

DNA Methylation Level of Transcription Factor Binding Site in the Promoter Region of Acyl-CoA Synthetase Family Member 3 (*ACSF3*) in Saudi Autistic Children

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Background: DNA methylation (DNAm) is one of the main epigenetic mechanisms that affects gene expression without changing the underlying DNA sequence. Aberrant DNAm has an implication in different human diseases such as cancer, schizophrenia, and autism spectrum disorder (ASD). ASD is a neurodevelopmental disorder that affects behavior, learning, and communication skills. Acyl-CoA synthetase family member 3 (*ACSF3*) encodes malonyl-CoA synthetase that is involved in the synthesis and oxidation of fatty acids. The dysregulation in such gene has been reported in combined malonic and methylmalonic aciduria associated with neurological symptoms such as memory problems, psychiatric diseases, and/or cognitive decline. This research aims to study DNAm in the transcription factor (TF) binding site of *ACSF3* in Saudi autistic children. To determine whether the DNAm of the TF-binding site is a cause or a consequence of transcription regulation of *ACSF3*.

Methods: RT-qPCR and DNA methyl light qPCR were used to determine the expression and DNAm level in the promoter region of *ACSF3*, respectively. DNA and RNA were extracted from 19 cases of ASD children and 18 control samples from their healthy siblings.

Results: The results showed a significant correlation between the gene expression of *ACSF3* and specificity protein 1 (*SPI*) in 17 samples of ASD patients, where both genes were upregulated in 9 samples and downregulated in 8 samples.

Conclusion: Although this study found no DNAm in the binding site of *SPI* within the *ACSF3* promoter, the indicated correlation highlights a possible role of *ACSF3* and *SPI* in ASD patients.

Keywords: autism, DNA methylation, *SPI*, *ACSF3*, Saudi autistic children, DNAm

Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by impairment in social communication and interaction, restricted and repetitive interest, and behavior.¹ However, attention-deficit hyperactivity disorder (ADHD), anxiety, bipolar disorder, and depression are comorbid psychiatric illnesses that appear in a high percent of ASD patients.² It is not easy to find an accurate and updated record on the global prevalence of ASD because the numbers are unknown in many low- and middle-income countries, while the reported prevalence varies substantially across studies. However, one study (cited by the World Health Organization) estimates that about one in 160 children has ASD worldwide.³ Despite the fact, genetic and environmental factors were found to influence ASD^{4,5} but the

relationships are still not well understood.⁶ According to King Salman's Center for Disability Research (KSCDR), the Statistics of ASD were around 50,714 cases in Saudi Arabia in 2017.⁷

Gene expression can be regulated through various transcription factors (TFs) and DNA methylation (DNAm).⁸ The binding of TFs to the promoter of a particular gene is influenced by many factors such as mutation and DNAm.^{9–11} DNAm is one of the epigenetic mechanisms that alters the gene expression without changing the underlying DNA sequence.¹² It plays a critical role in regulating normal development by affecting the expression of selected genes.¹³ Moreover, the abnormality in the DNAm process in the promoter region of a gene, especially around the TF binding site, has reported different diseases, including ASD.^{14–17} Evidence suggests that DNAm signatures can distinguish between pathogenic and benign mutations or copy number variants (CNVs) in various syndromic forms of ASD. This is because that pathogenic variants produce unique epi-signatures when combined with genetic or cytogenetic findings can provide an accurate diagnosis.¹⁸

ACSF3 is the gene encodes a mitochondrial enzyme that is involved in fatty acid synthesis. *ACSF3* enzyme performs a chemical reaction to convert malonic acid (toxic metabolite that inhibits mitochondrial metabolism) to malonyl-CoA, which is the first step of fatty acid synthesis.¹⁹ Previous studies of exome sequencing showed that *ACSF3* implicated in combined malonic and methylmalonic aciduria which associated with neurological symptoms such as memory problems, psychiatric disease and/or cognitive decline. Many studies revealed that methylation levels of the promoter regions of *ACSF3* play a critical role on its expression.²⁰ Moreover, the abundance of CpG sites in the *ACSF3* promoter suggested that methylation could be a regulatory factor. In addition, *ACSF3* displayed a differential hypermethylation in region cg04033022 of *ACSF3* in B-cells which is implicated in multiple sclerosis disease (MS), neurodegenerative diseases, Rheumatoid arthritis (RA), and in systemic lupus erythematosus (SLE).²¹ However, to the best of our knowledge no previous study has looked at the effect of DNAm on the TF binding site of this gene. There are few studies about the role of DNAm in the expression of the genes associated with ASD, specifically in Saudi Arabia. Therefore, this research aimed to determine the role of DNAm in the TF binding site of the *ACSF3* gene in Saudi ASD children through bioinformatics tools and molecular techniques.

