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Down-Regulation of *Shank3* Gene in Patients with Autism Spectrum Disorders and its Reflects in Ability to Speech, Education and Social Communication

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Abstract: The objective of this study was to detect the effect of *Shank3* gene expression levels and their related to speech ability, education and social communication in children with Autism Spectrum Disorders. Forty ASD patients who were admitted to the (Rahman Specialist Centre for the care and service autistic children/ Baghdad/Iraq) and ten children apparently healthy group. Patients and healthy control group aged from (3-10) years. According the sex the results showed a significant increase in male (77.5%) compare to female (22.5%). Patients with ASD recorded a weak ability to speech in high significance increase (72.5%) compared to another group. While the ability to education and ability to social communication were moderate in high significant increase (65.5%) in patient with ASD. The quantitative Real-Time PCR Results showed a significant decreased (0.374 ±0.18) in ASD patients when compared to the healthy control group (1.00 ±0.00). The down-regulation of *Shank3* appear a positive correlation to Ability to Speech, Ability to Education and Ability to social communication.

Key words: Autism Spectrum Disorders, Quantitative Real-Time PCR, *Shank3* gene.

1. Introduction:

Numerous sensory impairments, particularly in the area of tactile sensitivity, are indicative of autism spectrum disorders (ASD) [Tomcheck et al., 2007][1]. Among these, aberrant nociception—which shows up as either hypersensitivity or hyposensitivity to painful stimuli—is remarkably prevalent in ASD. the prevalence of unreported wounds, self-mutilation (including self-extraction of teeth), and self-harm (Moore, 2015). Neonatal hypotonia, moderate to severe intellectual disability, absent to severely delayed speech, and mild dysmorphic characteristics are common in these patients(Phelan and McDermid,2012). Autism is a lifelong condition that first appears before the age of three. Additionally, synaptic connections between neurons are formed, reinforced, and altered during the first few years of life. In recent years, a large number of genes essential for synaptogenesis and synapse function have been linked to a variety of neurological and psychiatric conditions, including ASD (Mitcheel, 2010). One copy of *Shank3* losing its function is known to induce Phelan-McDermid syndrome (PMS), which in turn causes ASD and intellectual disabilities (Tachibana et al., 2017; Mitz et al., 2018). Specifically, it has been frequently documented that people with ASD have mutations in genes that code for scaffold proteins such neuroligins, neurexins, and SHANK as well as synaptic cell adhesion molecules (Geschwind, 2009). These proteins are essential for the development and maintenance of synapses (Huguet et al., 2013). Thus, the several genetic alterations that impact chromatin remodeling, synaptic translation, formation, and function have made the synapse a common target (Sudhof, 2008). The scaffold proteins found at the post-synaptic density of glutamatergic synapses are encoded by shank genes (Toro et al., 2010). While SHANK1 causes the expansion of spine heads, Shank2 and *Shank3* positively influence the induction and maturation of dendritic spines in neurons. Autism spectrum diseases have been linked to shank gene mutations

(Leblond et al., 2014). The 57 kb genome of the human *Shank3* gene, which is found on chromosome 22q13.3, has 24 exons. Exon 18, which is mostly found in the brain, is one of seven exons that are alternatively spliced (Leblond et al., 2014). The purpose of this study was to determine how the *Shank3* gene's level of expression affected the speech, social communication, and educational abilities of children with autism spectrum disorders.

2. Materials and Methods:

2.1. Ethical Approval:

Prior to their inclusion in the study tests, the Iraqi Ministry of Health and the Ethics Committee of the Department of Biology, College of Science, University of Wasit, gave their approval. Every participant in the study had their father's signed written consent secured.

2.2. Sample Collection:

Ten children who appeared healthy served as a control group, while 40 ASD patients who were admitted to the Rahman Specialist Centre for the Care and Service of Autistic Children in Baghdad, Iraq, had three milliliters of blood drawn. Patients ranged in age from three to ten, as did the healthy control group. The center's consultant medical staff has made a clinical diagnosis of the illness. For molecular analysis, EDTA tubes have been filled with each obtained blood sample.

