age-matched to the patient group. They were all evaluated by a Neurologist and had no history or current diagnosis of physical or psychiatric disorders. Medicated subjects were excluded from the control group.

Peripheral blood was used to extract total genomic DNA using GPP Solution Kit (Gen Pajoohan Pouya, Tehran, Iran) in accordance with the manufacturer's instructions. The primer sequences are shown in Table 1.

Table 1 The sequence of forward and reverse primers.

Gene symbol	Primer sequence (5'→3')
EGF	EGF-F: 5'TCCTCTTTGGCAGTCATCCC3'
	EGF-R: 5'CATTTCCTGCGAGAGTACCTT3'

EGF - Epidermal growth factor.

Primers were designed using Oligo Primer Analysis software (Molecular Biology Insights Inc., USA), based on nucleotide sequences from the Ensembl genome database. Polymerase chain reaction (PCR) amplification was done in a 20 µL reaction volume comprising 5 µL DNA, PCR master mix (Pishgam, Iran), 3 µL distilled water and 1 µL of both primers with the following PCR cycling program: initial denaturation at 94°C for 5 min, then repeated for 35 cycles, denaturation at 94°C for 45 sec, annealing at 57°C for 45 sec, extension at 72°C for 45 sec, and 72°C for 5 minutes and final elongation step of 72°C for 5 minutes. The restriction enzyme (*CspCI*) (New England Biolabs Inc, England) was used to digest the 708 bp DNA fragment, which generated 196 bp and 477 bp fragments. A 2% agarose gel was used to separate DNA fragments. Genotyping was performed and interpreted independently by two investigators at the Biology Laboratory of Guilan University.

To minimize pre-analytical and batch-related variability, peripheral blood samples from both ASD patients and control subjects were collected via standard venipuncture during routine morning clinical visits and processed promptly under standardized conditions. Serum was separated by centrifugation at 3000 rpm for 15 minutes, immediately aliquoted into microcentrifuge tubes, and stored at -80°C until analysis to avoid repeated freeze–thaw cycles. Serum EGF concentrations were determined using a commercial Human EGF ELISA Kit (ab217772; Abcam, UK) according to the manufacturer's instructions. Briefly, a standard curve was generated using serial dilutions of the provided standards across the recommended concentration range, and all samples and standards were analyzed in duplicate wells to minimize technical variability. Mean replicate values were utilized for statistical analysis, with appropriate sample dilution factors applied as needed to ensure measured concentrations fell within the linear range of the assay. Assay accuracy and reliability were strictly monitored, with both intra-assay and inter-assay coefficients of variation (CVs) maintained below 10%, ensuring high reproducibility and comparable experimental conditions across all cases and controls.

The required sample size was calculated using the OSSE software (www.osse.bii.a-star.edu.sg) based on the MAF of SNPs. In this study, there was 92.2% power to determine the relation of the 61A/G (rs4444903) SNP and ASD Risk. Analysis was performed using χ^2 by MedCalc Software v23.2.0 (Belgium). To evaluate deviations between observed and expected genotype frequencies in both the ASD and normal groups, the Hardy-Weinberg equilibrium was calculated by the Oege online server.

3. Results

This research contains 200 children with ASD and 200 controls (Table 2). Genotyping of +61A/G of EGF was done in all subjects enrolled in this project. The observed genotype frequencies in ASD and controls were in the Hardy-Weinberg equilibrium (χ^2 = 2.19, P = 0.13; χ^2 = 0.78, P = 0.37, respectively). The prevalence of EGF GG, AG, and AA genotypes in ASD children was 15%, 41%, and 44%, while in healthy controls it was 5%, 30%, and 65%, respectively (Table 3). In addition, genotypes of EGF 61A>G under co-dominant (GG versus AA), recessive (GG versus AA+AG), dominant (AG+GG versus AA) models have influential contribution to the susceptibility to ASD (OR = 4.43, CI = 2.06-9.52; P = 0.0001; OR = 3.35, CI = 1.59-7.06; P = 0.001; 2.36, CI = 1.57-3.53; P < 0.0001) (Table 1). Furthermore, the frequencies of the A and G alleles were 68.8% and 31.2% in ASD patients and 79.8% and 20.2% in the control group. We also showed a significant change in allele frequencies between the ASD and control groups (P < 0.0001). Furthermore, we showed that the G allele was associated with the risk of ASD (OR = 1.79, CI = 1.34-2.39, P = 0.0001) (Table 3).

