



Research Paper: Association of Hepatocyte Growth Factor Genetic Variation (S3735520) and Its Concentration in Autism Spectrum Disorders: A Case-control Study



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Running Title HGF Gene Polymorphism and Serum Levels in Autism

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ABSTRACT

Background: Hepatocyte Growth Factor (HGF) was shown to play a key role in synaptogenesis, survival, maturation, and reconstruction of neuron cells and was shown to be implicated in Autism Spectrum Disorder (ASD).

Objectives: Assessing the relationship between HGF (rs3735520) gene polymorphism and its circulating levels in ASD.

Materials and Methods: A total of 140 ASD patients and 120 children healthy controls referred to Shahid Rajaei Hospital, Mazandaran, Iran from September 2017 to January 2019 were enrolled in the study. Genomic DNA was extracted from blood samples, HGF polymorphism was determined by polymerase chain reaction-restriction fragment length polymorphism and HGF serum concentration was measured by enzyme-linked immunosorbent assay. Statistical analysis was done by ANOVA and the Chi-square test, using χ^2 in MedCalc statistical software ver. 12.1.4.

Results: Genotype frequencies of CC, CT, and TT in the ASD group were 25.71%, 52.86%, and 21.43%, and in controls were 26.67%, 68.33%, and 5%, respectively ($P=0.003$) and C and T alleles frequencies in patients were 53% and 47% and in controls were 61% and 39%, respectively ($P=0.046$). Moreover, the Mean \pm SD serum HGF levels in the controls and ASD patients were 363.33 \pm 118.44 and 219.95 \pm 73.61 pg/mL, respectively ($P=0.009$). Furthermore, ASD patients carrying TT genotype had lower serum HGF levels than CT and TT carriers (CC, CT, and TT Mean \pm SD serum levels were 271.88 \pm 30.47, 217.77 \pm 33.59 and 156.33 \pm 22.72 pg/mL, respectively).

Conclusion: There was a significant relationship between HGF gene polymorphism and its serum levels with ASD in an Iranian population. We also suggest that TT genotype may be associated with a decrease in HGF circulation levels in ASD.

Keywords: Polymorphism, Genetic; Hepatocyte Growth Factor; Serum; Autism Spectrum Disorder

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Highlights

- HGF rs3735520 gene polymorphism and HGF serum levels are associated with the susceptibility to autism spectrum disorder.
- TT genotype of HGF gene may be associated with decreased HGF circulation levels in patients with autism spectrum disorders.

Introduction

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utism Spectrum Disorders (ASDs) are a group of neurological disorders that are characterized by deficits in verbal and nonverbal communication, stereotyped behavior, and interacting with others [1].

According to the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), ASD contains a range of disorders, including Asperger syndrome, pervasive developmental disorders not otherwise specified, and autistic disorder and is principally diagnosed at the first three years of life [2]. ASD is more common in males but in a recent meta-analysis, the exact male-to-female ratio is closer to 3:1 than the previously reported 4:1 [3]. Studies during the previous years have shown a significant increase in ASD prevalence, which has reached as much as 1%-2% of children in recent years [4]. Several environmental factors are involved in the pathogenesis of ASDs, including nutritional factors, hormones, viral infections with rubella, and cytomegalovirus [5].

Growth factors, including hepatocyte growth factor (HGF) has been shown to play an important role in pathophysiology and clinical manifestations of ASD [6]. HGF is a polypeptide growth factor that acts via binding to MET receptor tyrosine kinase. HGF gene is located on human chromosome 7 (7q21.11) and includes 18 exons and 17 introns encoding a protein of 728 amino acids. Active HGF is a heterodimer composed of an alpha-chain subunit (69 kDa) and a beta-chain subunit (34 kDa), which are linked by a disulfide bond [7].

HGF influences the growth, motility, and morphogenesis of various epithelial and endothelial cells and functions as a trophic factor for organ regeneration. Accumulating evidence suggests that HGF and its receptor MET play a role in neuronal cell development. Taken together, these findings suggest that HGF may be a candidate for mediating interneuron development in vivo [8]. HGF signaling is necessary for neurodevelopmental and neurophysiological processes, including neuron survival, migration, enhance axonal outgrowth, synapse maturity, and activity [9]. Defi-

cits in any of these processes can damage brain circuits and causing impairment of interneuron migration and neuronal growth in the cortex, and also conduce to a decreased reproduction of granule cells, causing a reduction in the size of the cerebellum [10]. Given the role of HGF/MET signaling in autism susceptibility, the purpose of this study was to investigate the association between HGF (rs3735520) gene polymorphism and its circulating levels with ASD.