2.3. Gene Expression:

Total RNA of all samples was extracted using the TRIzol®LS Reagent according to the manufacturer's instructions. Total RNA was reversely transcribed to complementary DNA (cDNA) using WizScript™ RT FDmix Kit. The procedure was carried out in a reaction volume of 20µl. The reveres transcription step was carried out one cycle using the next program: 25°C for 10 min,42 °C for 10 min,85°C for 5 min and 4°C to the end of the run. The expression levels of *Shank3* gene were estimated by quantitative real time-PCR (qRT-PCR). To confirm this expression EVA Green was used. The mRNA levels of reference gene Glyceraldehyde 3-phosphaee dehydrogenase (gapdh) were amplified and used to normalize the mRNA levels of the shank gene.

2.4. PCR Reaction and Program:

Quantitative Real-Time PCR reaction was performed using specific primers. Lyophilized primers were dissolved in a free DNase/RNase water to give a final concentration of (100 pmol/µl) as stock solution, to prepare 10µM concentration as work primer resuspended 10 pmol/µl in 90 µl of demonized water to reach a final concentration 10µM as work solution, the program of the reaction was : Initial denaturation: 95°C for 5 min (on cycle), Denaturation: 95°C for 40 sec, annealing (gapdh =58°C *shank3*=55°C) for 40 sec, Extension :72 °C for 1 min , the run carried out with 35 cycles then holding with 4 °C for1cycle).The sequences of gapdh gene primers was F:5′-AACTTTGGCATTG TGGAAGG-3′, R:5′-ACACATTGGGGGTAGAACA-3′ [9] and *Shank3* gene was (F:5′-CTGCGCTCCAAGTCCATGACA-3,R:5′-GGCCCTGG CGTTCAAACAATG -3′.

2.5. Statistical Analysis:

ΔCT and ΔΔCT were calculated according to Livak and Schmittgen equation [2001]. The statistical analysis system –SAS program [2004] was used to the effect of difference factors in traits in this study. Least significant difference (LSD) test was used to the significant compare between means.

3. Results and Discussions:

3.1. Distribution according ASD features:

Distribution of patients according the gender showed there were a significant ($P \leq 0.05$). increase in male (77.5%) compare to female (22.5%). The patient with ASD recorded a weak ability to speech in high significance ($P \leq 0.01$) increase (72.5%) compared to other group. While the ability to education and ability to social communication were moderate in high significant ($P \leq 0.01$) increase (65.5%) in patient with ASD. Table (1)

Table (1): Distribution of Case according to Ability to Speech, Education and Social communication

Factor		No	%	P-value
Gender	Male	31	77.50	0.0005 **
	Female	9	22.50	
Ability to Speech	Good	1	2.50	0.0001 **
	Moderate	10	25.00	
	Weak	29	72.50	
Ability to Education	Good	6	15.00	0.0001 **
	Moderate	26	65.00	
	Weak	8	20.00	
Ability to Social communication	Good	6	15.00	0.0001 **
	Moderate	26	65.00	
	Weak	8	20.00	
Age (year)	Mean ±SE	530 ±0.31		---
**** (P≤0.01).**				

Two primary characteristics of autism spectrum disorders are limited interests and repetitive activities, as well as deficiencies in social interaction and communication. Patients may exhibit signs of ASD as early as 12 months, however it may take until later in childhood for these symptoms to be officially diagnosed as ASD (Tachibana et al., 2017). According to research on ASD, some patients have trouble updating links between unpleasant unconditioned stimuli and environmental signals (South et al., 2012). Lee and Odom (1996) study comparing the social interaction of two children, one with ASD and the other with a mental disability, who were both between the ages of 7 and 8. We watched how they interacted with their peers. The study's findings demonstrated that compared to other mentally challenged children, the ASD youngster was more reclusive while interacting socially with classmates.