Methods

Study Population

The ethics of this study was approved by the Center of Excellence in Genomic Medicine Research (CEGMR). Parents or legal guardian of all participants provided informed consent to participate in the study after explaining the aim of research to them. The study group included 19 ASD children and 18 of their siblings, (see [Table 1](#)). The children were diagnosed based on Diagnostic and Statistical Manual of Mental Disorders version 5 (DSM-5) and showed no symptoms of malnutrition, active infection, or known genetic disease (such as Down syndrome). To detect chromosomal abnormalities and copy number variations, Agilent Cytogenomics version 4.0 software (Agilent Technologies, USA) was used to analyze array-CGH data (Agilent sure print G3 Hmn CGH 2x 400K array chips). Bioinformatics analysis of array-CGH data did not detect any alteration in the gene/loci that have been characterized as genetic factors for syndromic ASD. Blood samples were collected in EDTA tubes for DNA extraction.

DNA Extraction

Genomic DNA was extracted from a blood sample in vitro at the CEGMR using DNeasy Blood and Tissue Kit (Qiagen, UK) according to the manufacturer's protocol. First, the purity and concentration of DNA were measured using a NanoDrop 2000c spectrophotometer (Thermo Scientific Inc.). Then, samples were stored at -20°C until use.

RNA Extraction

According to manufactures instructions, total RNA was extracted from blood using an RNeasy kit (Qiagen, UK). RNA concentration and purity were measured using a NanoDrop 2000c spectrophotometer (Thermo Scientific Inc.). Then, RNA reverse-transcribed into cDNA was applied using ImProm-IITM Reverse Transcription System (Promega, USA) according to the provided protocol.

Table 1 Demographic Characteristics of ASD Children and Their Siblings

Case	Gender	Patient/ Normal	Age (Years)	Birth	Severity of ASD	Onset of Symptoms	Family History
1	Male	P	12	Caesarean	Simple/level I	After 2 years	Yes
	Female	N	5				
2	Male	P	9	Natural	Mild/level 2	After 2 years	No
	Female	N	6				
3	Male	P	10	Natural	Simple/level I	After 2 years	Not known
	Female	N	5				
4	Male	P	8	Caesarean	Mild/level 2	After 2 years	No
	Male	N	4				
5	Male	P	8	Caesarean	Mild/level 2	After 6 months	No
	Male	N	6				
6	Male	P	6	Caesarean	Mild/level 2	After 2 years	No
	Male	N	7				
7	Male	P	7	Caesarean	Simple/level I	After 2 years	Yes
	Female	N	8				
8	Male	P	8	Caesarean	Sever/level 3	After 2 years	Yes
	Female	N	6				
9	Male	P	7	Natural	Mild/level 2	After first year	Yes
	Male	N	3				
10	Male	P	12	Natural	Not specified	Not specified	
	Male	N	10				
11	Female	P	12	Caesarean	Mild/level 2	After 2 years	No
	Male	N	10				
12	Male	P	6	Natural	Simple/level I	After 2 years	Yes
	Female	N	11				
13	Male	P	7	Natural	Simple/level I	After 2 years	No
	Female	N	5				
14	Male	P	10	Caesarean	Mild/level 2	After 2 years	No
	Male	N	6				
15	Male	P	10	Natural	Simple/level I	After 2 years	No
	Female	N	7				
16	Male	P	7	Caesarean	Simple/level I	After 2 years	No
	Male	N	5				

(Continued)

Table 1 (Continued).

Case	Gender	Patient/ Normal	Age (Years)	Birth	Severity of ASD	Onset of Symptoms	Family History
17	Female	P	6	Caesarean	Simple/level I	After 2 years	No
	Female	N	4				
18	Male	P	7	Natural	Simple/level I	After 2 years	No
	Female	P	8	Natural	simple/level I	After 2 years	No
	Male	N	7				