Materials and Methods

Study Samples

A total of 140 autistic children who were referred to Shahid Rajaei Hospital, Tonekabon City, Mazandaran Province, Iran from September 2017 to January 2019 were enrolled in the study. A total of 120 controls matched for age, sex, and ethnicity were recruited from the same geographic region. These were children attending a clinic for a routine examination. The controls were investigated to determine whether they or their first-degree relatives had psychiatric disturbances or previous psychiatric treatment through personal interviews.

The diagnosis of ASD was made by a psychiatrist according to DSM-5 criteria for ASD. All cases with a neurological, inflammatory, endocrine, or immune disorder and patients with fragile X syndrome, tuberous sclerosis, a formerly distinguished chromosomal variation abnormality, dysmorphic highlights, or some other neurological condition suspected to be related to ASD were excluded from this study. Written informed consent was obtained from the parent(s) or legal guardian(s) of each study participant. Three milliliters of blood was collected from each case and control sample and was used for genomic DNA extraction and serum preparation.

Genotyping

Genomic DNAs were extracted from blood white cells using a protocol of Triton X100. The extracted DNAs were analyzed by electrophoresis on 1% Agarose gel. We used the polymerase chain reaction-restriction fragment

length polymorphism (PCR-RFLP) method to define the rs3735520 polymorphism of HGF gene.

Peripheral blood samples (2 mL) were collected in EDTA contained Venojects and genomic DNA was extracted using Triton X-100 extraction method and was stored at -70°C for genotyping. Polymerase chain reaction (PCR) was performed to amplify a 500 bp fragment containing the target single-nucleotide polymorphism (SNP) using specific primers. Primers used were designed using Oligo primer analysis software. The following primers were used to amplify a 500-bp fragment containing the loci: forward (F) primer: 5'-TAGGCCCTTTAAATACAGCTT-3'; reverse (R) primer: 5'-TCTCCAGCCCCAATTATCACA-3'. In each reaction, 20 µL, containing 10 µL of Master mix, 1 µL of F primer, 1 µL of R primer, 4 µL of deionized H₂O, and at the end 4 µL of the template was added to each reaction. Details of primers are presented in Table 1.

PCR protocol

The PCR conditions were as follows: initial denaturation at 94°C for 5 min, 35 cycles of secondary denaturation at 94°C for 45 s, annealing at 62°C for 45 s, initial extension at 72°C for 45 s, and a final extension at 72°C for 5 min. Then 5 µL of PCR product was digested with 0.2 of BglIII at 37°C for 4 h. The PCR product with the fragment of 406 bp was broken down into fragments of 329 and 77 bp and the products were verified on 2% agarose gels, containing 0.5 µg/mL safe stain and observed under ultraviolet light. At least 15% of samples were randomly selected for repeat analysis, yielding 100% concordance.

HGF serum concentration by ELISA

One milliliter of blood was collected from each patient and control for HGF protein analysis by ELISA. Human HGF ELISA kit (ab100534) (Abcam, Cambridge, UK) was used for the measurement of HGF serum concentration according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was done using χ^2 in MedCalc version 12.1.4 software (Mariakerke, Belgium) for analysis of the relationship between HGF genetic polymorphism and the risk of ASD. Odds ratios were calculated together with their 95% confidence intervals. The obtained data were analyzed by the Chi-square test, analysis of variance (ANOVA), and by calculating the odds ratio (OR) with 95% confidence intervals (CI). A P value of less than 0.05 was considered statistically significant.

Results

This research contains 140 ASD patients (108 boys and 32 girls, Mean±SD of age: 10.0±3.8] years) and 120 healthy controls (89 boys and 31 girls, Mean±SD of age: 9.2 [3.7] years). ASD cases had no family history of autism. The exclusion criteria included patients with suspected genetic, metabolic, or chronic disease. The patients did not take any medication and the samples were taken before starting ASD treatment. In the ASD group, 64 patients were in level 1 (requiring support), 48 patients in level 2 (requir-

Table 1. Primer sequences and different genotypes after BglIII enzymatic digestion of HGF rs3735520 polymorphism