The symptoms of ASD are defined in the following ways: anxiety related to language and social skills, variations in how the senses react to stimuli, Incapacity to interact with others and lack of speech and communication, even though linguistic skills are present (American Psychiatric Association, 2013). Out of 80 cases, Kadhim et al. (2015) reported: There were 40 children with ASD, 20 children without ASD (siblings), and 20 healthy children without relations. The risk of ASD males was larger in 31 (77.5%) than in ASD females (9 (22.5%), with a 4:1 ratio. Prior research used a mouse model lacking a subset of the key *Shank3* isoforms (*Shank3*E13), which showed behavioral abnormalities such as excessive grooming, impaired social interaction, reduced rearing, locomotor impairments, and learning and memory deficits (Jaramillo et al., 2017). Along with finding reduced hippocampal long-term potentiation, morphologic and structural abnormalities such as reduced spine density and longer dendritic length, and a decrease in synaptic proteins that associate with *SHANK3*, other studies have also confirmed these findings in similar *Shank3* mouse models (Yang et al., 2012; Kouser et al., 2013).

3.2. Molecular Study:

Since the blood was processed right away, total RNA was successfully recovered from every sample. The range of total RNA concentration and purity was 65–98 ng/µl and 1.68–1.97 ng/µl, respectively. Following the RNA extraction procedures, cDNA reverse transcription was performed. Random hexamer primers that cover all RNA regions to produce cDNA were included in the kit.

3.2.1.Quantitative Real-Time PCR Results:

In this experiment, the EVA green was used for real-time PCR quantification. Any double-stranded DNA, including cDNA, was detected by the fluorescent dye, and the amplification was quantified as a Ct value. Higher copies of the target are present when the Ct value is smaller, and vice versa. A low

level of gene expression is indicated by high Ct values. The Ct value of gapdh did not significantly differ between the individuals and the healthy control group (1±0.00). According to Table (2) and Figure (1), the patient groups' mean fold of gapdh gene expression was 0.98 ± 0.08.

Table (2): Comparison of gapdh fold between study groups depending on 2- ΔCt Method.

Group	Mean Ct of gapdh	2^-ct	Expression group/ control group	mean fold of gapdh expression
Patients	29.977	9.5 E10	9.5 E10/9.7 E10	0.98 ± 0.08
Control	29.946	9.7 E10	9.7 E10/9.7 E10	1 ± 0.00
LSD value	---	---	---	0.217 NS
NS: Non-Significant.				

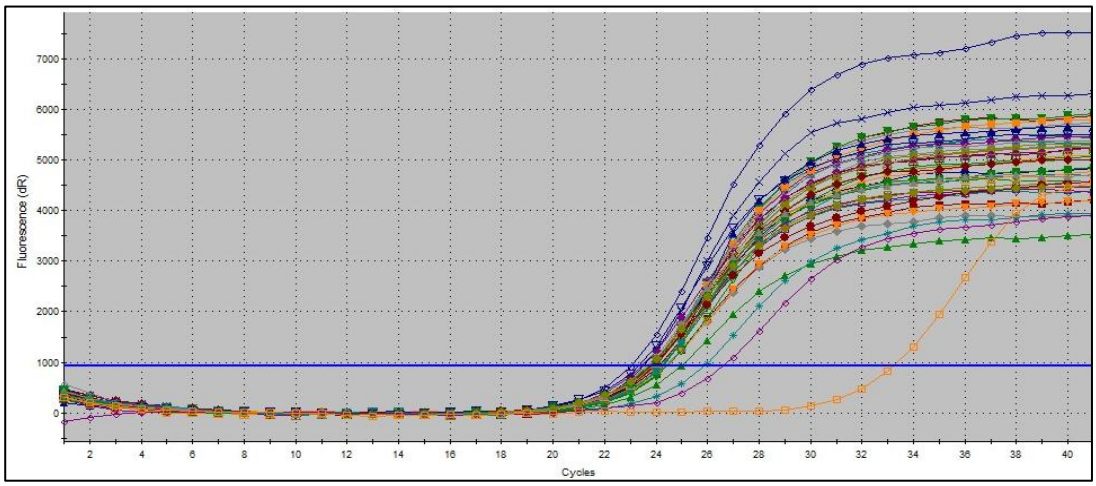

Figure (1): gapdh genes amplification plots by qPCR .Ct values was ranged from 23.32 to 25.4.The photograph was taken directly from Qtower2.0/2.2

The usage of housekeeping genes in molecular research is predicated on the underlying premise that the cells' expression of these genes is constant (Reboucas, 2013). According to the gene expression data, gapdh is one of the most often used housekeeping genes (Barber et al., 2005). Using qRT-PCR, Robert et al. (2005) investigated the expression of 1718 genes. They used the gapdh gene as a reference gene in seventy-two different types of healthy human tissues. When used in clinical investigations, they discovered that gapdh is a very dependable method for qRT-PCR normalization.