Real-Time Quantitative PCR (RT-qPCR) for *ACSF3* and *SPI*

For RT-qPCR, a master mix was prepared by mixing 5 μ L of QuantiFast SYBR[®] Green 2x master mix buffer (Qiagen, USA), 0.5 μ L of forward Primer, 0.5 μ L of reverse Primer, and 2 μ L of H₂O. Master mix (8 μ L) was loaded into 96-Well Reaction Plate (MicroAmp Fast Optical, Applied Biosystem) then cDNA sample (2 μ L) was added. RT-qPCR was carried out using QuantiFast SYBR[®] Green PCR Kit (Qiagen, USA) on StepOnePlus Real-Time PCR System (Applied Biosystem). The PCR program consisted of an initial denaturation step of 5 min at 95°C, followed by 40 cycles of denaturation at 95°C for 10 sec, and annealing/extension at 58°C for 30 sec. All PCR reactions were applied in duplicate and actin beta (*ACTB*) was used as a reference gene to normalize the expression of target genes. The sequences of the forward and reverse primers used for RT-PCR were: *ACTB* (forward: 5'- AAAATCTGGCACCACACCTT-3' and reverse: 5'- GCCTGGATAGCAACGTACAT-3'), *ACSF3* (forward: 5'- CAG TGC TGG AGA AGT GGA AG-3' and reverse: 5'- GGT TTT CTG AGA CAA TGC GC-3') and *SPI* (forward: 5'- AGTTGGTGGCAATAATGGGG-3', and reverse: 5'- CTGGGAGTTGTTGCTGTTCT-3'). The PCR products were confirmed via 2% agarose gel electrophoresis.

Identification of Promoter Region of *ACSF3*

Eukaryotic Promoter Database was used to identify the promoter region of *ACSF3*, choosing the length of the promoter region ranges from -499 to 100. (https://epd.epfl.ch/cgi-bin/get_doc?db=hgEpdNew&format=genome&entry=ACSF3_1)

>FP022318 *ACSF3_1*:+U EU:NC; range -499 to 100.

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ccagctaattgggaggttgaggcaggagaatcactgaaccgggaggcggaggttgcgg
tgagccgagatcacaccactgcactctagtctggcgacagagcgagactccatctcaa
ataataataataaagaaaaagtaacaacaataataataataataaaatcacccaggag
ctcagcgtgcagacccccagccgaaagcccagagagcccctttgggaggacggggcga
ggcccgcaatccgcgtgggagtggtcccgcgcgcccgttctgatctgggcagccctggg
ctagcggagaccacggcgccttgcctcgccacggctcacgagacggcggcggcccgac
cccgccagacgggacggcgggacaggggaacacgcgcgcccgaaccgcccgcacgg
cgccgcgggggggacgccttggcgacggacgcgcgcccggggaagctgttgggc
gcccgaactgtccggcccGACTCAGACCCCGCGGGACCCGCGCGGAACCCGGCCCGAC
CCCGCGCGCGCGCGGCGGAGGACGAGGAAGAGTTGTGGCGAGGCAGATCCTGCCCCGTG
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Bisulfite Modification and MethyLight qPCR

According to manufactures instructions, all DNA samples were bisulfite-converted using EpiTect Bisulfite Kit (Qiagen, Germany). MethyLight assay (quantitative real-time PCR) was performed in a 10 μ L reaction volume containing 5 μ L 2x EpiTect MethyLight Master Mix, 1 μ L *ACSF3* primer/probe mix, 1 μ L COL2A1 (collagen type II alpha one chain) primer/probe mix, 2 μ L nuclease-free water and 1 μ L of bisulfite-treated DNA template. COL2A1 was used as a reference gene for normalization. Reactions were performed in duplicate with the positive control (methylated human bisulfite-converted DNA), negative control (unmethylated human bisulfite-converted DNA), and unconverted human

genomic DNA from healthy human blood samples. The MethyLight assay was performed in the StepOnePlus Real-Time PCR System (Applied Biosystems). The PCR program consisted of an initial denaturation step of 5 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15s and annealing/extension at 58°C for 1 min. Two sets of primers and probes were designed specifically for bisulfite-converted DNA in this study for both (*ACSF3*, *COL2A1*). Collagen was used as the endogenous internal reference to normalize the amount of input DNA. The primers were purchased from (Integrated DNA Technologies, USA), and probes were purchased from (Macrogen, Korea). The primers and probes sequences used for MethyLight qPCR were:

COL2A1: (forward: 5'-TCTAACAAATTATAAACTCCAACCACCAA-3', reverse: 5'GGGAA GATGGG ATAGAAGGGAATAT-3', and probe: 5'-CCTTCATTCTAACCCAATACCTAT CCC ACCTCTAAA-3') and *ACSF3*: (forward: 5'-TTTTGGGTTAGCGGAGATTA -3', reverse: 5'-TAACGACGAATTCCGAAC-3', and probe: 5'-TTTTGTTTCGGTTACGGTTT-3').

