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Association of *CNTNAP2 gene* Variants with Palestinian Autism Patients

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Jerusalem- Palestine

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Dedication

To my family…

To my friends…

To my Teachers...

To all the people who supported me

Muhanad Said Suleiman Al-Qiq

Declaration:

I certify that this thesis submitted for the degree of master degree in Biochemistry and molecular biology is the results of my own research work, except where otherwise acknowledged. The results in this study has not been submitted for any other degree or publication in other universities or institution

Signed:

Muhanad Said Suleiman Al-Qiq

Date: 26/10/2018

Acknowledgment

Abstract

Autism is a neuro developmental disorder that involve various defects with vary severity such as lack of communication, social behavior or speech development. So far, the cause of autisms is still unknown; however, it is thought that genetic background might play a major role in the disorder. Various variants within the CNTNAP2 gene were linked to autism in many populations. Our objective focused on investigating the association of selected polymorphisms in the CNTNAP2 gene with Autism and its clinical symptoms.

A case control study was conducted between 45 autism patients and 145 healthy individuals. Using PCR-RFLP technology and direct DNA sequencing regarding to specific studied single nucleotide polymorphisms within the gene. The correlation between the genotype distribution and allele frequency between autism patients and healthy individuals and the clinical symptoms of the disease was analyzed using Pearson chi squared test (SPSS 22).

No significant association could be detected between the rs 2710102 and rs 7794745 variants between the autism patients and control subjects .significant correlation was evident between specific genotype interaction between the two SNP sites and the disease. Specific genotype association between one specific SNP variant in rs 2710102 and the ability for self-expression was evident.

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List of Abbreviations:

Abbreviation	Term
ASA	American psychiatric association
ASD	Autism spectrum disorder
ABA	Applied behavioral analysis
GWA	Genome wide association study
CNTANP2	Contactin associated protein gene
SNP	Single nucleotide polymorphism
DSM	Diagnostic and Statistical Manual of Mental Disorders
CNTNAP2	Contactin associated protein gene
STOX1A	Storkhead box A1
TCF4	Transcription factor 4
FOXP1	Foorkhead box P1
FOXP2	Foorkhead box P2

Chapter One

Introduction

1.1. Background

Autism came originally from a Greek word means "self". A physician named Eugen Bleuler first used the autism term in 1911 referring to symptoms in schizophrenia. Later, in 1944 and 1943 two American psychiatrics Leo Kanner and Hans Asperger took the pioneering step in redefying autism as individual disease with varying onset of psychological symptoms, being what is called Asperger syndrome is the milder case ("Autismus Hamburg - Was ist Autismus", 2018).

Recently Autism spectrum disorder ASD is defined according to American Psychiatric Association by deficits in social communication and social interaction on many levels, such as deficits in social interchange, nonverbal communicative behaviors used for social interaction, and skills in developing, maintaining, and understanding relationships (American Psychiatric Association, 2013). Most of autism patients fail to achieve independency in adult life. In fact, minor percentage lived on their own or had constant jobs. The most patients needed help from their families and failed to be independent (Howlin, Goode, Hutton & Rutter, 2004). About 15-47% of patients achieved improvement in symptoms upon development, however at 2 years of age the symptoms begin to appear again and lose the improvement in speech and social skills. (Stefanatos, 2008) For many years, Autism and other closely related neurodevelopmental disorder, which are also considered subtypes of Autism such as Asperger syndrome, Rett syndrome, childhood disintegrative disorder, and pervasive developmental disorders, were diagnosed individually as a single condition according to the Diagnostic and Statistical Manual of Mental Disorders (DSM IV). In the last edition of the DSM , all of these subtypes are included under one classification known as Autism spectrum disorder (ASD) and the indicated subtypes are no longer diagnosed separately as individual disorder but under one condition named ASD with varying severity (American Psychiatric Association, 2013).

1.2. Diagnosis:

According to DSM 5, ASD diagnosed according to the following criteria:

A. Permanent lack of ability in social communication and interaction that include:

1. Difficulties in social and emotional contact such as, showing no interest in expressing feeling or interest or emotions. Also showing malfunction in responding to social interaction.