3.2.2. *Shank3* Gene Expression:

Expression of the *Shank3* gene was a significant decreased (0.374 ±0.18) in ASD patients when compared to the healthy control group (1.00 ±0.00) as shown in table (3), Figure (2).

Table (3): Comparison of *Shank3* gene fold between groups depending on $2^{-\Delta Ct}$ Method.

Group	Mean Ct of *Shank3* gene	Mean Ct of gapdh gene	ΔCt	ΔΔ Ct	*Shank3* Fold change
Patients	20.01	20.94	-0.9379	-2.3461	0.421 ±0.19
Control	19.58	18.16	1.5256	1.5256	1.00 ±0.00
T-test	--	--	--	--	0.518 *
P-value	--	--	--	--	0.0476
*** (P≤0.05).**					

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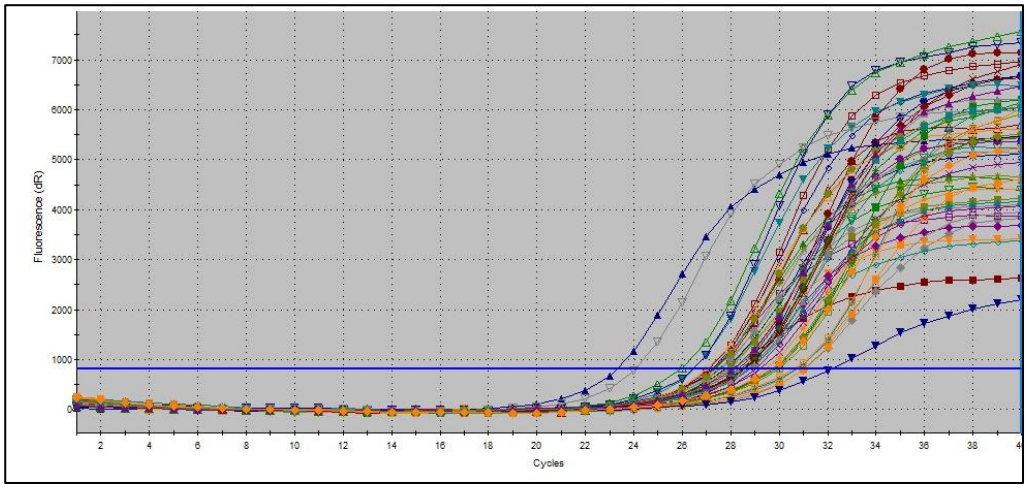

Figure (2): *Shank3* gene amplification plots by qPCR .Ct values was ranged from 28.34 to 32.13.The photograph was taken directly from Qtower2.0/2.2,

The substantial reduction in gene expression caused by gene mutations, particularly *Shank3* mutations in three families were examined by Durand et al. (2007), who found that frameshifts and translocations were linked to ASD. *Shank3* mutations are present in about 0.5–1% of all people with ASD (Jiang and Ehlers, 2013). In patients with autism and speech delay, Boccuto et al. (2013) discovered three instances of amino acid deletion leading to a frameshift mutation in *Shank3* and one instance of insertion resulting in an early stop codon. One of the best-studied genes linked to ASD is *shank3*. There are more than 40 known mutations in the *Shank3* gene that either result in truncating variations or are anticipated to be harmful, according to genetic studies conducted on patients with ASD. (Leblond et al., 2014).

Previous study showed that targeted disruption of the PDZ domain in the *Shank3* gene in mice led to a number of behavioral abnormalities (Jaramillo et al., 2017).