Bioinformatics Tools

Eukaryotic-Promoter-Database (EPD) (<https://epd.epfl.ch/index.php>) was used to identify the promoter region of *ACSF3*. Genomatix MatInspector (<http://www.genomatix.de/matinspector.html>) and GeneCards (<https://www.gene-cards.org/>) were used to determine the most common TFs that may influence the expression of *ACSF3* gene. Moreover, Genomatix MatInspector was used to identify TF binding sites (TFBSs) of the TF (*SPI1*). MethPrimer online software (<http://www.urogene.org/methprimer/>) was used to predict CpG islands in the promoter region of *ACSF3*.

Statistical Analysis

IBM SPSS Statistics for Windows, version 23 (IBM SPSS, IBM Corp., Armonk, NY, USA) was used to analyze the correlation between the expression level of *ACSF3* and its TF (*SPI1*) in ASD. The correlation between the two variables was determined using Pearson's test and P values of < 0.05 were considered as significant.

Results

Prediction of CpG Island in the Promoter Region of *ACSF3*

MethPrimer online software (<http://www.urogene.org/methprimer/>) was used to predict CpG islands. One CpG island in the *ACSF3* promoter was detected with size of 350 bp (196–545) shown in Figure 1.

Identification of TFBSs of *ACSF3*

Genomatix MatInspector (<http://www.genomatix.de/matinspector.html>) was used to identify TFBSs found in the promoter region of *ACSF3*. The 600 bp of *ACSF3* promoter was uploaded in MatInspector (Genomatix software) (April

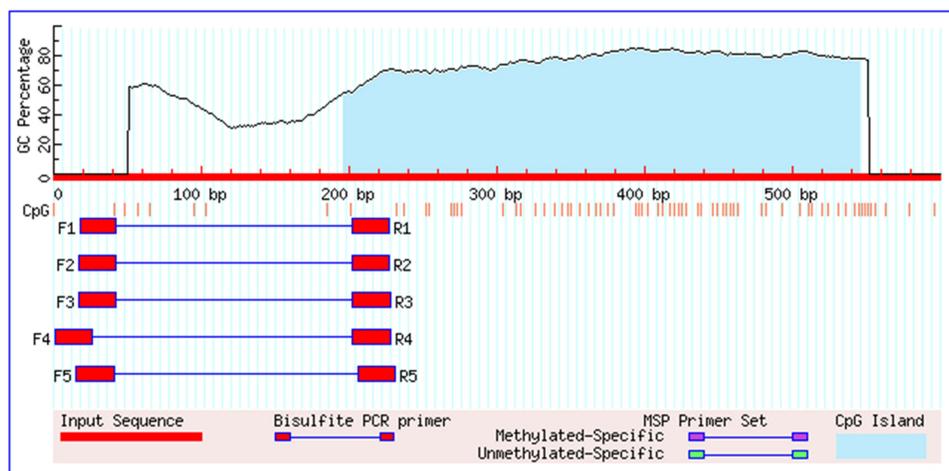


Figure 1 The predicted CpG Island in the promoter of *ACSF3*. The island appears in the light blue region.

Table 2 *ACSF3* and *Sp1* Expressions in ASD Children

ACSF3 Expression in ASD Children				SP1 Expression in ASD Children			
Upregulation		Downregulation		Upregulation		Downregulation	
Sample	Fold Change	Sample	Fold Change	Sample	Fold Change	Sample	Fold Change
3	1.4	1	-2.1	3	1.5	1	-1.6
4	1.5	2	-2	4	1.8	2	-16.4
5	27.4	9	-2.2	5	14.7	7	-1.4
6	34.6	10	-1.7	6	46.6	8	-1.7
7	1.3	11	-1.4	15	1.5	9	-1.4
8	1.6	12	-1.2	16	176.8	10	-1.9
15	1.3	17	-1.6	17	3.5	11	-1.2
16	1.9	18	-1.7	18	245.1	12	-1.3
19	1.8		-			13	-1.1
						14	-13
						19	-1.2

2019). Numerous TFs families were found in *ACSF3* promoter. According to GeneCards, one of the potential TFs that binds to *ACSF3* promoter is *SP1*. The sequence of TFBS: gggacGGGCgggacagg.

Gene Expression Results of *ACSF3* and *Sp1*

RT-qPCR was used to quantify the expression level of *ACSF3* and *SP1* and *ACTB* used as a reference gene to normalize the expression of target genes. The results showed that the expression level of *ACSF3* was upregulated in 9 out of 19 ASD patients with fold change ranged from 1.3 and 34.6 while downregulated in 8 samples (fold change from -1.2 to -2.2, see Table 2). The analysis also showed that *SP1* was upregulated in 8 out of 19 ASD patients as fold change ranged between 1.5 and 245.1 while downregulated in 11 samples, see Table 2.