Table 2 Characteristics of the study samples.

Variables	Cases (n = 200)	Controls (n = 200)	P value
Gender			
Male	171 (85.5%)	169 (84.5%)	0.78
Female	29 (14.5%)	31 (15.5%)	
Age (Range, years)	2-7	2-7	
Mean ± SD	(6.23 ± 2.91)	(5.92 ± 2.98)	0.18
Maternal age at conception (years)	28.77 ± 5.65	26.9 ± 4.80	0.09
Maternal diabetes	12 (6%)	7 (3.5%)	0.29
Immune system disorder during pregnancy	5 (2.5%)	3 (1.5%)	0.72
Family history of ASD			
Positive	8 (4%)	0	0.07
Negative	192 (96%)	200 (100%)	
Birth complications	32 (16%)	21 (10.5%)	0.10
Low birth weight	19 (9.5%)	4 (2%)	0.08
Gestational age (weeks)			
<37	18 (9%)	12 (6%)	
37-42	169 (84.5%)	179 (89.5%)	0.41
>42	13 (6.5%)	9 (4.5%)	

Table 3 Genotype and allele frequencies of epidermal growth factor (EGF) (rs4444903) gene polymorphism in ASD patients and controls.

Genetic model	Genotype	ASD n (%)	Controls n (%)	OR[a] (95% CI, P value)	OR[b] (95% CI, P value)
Codominance	AA	88 (44)	130 (65)	1.00	1.00
	AG	82 (41)	60 (30)	2.01 (1.31-3.10, 0.001)	1.97 (1.30-2.96, 0.0012)
	GG	30 (15)	10 (5)	4.43 (2.06-9.52, 0.0001)	4.46 (2.10-10.05, 0.0001)
Dominance	AA	88 (44)	130 (65)	1.00	1.00
	GG+AG	112 (56)	70 (35)	2.36 (1.57-3.53, 0.0001)	2.29 (1.46-3.59, 0.0001)
Recessive	AA+AG	170 (85)	190 (95)	1.00	1.00
	GG	30 (15)	10 (5)	3.35 (1.59-7.06, 0.001)	3.31 (1.55-7.01, 0.0011)
Overdominance	GG+AA	118 (59)	140 (70)	1.00	1.00
	AG	82 (41)	60 (30)	1.62 (1.07-2.45, 0.02)	1.68 (1.12-2.49, 0.017)
Allele	A	344 (68.8)	399 (79.8)	1.00	1.00
	G	156 (31.2)	101 (20.2)	1.79 (1.34-2.39, 0.0001)	1.63 (1.11-2.28, 0.0001)

ASD - Autism Spectrum Disorder; OR - Odds Ratio; CI - Confidence Interval.

[a] crude, [b] adjusted odds ratio.

Serum EGF levels were measured by ELISA. Serum samples from the ASD group were shown to have lower EGF levels than the control group. The mean EGF serum levels in the ASD and the control group were 792.65 ± 178.19 pg/ml and 1265 ± 213.32 pg/ml, respectively (Figure 1). The results showed that serum EGF concentration was significantly reduced in the ASD children compared to normal subjects (P = 0.0001).