Gauthier et al. (2009) found two *SHANK3* mutations in a group of 427 individuals with autism spectrum disorder (ASD) including a de novo deletion that occurred at an intronic donor splice site, and a one-base-pair (BP) missense mutation that was transmitted by a father with epilepsy. A study of recurrent breakpoints in the *SHANK3* gene lead to the hypotheses that the autistic symptoms found in some patients with 22q13 deletion syndrome are most likely due to defects in *SHANK3* (Bonaglia et al., 2006). The 22q13 microdeletion syndrome is one of the most reported genomic rearrangements commonly related to cognitive impairment and it is associated with postnatal hypotonia, global development delay, normal or rapid somatic growth, non-development or severely retarded speech, the autistic phenotype, and minor dysmorphic features..

3.3. Correlation between *Shank3* Expression and Ability to Speech, Education and social communication.

Table (4) showed there were a positive Correlation between *Shank3* gene expression to Ability to Speech, Ability to Education and Ability to Communication

Table (4): Correlation between *Shank3* gene expression to Ability to Speech, Education and Communication

Features	Correlation coefficient-r with fold of *Shank3* gene	P-value
Ability to Speech	0.010 *	0.953
Ability to Education	0.147 *	0.400
Ability to social Communication	0.314 *	0.070
* (P≤0.05) , NS: Non-Significant.		

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Peca et al. (2011) created a model of *SHANK3* knockout mouse, in which a mutation in the PDZ domain created a range of behavior disturbances, such as deficiency of the social interaction, increased anxiety, and a set of resounding degrees of repetitive behaviors. Later on, Rendall et al. (2017) has reported that the knockout model shows an extended grooming period, augmented aggression, cognition, and better discrimination in pitch, which is a common phenomenon in persons with ASD (Remington and Fairnie, 2017). In a second study, Mei et al. (2016), showed that restoring expression of *SHANK3* in adult knockout mice is effective to normalize repetitive grooming and social behavior, indicating the role of this gene to produce modulation in behavioral phenotype even in later life.

Together, results in different *SHANK3* mouse models indicate that defect in the gene may affect features related to speaking as well as bipolar disorder and autism spectrum disorder (ASD). Both ASD and PhelanMcDermid syndrome (PMS) are characterized by speech and language issues, such as the absence or delayed development of the speech (and the use of simplified language), as well as their impairment (De Rubeis et al., 2018). According to these findings, it is postulated that PMS is an essentially a language, cognitive disorder with different neurophysiological processes being involved, unlike idiopathic ASD (Ponson et al., 2018). However, shared symptoms including engaged interests and repetitive therapy sustain in the two disorders (Soorya et al., 2013).

There is an increasing number of studies that confirm the correlation of *SHANK3* disruption with neurodevelopmental and psychiatric diseases and conditions, such as ASD and schizophrenia (De Sena Cortabitarte et al., 2017). As an example, de novo mutation in *SHANK3* was found in one person, and there were nine inherited nonsynonymous mutations found in *SHANK3* out of 400 persons having autism (Moessner et al., 2007). On the same note, a de novo splice-site deletion and a heritable missense mutation of *SHANK3* was also reported in relation to ASD by Gauthier et al. (2010). In a different study, a genetic analysis undertaken by Waga et al. (2011) using 128 individuals with ASD revealed multiple variants of the *SHANK3* gene as a six-amino-acid deletion upstream of the SH3 domain, missense mutation (arginine to histidine at position 656) within the PDZ domain, and a 10-basepair GC insertion/deletion polymorphism at 9 bp downstream of the 3 end of exon 11. The variants were not identified in 228 control subjects and the alterations were related with a harsh speech development.

Conclusions:

This study confirms the *Shank3* gene that is an important causable gene for the ASD and related to the autistic features. The evidence from previous studies has shown the different type of *Shank3* gene mutation, but this study concludes the down-regulation of *Shank3* is associated to Speech, Education and Social Communication ability. The results of recent study it may be promote the therapeutic approaches for the treatment of ASD, such as restoration of normal *Shank3* gene expression.

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