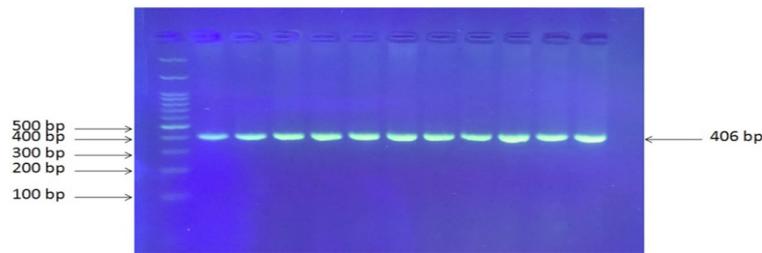
Primer Sequences	CC Genotype	CT Genotype	TT Genotype
F:5'-TAGTTTCTATGCCTCGCCTTG-3'	406bp	406bp	-
R:5'-CAACCTGCCTGATAAGTCC-3'	-	329bp	329bp
-	-	77bp	77bp



Table 2. Genotype and allelic distribution of HGF rs3735520 polymorphism in the patients with ASD and control groups

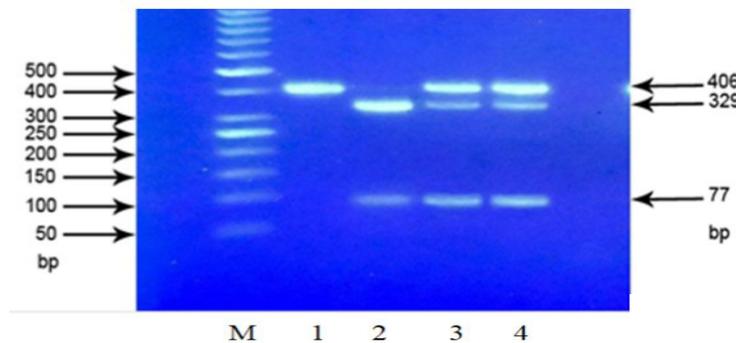
Variables	No. (%)		OR (95% CI)	P	
	Control n=120	Experimental n=140			
Alleles	C	146 (61)	146 (53)	1.00 (reference)	-
	T	94 (39)	134 (47)	0.36 (0.22-0.56)	0.0001*
Genotype	CC	32 (26.67)	36 (25.71)	1.24 (0.70-2.20)	0.4
	CT	82 (68.33)	74 (52.86)	1.00 (0.reference)	-
	TT	6 (5)	30 (21.43)	5.54 (2.18-14.05)	0.0003*





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Figure 1. Agarose gel electrophoresis of the HGF gene PCR amplification products. Fragments of 406 bp indicate the HGF gene



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Figure 2. Agarose gel electrophoresis of the HGF gene PCR-RFLP amplification products

CC homozygote had a single band of 406 bp (band 1). TC heterozygote had three bands of 406, 329, and 77 bp (3, 4), and TT homozygote had two fragments of 329 and 77 bp (3). M: Molecular marker.

ing substantial support), and 28 patients in level 3 (requiring very substantial support).

The undigested PCR product size was 406 bp for HGF s3735520 in all samples without the mutation (genotype C/C), and for the C/T genotype, PCR products had 3 bands, including 406 bp and 329 bp and 77bp. Moreover, for the TT genotype, there were two 329 bp and 77 bp bands (Figures 1 and 2).

Genotype frequencies of the rs3735520 were in Hardy Weinberg equilibrium in both groups ($P > 0.05$). The frequencies of HGF heterozygous (CT), homozygous (TT) and normal homozygous (CC) genotypes in healthy controls were 68.33%, 5%, and 26.67%, while in the patients' group, they were 52.86%, 21.43%, and 25.71%, respectively (Table 2). A higher prevalence of T allele was observed in ASD cases (47%) compared with the controls (39%), which showed a statistically significant association between rs3735520 T

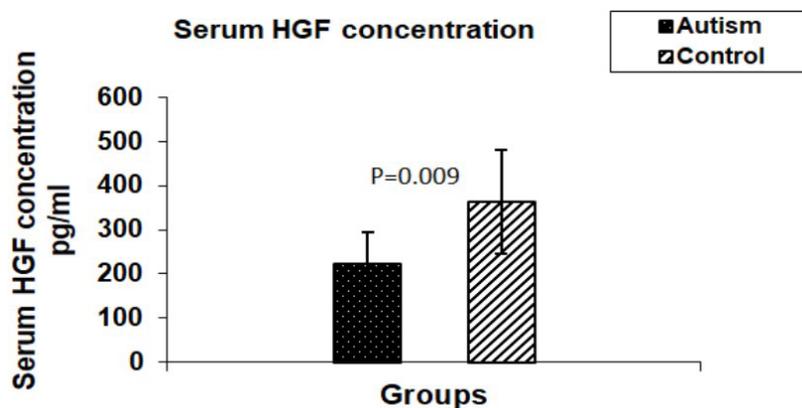


Figure 3. Serum HGF concentrations in the controls and patients' ASD

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Measured in a typical ELISA (pg/mL). Serum HGF concentration (pg/mL) of individuals with ASD was significantly lower than that in the healthy controls ($P = 0.009$).