In order to assess whether the expression of *ACSF3* and *SP1* was correlated in ASD samples, a Pearson's correlation was used to determine the relationship. The analysis indicated a significant correlation in the expression level between *ACSF3* and *SP1* (r -value = 0.454, P value = 0.05, Pearson's correlation). The results is shown as scatter plot in Figure 2. This result suggests that *SP1* may contribute to the regulation of *ACSF3* expression.

MethyLight qPCR Results of *ACSF3* Promoter Region

MethyLight technology was applied to detect the DNAm level in the promoter region of *ACSF3* among autistics and controls. The methyLight PCR did not show any methylation pattern in all samples, indicating the absence of DNAm at the *ACSF3* promoter. The amplification plot of methylated human bisulfite-converted DNA is shown in Figure 3, unmethylated human bisulfite-converted DNA (Figure 4), unconverted human genomic DNA (Figure 5). The specificity of the MethyLight PCR reaction was assessed by including control reactions to ensure that MethyLight PCR probes and primers specifically bind, also to exclude any false-negative results. The three DNA control templates were used in methyLight qPCR to determine the accuracy and specify of primers and probes as follow: 100% methylated converted DNA (positive control), and 100% unmethylated converted DNA as well as 100% unmethylated unconverted DNA (negative controls). The amplification plots of positive control showed signals for both probes of two genes (*ACSF3* and *COL2A1*) which indicated the probe's specificity to detect only the methylated converted sequence. While the amplification plots of negative control (unmethylated converted DNA) showed signals for *COL2A1* probe (reference) but no signals for *ACSF3* probe because the reference probe does not have CpG sites. Furthermore, the amplification plots of

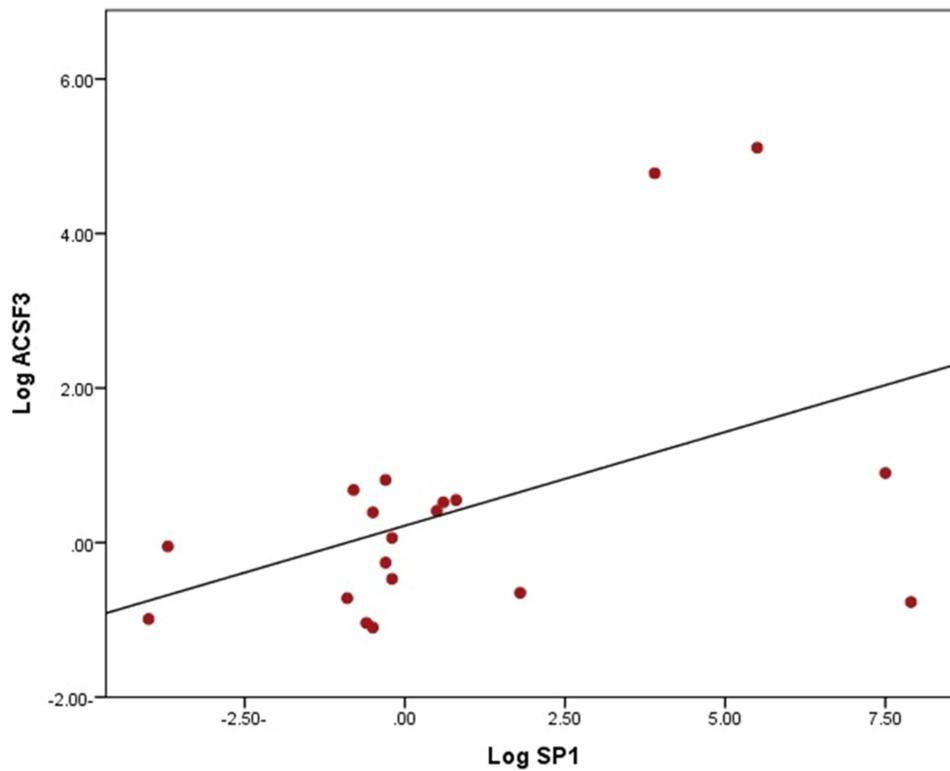


Figure 2 The positive correlation between expression of ACSF3 and transcription factor (SP1) by SPSS software. ($r = 0.454$, $P = 0.05$).