2. Difficulties in nonverbal communication like failure to maintain eye contact or proper body language.

3. Difficulties in forming and comprehending relationship, like showing no interest in making friends.

B. Restrained and repetitive form of behaviors, showing interest or activities as specified by at least two of the followings:

1. Repetitive motor movement like stereotyped playing style or using of objects or pattern of speech

2. Persistent attachment to certain routine or forms of verbal or non-verbal behavior Like eating the same food or taking the same route every day and showing extreme rage for small changes and modifications

3. Intense and abnormal concentration in certain interests in unusual objects and showing strong attachment

4. Showing hyper or hypo reactivity in sensory input or abnormal reaction to sensory changes in environment, like abnormal response to sound, temperature or pain

C. Symptoms must exist in early childhood and become fully visible later until social demands overwhelm the limited capability

D. Symptoms cause clinically significant deficits in social functioning.

Severity levels for the above-mentioned symptoms ranges from level 1 "requiring support"
Level 2 "requiring subnational support" and level 3 "requiring very substantial support".
A major percentage of ASD cases have a comorbidity with many psychiatric disorders such Attention-defective/ hyperactivity activity disorder (ADHD), or obsessive-compulsive disorder (OCD), or depression (Belardinelli & Raza, 2016).

1.3. Epidemiology

The prevalence of autism spectrum disorder across the U.S. and other developed countries have approached 1% of the population, with similar results in child and adult patients (American Psychiatric Association, 2013). A recent study in USA reported the prevalence of ASD among children was 14.6 per 1,000 (1 in 68), with 23.6 per 1,000 boys and 5.3 per 1,000 girls (Christensen et al., 2016). Very limited Studies regarding autism prevalence were performed in the Middle East. One study in the Saudi Arabia estimate the prevalence of autism is 18 per 10000 (Al-Salehi, Al-Hifthy & Ghaziuddin, 2009). In UAE until 2007, the estimate is 58 per 10000 (Eapen, Mabrouk, Zoubeidi & Yunis, 2007). The prevalence in Oman is 1.4 ASD patients per 10000 with boys representing 75% of the cases (Al-Farsi et al., 2010). A study published in Israel reports the prevalence of children with ASD was 3.6 per 1000 in 2003 with an increase of three times, comparing with data from 1986, which was 1.2 per 1000. In addition, higher prevalence was seen in males with 5.7 per 1000 then females with 1.2 per 1000 (Gal, Abiri, Reichenberg, Gabis & Gross, 2011). A Later study regarding Israeli population in 2010 found a prevalence of 0.48 % of children aged between 1-12 years old and 0.65 % of children aged 8 years old (Davidovitch, Hemo, Manning-Courtney & Fombonne, 2012). In Palestine, no published data is available regarding the prevalence of autism among the population. Figure 1.1 summarizing a meta-analysis study reviewing autism prevalence studies worldwide from 1966 to 2004 as noticed the increase in autism incidence over the years, which is in part could be due to changing of diagnosing criteria over the years (Williams, 2005).
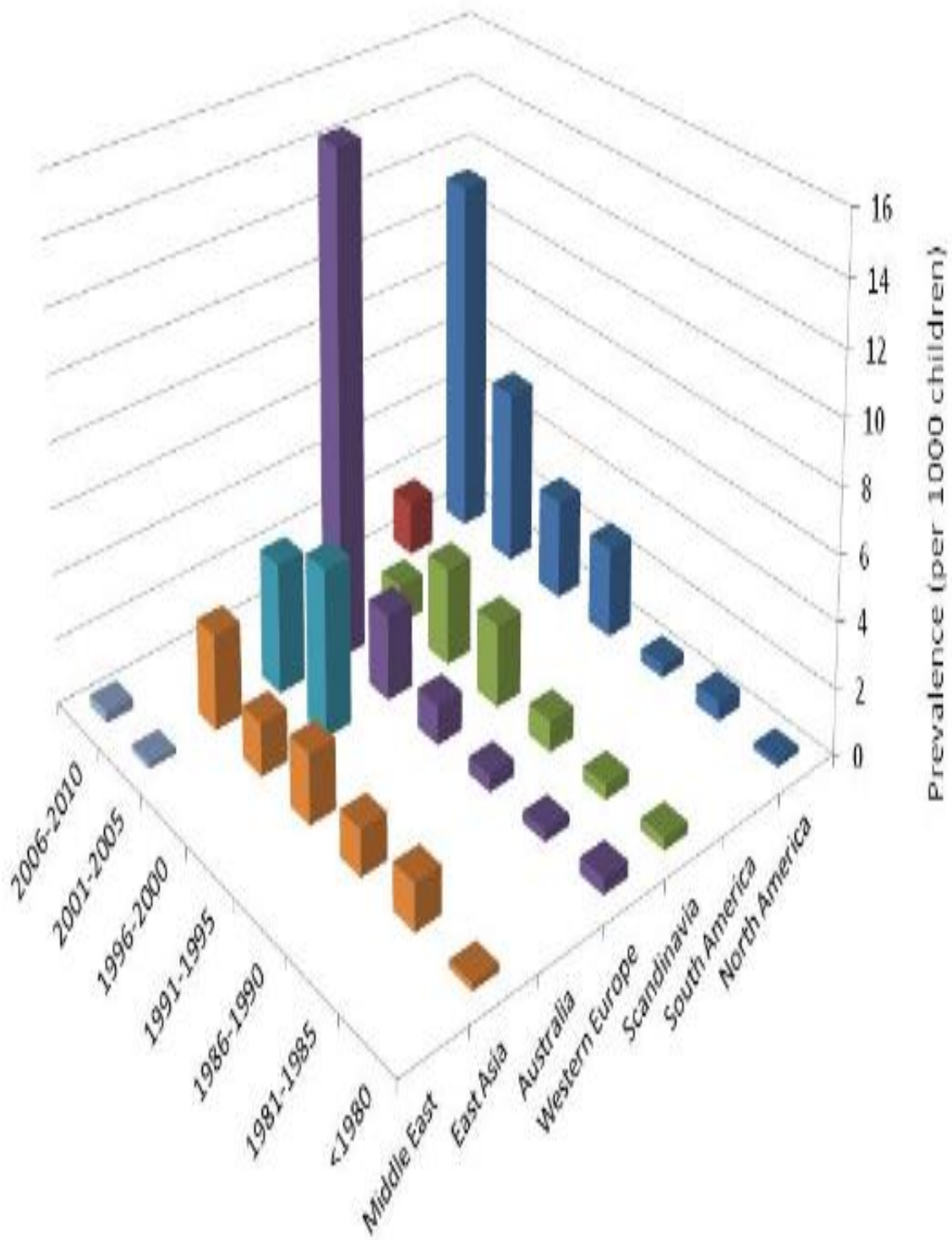
Figure 1.1: diagram summarizing prevalence study in autism from studies older than 1980 to 2010, designated at 5 years intervals ("How prevalent is autism? - Autism Reading Room", 2018)