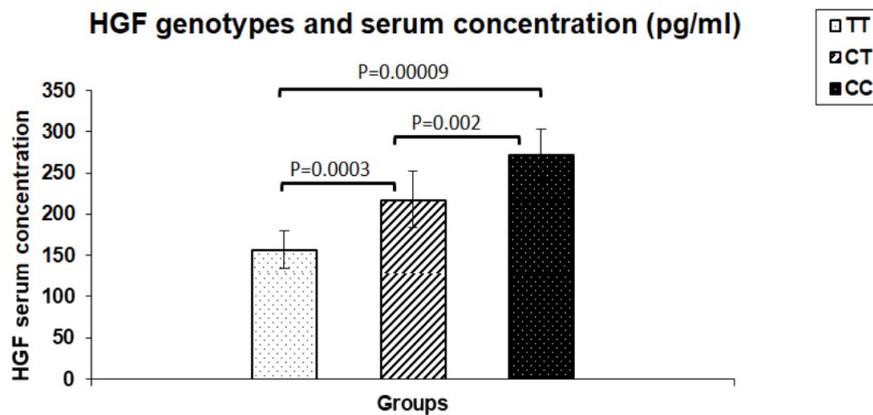


Figure 4. Association of HGF serum concentration and genotype in the ASD group

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TT genotype is associated with decreased HGF expression in ASD (Mean±SD) TT, CT and CC serum levels were 156.33 [22.72], 217.77 [33.59] and 271.88 [30.47] pg/mL, respectively).

allele and ASD risk (OR=1.42, CI=1.005-2.02; P=0.046). Besides, statistical analysis showed that TT carriers have a 5.5-fold higher risk of ASD susceptibility compared to the controls (OR=5.54, CI=2.18-14.05; P=0.0003).

Using ELISA, we found that the concentration of HGF in the serum samples of the control group was higher than that in the ASD patients. Mean±SD HGF concentration in the serum of the control and ASD patients were 363.33 (118.44) pg/mL and 219.95 (73.61) pg/mL, respectively (P=0.009) (Figure 3). We have also shown that TT genotype is significantly associated with decreased serum HGF levels in ASD ([Mean±SD], TT, CT, and CC serum levels were 156.33 [22.72], 217.77 [33.59], and 271.88 [30.47] pg/mL, respectively) (Figure 4) and in the control group, Mean±SD

TT, CT, and CC serum levels were 307.88 (20.86), 349.88 (16.79) and 410.44 (26.20) pg/mL, respectively (Figure 5).

Discussion

Development of the CNS is a complex process set by interfering with countless factors, including a large family of growth factors and their receptors. HGF with its receptor is one of the growth factors that is expressed in the nervous system and contributes to the development of the nervous system [11, 12]. Previous studies suggest that HGF/MET signaling is also necessary for numerous neurodevelopmental events, including neural induction, cell migration, neurotrophic and chemotrophic effects of developing axons, dendritic and axonal development, motogenic effects of specialized central neuron populations, and synaptogenesis

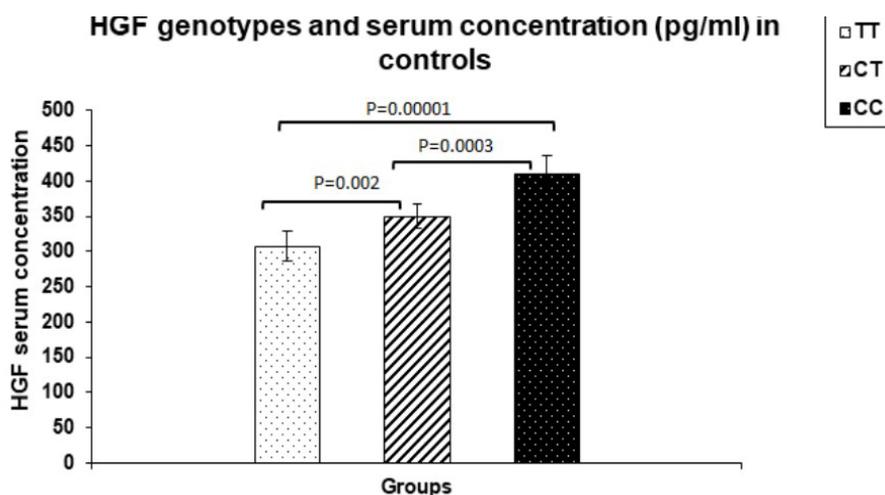


Figure 5. Association of HGF serum concentration and genotype in the control group

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TT genotype is associated with decreased HGF expression in the controls ([Mean±SD] TT, CT, and CC serum levels were 307.88 [20.86], 349.88 [16.79] and 410.44 [26.20] pg/mL, respectively).