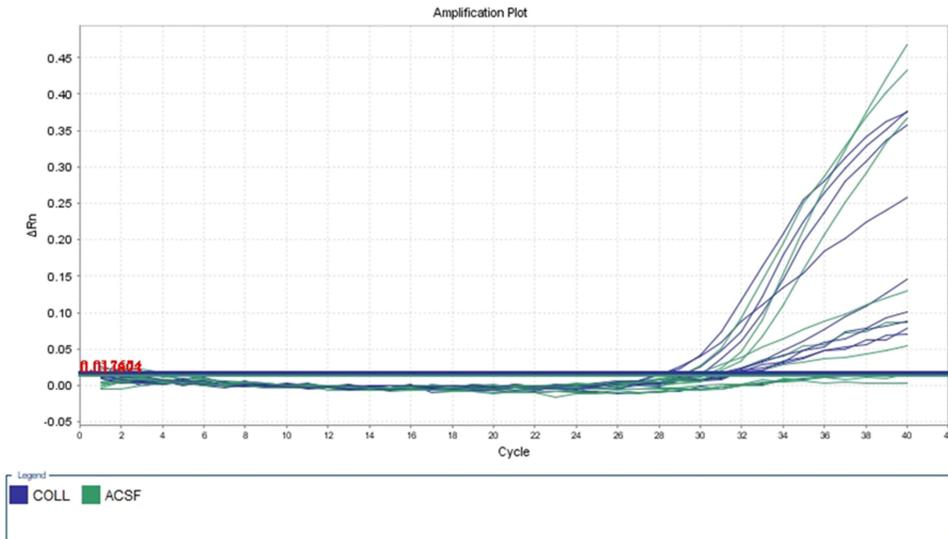


Figure 3 Five dilutions of positive control (methylated human bisulfite-converted DNA).

negative control (unmethylated unconverted DNA) showed no signals for both probes which confirmed the conversion status of the sequence. These results of negative control confirmed that any negative reaction would not be a false negative result. However, the result of amplification plots of both probes showed no methylation in the *ACSF3* at selected CpG sites for control and ASD samples (Figure 6).

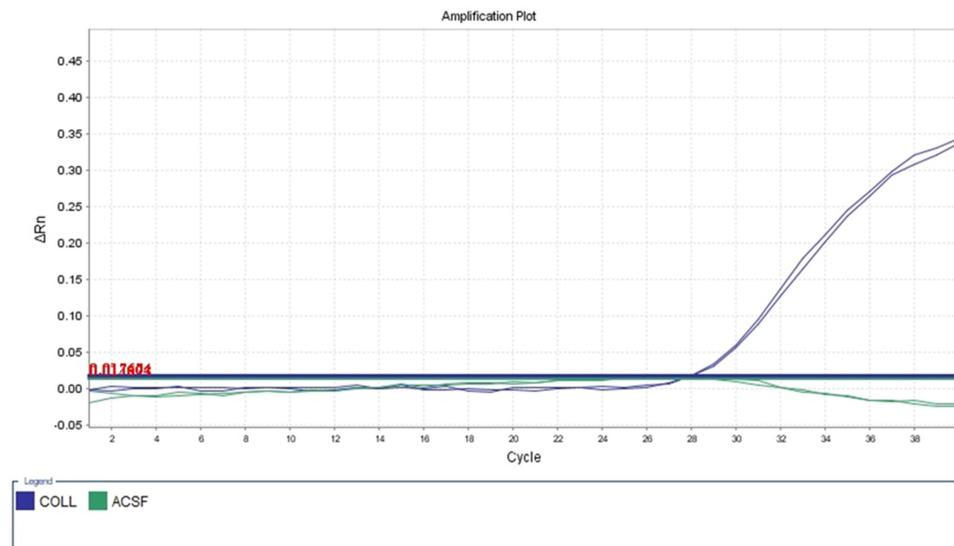


Figure 4 Negative control (unmethylated human bisulfite-converted DNA).