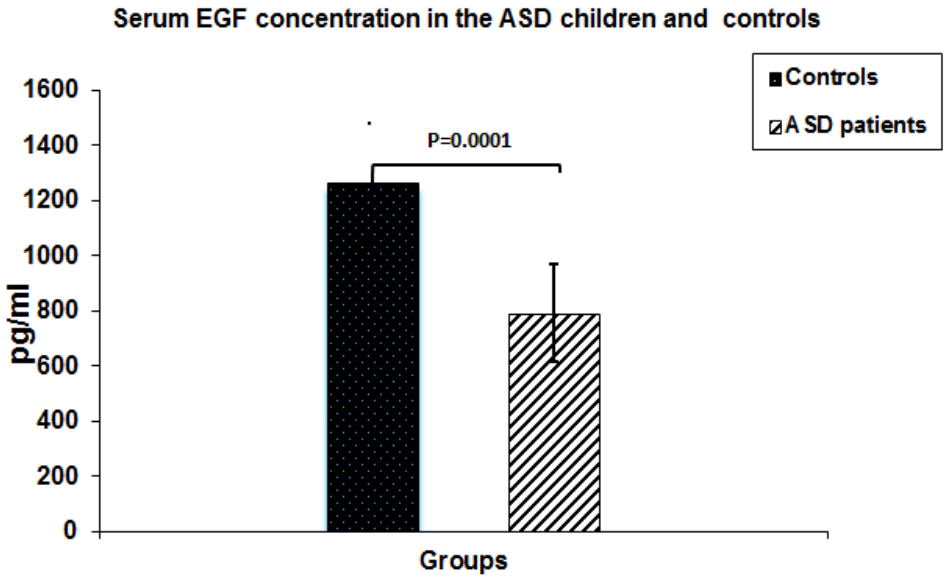

Figure 1 EGF serum level in the patients with ASD (792.65 ± 178.19 pg/ml) and control group (1265 ± 213.32 pg/ml). A significant decrease in serum EGF concentration was seen in the ASD serum samples as compared with the control group (P = 0.0001).

We also showed that the GG genotype was significantly linked to lower serum EGF levels in ASD (serum concentrations of GG, AG and AA carriers were 515 ± 109.63, 716.22 ± 102.26 and 886.11 ± 119.69, respectively) (Figure 2), and in control group were 757 ± 187.40, 1057.88 ± 191.68, and 1335.77 ± 199.04 pg/ml, respectively (Figure 3).

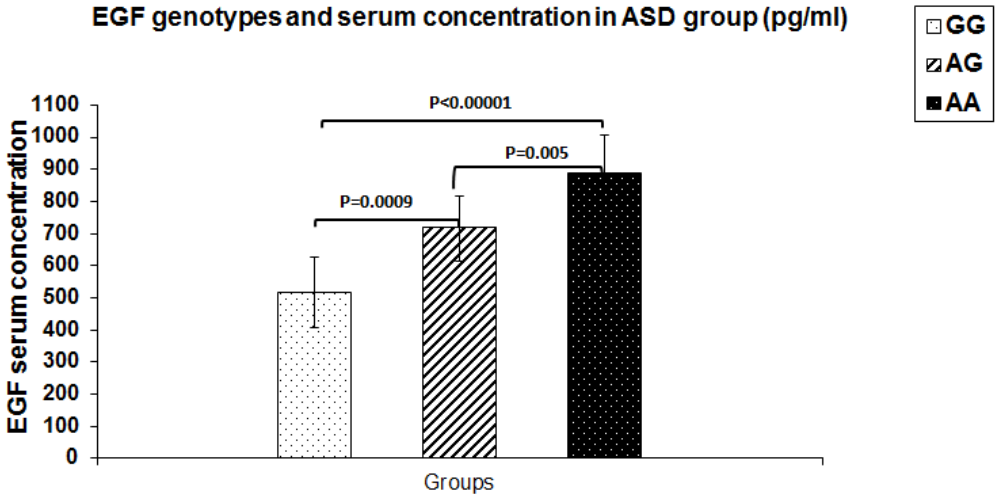

Figure 2 Association of EGF serum concentration and genotypes in ASD patients. GG genotype is associated with decreased EGF serum levels in ASD (GG, AG and AA serum levels were 515 ± 109.63, 716.22 ± 102.26 and 886.11 ± 119.69 pg/ml, respectively).