[13, 14]. Besides, they can modify neuronal connectivity by changing synapses. MET protein is localized at the postsynaptic dendritic place, so it is part of postsynaptic signaling though HGF and MET can enhance clustering of synaptic proteins [15, 16]. HGF/MET signaling was shown to be involved in autism susceptibility [9]. Altered expression of MET has been seen in ASD which suggests dysregulation of signaling that may contribute to altered circuit formation and function in ASD [16]. It has been suggested that there is an association between HGF serum concentrations and the presence of gastrointestinal (GI) disease in autistic children that explain a potential functional connection between the MET gene and autism. It has been suggested that the concentration of serum HGF may be a useful biomarker for autistic children, especially those with severe GI disease [17]. This study is the first study evaluating the association of HGF (s3735520) gene polymorphism and ASD susceptibility; however, the association between HGF and MET gene polymorphism with other diseases have been studied. It has been suggested that there is no association between carrier states of gene promoter polymorphisms and pathogenesis of retinopathy of prematurity [18]. It was shown that there is a strong association between mild to moderate myopia group and HGF SNP rs3735520 and HGF haplotypes rs2286194-rs3735520-rs17501108 and rs12536657-rs2286194, and a moderate association of extreme high myopia with rs2286194 [19].

A study in 2012 indicated a significant relationship between rs3735520 and myopia [20]. Yang et al. suggested that genetic variants in MET are related to high myopia in the Chinese population [21]. It was also suggested that an SNP in the HGF promoter region may modulate the severity of interstitial lung disease by controlling the transcriptional efficiency of the HGF gene [22]. Dudakova and colleagues showed an association between rs3735520 polymorphism with keratoconus in the European population [23]. It was suggested that there is no significant correlation between rs3735520 polymorphism with primary angle-closure glaucoma (PACG) patients in the Han Chinese population [24]. It has been reported that there is no significant correlation between HGF (rs3735520) gene polymorphism and ASD disease but the serum levels of HGF were shown to be lower in the autistic patients compared with the controls in the Japanese population [25].

The association between some genes with ASD occurrence has been studied in Iranian populations. It was suggested that methionine synthase (rs1805087), neuropilin-2, SHANK3 rs9616915, and contactin associated-like 2 rs7794745 polymorphisms are associated with an increased risk of autism [26-29]. However, it was suggested that methylenetetrahydrofolate reductase polymorphism

(rs1801133) is not associated with autism in a northern Iranian population [30].

It has been suggested that there is an association between serum HGF levels with neurological disorders, including CIND (cognitively impaired no dementia) and Alzheimer [31]. Another study in the Iranian population suggested that HGF concentration is higher in patients with Parkinson compared with the normal controls [32]. The results of a study reported the cerebrospinal fluid HGF levels of patients with meningitis were higher than that in the normal controls [33].

The results of this study show a significant difference in genotype frequencies underlying co-dominant and dominant models and allele distribution of HGF polymorphism between ASD and controls. Moreover, a significant decrease in the serum HGF levels in ASD patients have been seen when compared with the controls. Also, individuals carrying the TT genotype had lower serum HGF levels than CT and TT carriers. We also suggest that TT genotype may be associated with a decrease in HGF circulation levels in ASD patients.

Conclusion

There is a significant relationship between HGF rs3735520 gene polymorphism and serum levels of HGF with ASD in an Iranian population. It is also suggested that HGF may play a role in the pathophysiology of ASD. Larger studies with more patients and controls are necessary to confirm these results.

Ethical Considerations

Compliance with ethical guidelines

This project has been approved by the Research Committee of the University of Guilan (Ref. No.: 1397-106570). All study procedures were done in compliance with the ethical guidelines of the Declaration of Helsinki, 2013.

Funding

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Authors' contributions

Methodology: Masoumeh Khalili; Investigation: Masoumeh Khalili and Farhad Mashayekhi; Resources and funding acquisition, Writing the original draft: Farhad Mashayekhi; Conceptualization, Supervision, Writing - review and editing: Farhad Mashayekhi and Zivar Salehi.

Conflict of interest

The authors declared no conflict of interest.

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