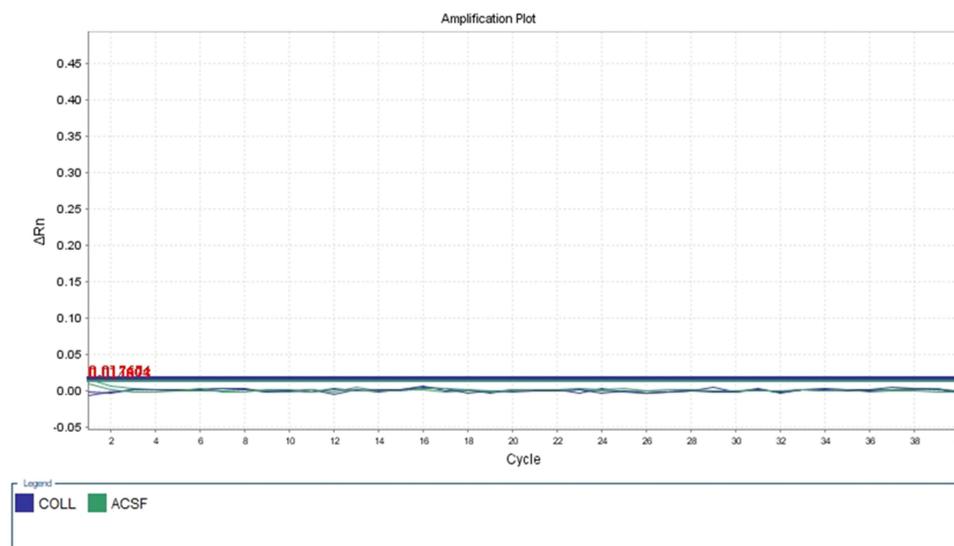


Figure 5 Unconverted human genomic DNA.

Discussion

ASD is a serious condition related to a neurodevelopmental disorders that can cause behavioral challenges and impairment in communication and social interaction with others.²² According to Autism and Developmental Disabilities Monitoring (ADDM), the prevalence of ASD in the USA was higher than the previous estimation in 2014 (1 in 59), whereas in 2016 (1 in 54) among children aged 8 years old, where boys were four times likely to be affected than girls.²³ In Saudi Arabia, the statistics of ASD in 2017 were around 50,714, whereas the actual prevalence has not been determined recently.⁷ The exact cause of ASD is still unclear. However, many studies suggested the implication of epigenetic mechanisms in ASD, specifically DNA methylation.^{24,25}

This study aimed to increase the understanding of the biological role of *ACSF3* promoter methylation in the binding ability of the TF by analyzing the correlation between the expression level of *ACSF3* and its candidate TF (*SPI1*) via