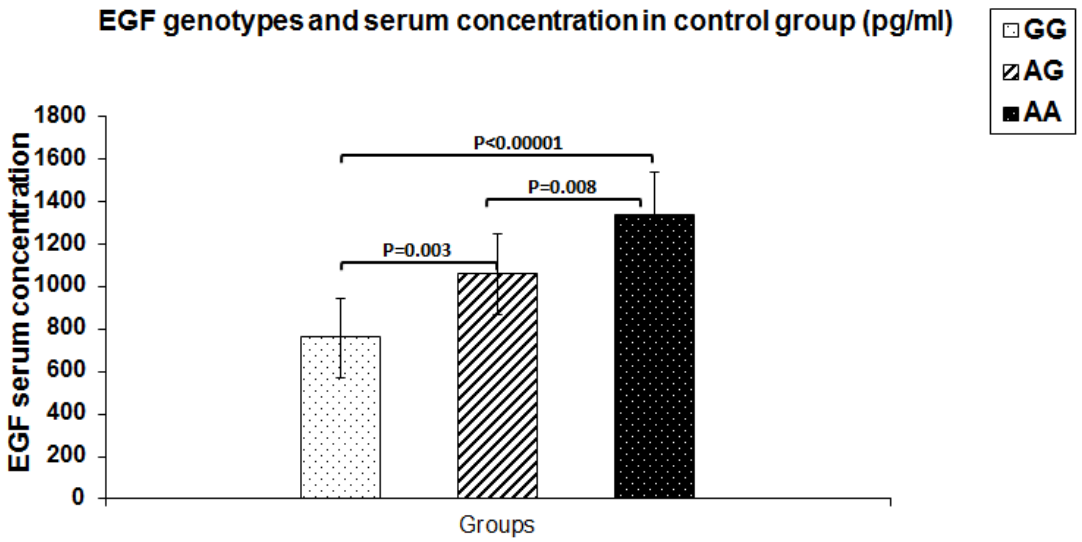

Figure 3 Association of EGF serum concentration and genotypes in controls. (GG, AG and AA serum levels in GG, AG and AA carriers in the control group were 757 ± 187.40, 1057.88 ± 191.68 and 1335.77 ± 199.04 pg/ml, respectively).

4. Discussion

For the first time in this project, we studied the relationship of the +61A/G (rs4444903) gene variant and its serum concentration and ASD. Many growth factors, including those involved in EGF signaling, have been implicated in the pathogenesis of ASD. EGF and its receptors (ErbB 1-4) maintain oligodendrocyte populations [27]. It has been reported that EGF crosses the blood-brain barrier [28]. It has been shown that there is a relationship between decreased blood EGF concentration and some symptom severity in children with Autism [29]. EGF has been suggested to play an important role in neuronal differentiation and survival [30].

It has been shown that EGF serum levels are altered in Parkinson's disease (PD) [31]. Toyoda and colleagues showed a significant relationship between EGF gene variation and ASD [21]. It has been shown that there is a strong association between EGF (rs11569017 and rs11569126) polymorphisms with major depressive disorder (MDD). Decreased serum EGF levels were shown in ASD patients as compared to normal controls [32]. Puttonen and colleagues suggested that EGF gene polymorphism is associated with temperament dimension of activity in the Finnish population [33]. It has also been suggested that the G allele of the EGF gene is connected to lower heart rate and greater respiratory sinus arrhythmia in women at rest [34]. It has been documented that the EGF promoter variation can be related to the susceptibility to the development of extra-axial nervous system tumors [35].

Previous studies suggest involvement of the EGF/EGFR signaling pathway in ASD, while findings remain heterogeneous. Toyoda and colleagues showed significant links between EGF gene polymorphisms and autism susceptibility in a Japanese cohort, supporting a genetic contribution of growth-factor signaling to ASD [21]. But Russo and colleagues mainly examined peripheral biomarkers and found reduced plasma EGF but elevated EGFR levels in ASD, with associations to behavioral severity and inflammatory markers [29]. Moreover, genetic polymorphism studies often differ across populations because allele frequencies and linkage patterns vary by ethnicity. Environmental exposures and gene–environment interactions also influence disease risk. In addition, differences in sample size, diagnostic criteria, and genotyping methods contribute to

inconsistent findings. For complex disorders such as ASD, biological heterogeneity and population stratification further reduce reproducibility across studies.

To our knowledge, this is among the first case-control studies to concurrently evaluate rs4444903 genotype and corresponding serum EGF levels in an Iranian cohort. However, the association of EGF +61A/G (rs4444903) genetic variation with other diseases has been reported. Almeida and colleagues suggested that there is a significant connection between EGF gene variation and glioma risk, and EGF mutation genotypes have contributed to increased susceptibility to glioma [36]. Moreover, a meta-analysis showed that the EGF +61 G/A gene variant is associated with both susceptibility and malignancy of glioma [37]. Aminmalek and colleagues suggested that EGF rs4444903 genetic variation and EGF serum concentrations are associated with male infertility in an Iranian population [25]. It was suggested that EGF rs4444903 GG genotype carriers are more susceptible to liver carcinoma in the Iranians [38]. James and colleagues showed that although the EGF +61A/G gene variation does not seem to predispose to the development of melanoma, its significant relationship with tumor size shows that it might be a valuable prognostic marker [39]. In addition, it was found that AA carriers of rs4444903 have a greater risk of schizophrenia [40].