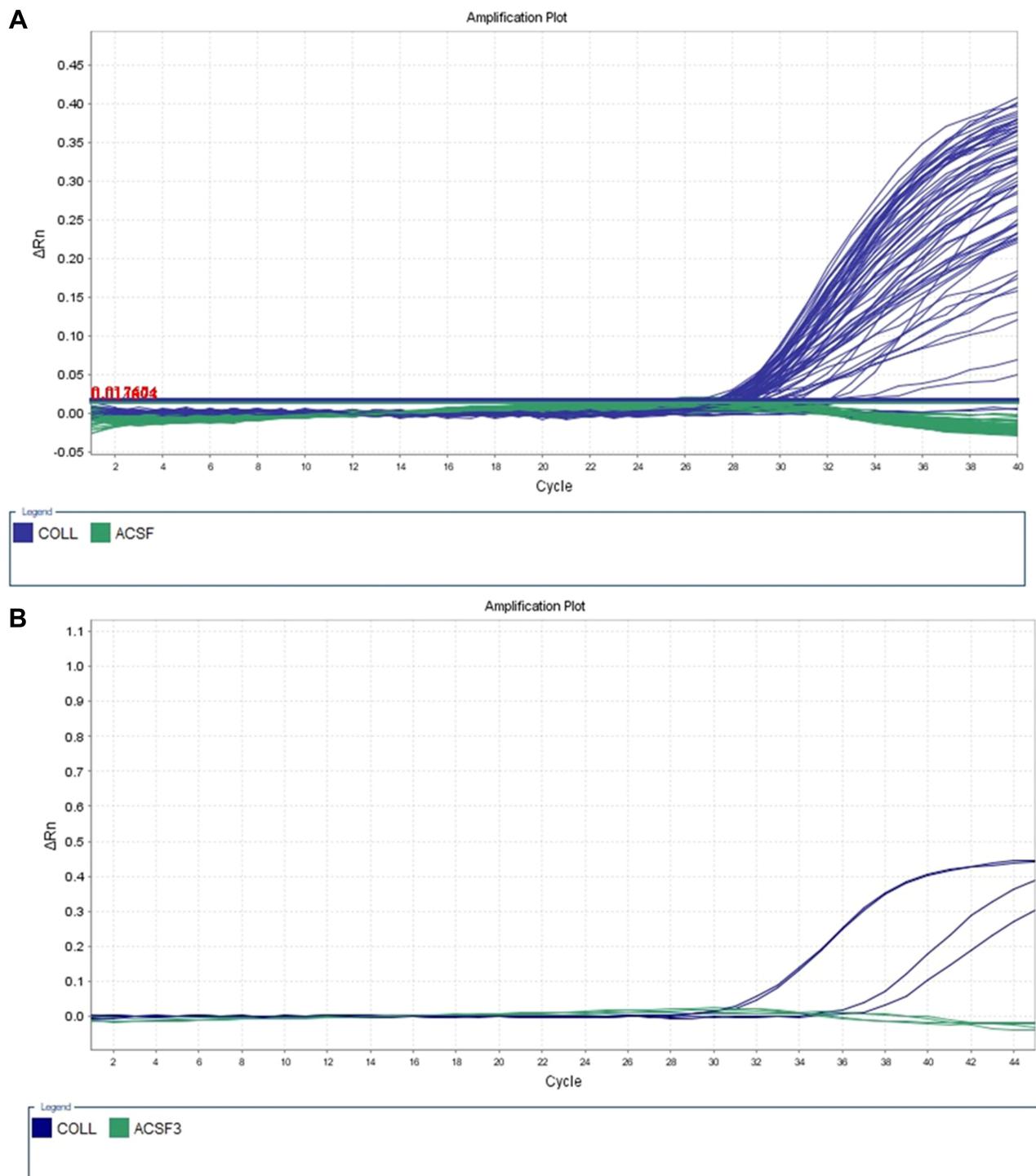


Figure 6 Converted DNA samples. (A) Sample (1–40). (B) Samples (41–42).

using qPCR. Then, detecting and quantifying the methylation level of specific binding sites of *SPI* in the promoter region of *ACSF3* was analyzed by MethyLight qPCR. *SPI* was chosen as a candidate TF because it has many recognitions binding sites in the promoter region of *ACSF3* that are rich with CpG sites, thus DNAm may interfere with the binding of Sp1 to the promotor of *ACSF3*.

Our results indicated that transcriptional regulation of *ACSF3* is associated with *Sp1* in ASD. Thus, *Sp1* could be essential to regulate transcription of *ACSF3* and any dysregulation of *SP1* is directly affect the *ACSF3* expression. These variations in the expression levels of *ACSF3* and *SP1* in ASD cases could be related to the genetic pathway or environmental factors. In regarding to methylation analysis, the result revealed that the methylation of the CpG sites in *ACSF3* does not affect the affinity of *SP1* to bind and regulate the promoter of *ACSF3* which is consistent with the finding of previous study which reported that the methylation level of CpG island does not affect the binding sites of *Sp1*.²⁶ Furthermore, another possible explanation of this result might be related to multiple binding sites of *SP1* within the promoter region and gene body. Supporting this assumption, Wang et al demonstrated that DNAm within *ITPKA* gene body modulates the binding of *SP1* TF to the promoter region of *ITPKA*.²⁷ Moreover, according to the study of Laubach et al, DNAm level that play role in expression of the *ACSF3* gene was low in the body not in the promoter region of participants who have low socioeconomic status during the prenatal period.²⁷

Despite the MethyLight qPCR result revealed no methylation pattern in the selected region of the promoter regions of *ACSF3* in all autistic samples, there was a positive correlation between expression levels of *ACSF3* and *SP1* in the same samples of patients with ASD. This result is in the line with those of previous study which reported increasing in *SP1* expression in autistic brains and the expression of related autistic candidate genes.²⁸ Therefore, the dysregulation in the expression of *ACSF3* and *SP1* indited that these genes could have a role in ASD which correlated to specific symptom or mechanism of ASD.

Conclusions

This study did not confirm the role of DNAm on the binding site of *SP1* within the *ACSF3* promoter in autism Saudi children. The results partially substantiated the correlation between the expression of *ACSF3* and *SP1* in ASD Saudi children. The positive expression correlation between *ACSF3* gene and its transcription factor (*SP1*) in all autistic samples pointed a potential role of *SP1* as a target for *ACSF3* in ASD. Furthermore, this correlation may be associated with certain mechanism or symptom of ASD etiology. However, studying the pathway mediated by the *Sp1-ACSF3* could explain the complex phenotypes associated with autism.

In addition, although the methylation analysis technique that was used in this study was shown to be sequence-specific, it may not have been enough to cover all CpG sites in transcription binding sites of *SP1* in *ACSF3*. Therefore, further studies are needed to analyze the potential interaction between *ACSF3* and *SP1* in ASD using advanced techniques and large samples to explore the pathophysiology of ASD.

Institutional Review Board Statement

The study was designed with correspondence to the codes of the guidelines for Ethics Committee of Biomedical Research-Centre of Excellence in Genomic Medicine Research at King Abdul Aziz University, ethical approval number (02-CEGMR-Bioeth-2018). The study was executed in consensus with the guidelines followed in King Fahd Center for Medical Research, KAU, Jeddah, Saudi Arabia, which were in accordance with declaration of Helsinki.

Informed Consent Statement

The informed consent was signed by boy's parents for the publication of his case details.

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Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests.

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