In this study, we investigated the potential association between the EGF +61A/G (rs4444903) promoter polymorphism and serum EGF concentrations in individuals with ASD and controls. Our results showed that blood concentrations of EGF were significantly lower in ASD patients compared with healthy controls. Furthermore, carriers of the GG genotype exhibited markedly reduced EGF levels compared with AG and AA genotype carriers, indicating that this promoter variant functionally affects EGF expression. Genotypic analysis revealed that the EGF G allele conferred a 4.43-fold increased risk for ASD relative to the A allele, supporting its potential contribution to ASD susceptibility. Interestingly, even within identical genotypes, ASD patients consistently displayed lower serum EGF levels than control subjects (e.g., GG carriers in ASD vs. GG carriers in controls). This finding suggests that the rs4444903 variant alone cannot fully account for the reduced EGF concentrations observed in ASD. Disease-specific mechanisms—such as chronic neuroinflammation, oxidative stress, epigenetic modification of the EGF gene or its regulatory network—are likely to further suppress EGF expression. Additional environmental and biological factors, including perinatal complications, immune activation, gut–brain axis disturbances, and interactions with cytokine pathways (e.g., TNF-α, IL-6, TGF-β), may also contribute to this down-regulation. Collectively, our data support a model in which rs4444903 sets a genetic baseline for EGF production. At the same time, ASD-related pathological processes further diminish EGF levels, intensifying the biological impact of this promoter polymorphism.

This study has several limitations that should be considered when interpreting the findings. First, the study was conducted at a single center and included only individuals from northern Iran, which may limit the generalizability of the results to other ethnic and geographic populations. Second, only one EGF SNP was analyzed; therefore, the potential contribution of other functional variants or haplotypes in the EGF gene cannot be ruled out. Third, ASD is a multifactorial disorder influenced by numerous genetic and environmental factors that were not comprehensively evaluated in the present study. In addition, standardized neurodevelopmental screening tools such as M-CHAT-R/F, SRS-2, or ADOS-2 Toddler Module were not applied to control subjects, raising the possibility of undetected subclinical neurodevelopmental abnormalities and potential healthy-volunteer bias. Moreover, detailed phenotypic characterization of ASD severity and subtypes was unavailable, limiting genotype–phenotype analyses. Finally, the cross-sectional design precludes establishing

causal or temporal relationships between EGF alterations and the onset of ASD. Furthermore, a key limitation of the present work is the lack of *in vitro* functional validation of the rs4444903 promoter polymorphism (e.g., allele-specific luciferase reporter assays), which will be an important next step to directly evaluate its impact on EGF transcriptional activity and to deepen the mechanistic understanding of our association findings. Future multicenter studies with larger cohorts and more comprehensive phenotypic assessments, alongside mechanistic functional studies, are warranted.

5. Conclusions

The results of this project suggest that there may be a relationship between the EGF +61A/G (rs4444903) polymorphism and its serum levels with the risk of ASD. Moreover, the GG genotype seems to be associated with decreased EGF expression in patients with ASD. However, more studies are necessary to confirm the results in larger-scale studies that are likely to show more significant results and highlight the role of EGF in the pathophysiology of ASD.

Abbreviations

ASD	Autism Spectrum Disorder
CNS	Central Nervous System
CNTNAP2	Contactin-Associated Protein-Like 2
DSM-5	Diagnostic and Statistical Manual of Mental Disorders
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbent Assay
GST	Glutathione S-Transferase
IGF-1	Insulin-Like Growth Factor 1
MDD	Major Depressive Disorder
MMP-9	Matrix Metalloproteinase
NRP2	Neuropilin-2
PD	Parkinson’s Disease
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
SHANK3	SH3 and Multiple Ankyrin Repeat Domains 3
SNPs	Single-Nucleotide Polymorphisms

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Author Contributions

Material preparation, data collection [MK, FM and ZS]. Writing of the manuscript [FM], edited by [FM]. Conceptualization [FM, ZS]. Methodology and Software [MK, ZS]. Supervision [FM]. All authors accepted the final form of the manuscript.

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

AI-Assisted Technologies Statement

During the preparation of this manuscript, no results or scientific content were generated by AI. AI tools were used solely for grammar checking and language enhancement. All AI-assisted text was reviewed and edited by the authors for accuracy, and the authors take full responsibility for the content of the manuscript.

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