1.4. Economic burden

Economical cost of autism in the UK and US was $2.4 million and $2.2 million respectively during the lifetime of autistic patients with intellectual disability. In addition, $1.4 million for those without intellectual disability. This economic burden was much higher in adults than children (Buescher, Cidav, Knapp & Mandell, 2014). Another study in the US calculated the economic burden of autism in 2015 to be $ 268 billion and expected to rise to $461 billion in 2025. This makes autism in line with economic burden for diabetes and ADHA, and higher than stroke and hypertension (Leigh & Du, 2015). Moreover, Autistic patients have low contribution in employment or education after high school. In fact, more than 50% have no enrolment in jobs or education after high school and only 34.7% enroll in college after high school, which makes them with the lowest enrolment rate in employment compared with other normal youth, and highest rate of no contribution in employment among other disabilities (Shattuck et al., 2012).

1.5. Risk factors

There are numerous risk factors regarding reports in literatures with the followings represent most significant.

1.5.1 Maternal and paternal age

 Many studies have confirmed a trend of higher maternal age associated in increased chances of having child with autism. One study in the US, which recruited 3013 children by age of 36 months, found that child of mothers aged younger than 24 years of age had lower chance of any early infant diagnosis, while mothers who aged older than 30 years old had a greater chance of having child with early autism diagnosis (Manning et al., 2011). A study in Sweden reported that maternal aged higher than 40 had a significant risk of having child with autism, with odd ratio 2.5 (Haglund & Källén, 2010). Regarding Paternal age many studies showed that advance paternal age is considered a major risk factor regarding autism. In fact, father's age linked to higher incidence of de novo mutation, which possibly linked to disease such as autism and schizophrenia (Kong et al., 2012). In one study done in Sweden showed that men aged more than 50 years old had 2.2 times

greater chance of having children affected with autism than children of men aged <29 years old (Hultman, Sandin, Levine, Lichtenstein & Reichenberg, 2010) . Another study found that fathers aged more than 40 years old were more likely to have offspring with autism by 3.3 times than of offspring of fathers aged less than 20 years old (Buizer-Voskamp et al., 2011). A study done in Israel has revealed that there is a significant association between paternal age and the risk of developing autism, in which Childs of fathers aged 40 years or older had increased chance of 5.75 times compared with Childs of men aged younger than 30 years old (Reichenberg et al., 2006). The mixture of maternal and paternal age was evaluated. and revealed that the increase by 10 years in maternal and paternal age correlated significantly with the risk of developing autism. Moreover, both of their age independently affects their offspring regarding autism. (Croen, Najjar, Fireman & Grether, 2007)

1.5.2 Offspring sex

 Child gender is considered one of the major risk factor regarding autism. In fact, males are 3.4 times more likely to be diagnosed with autism than females in US population. (Christensen et al., 2016). Another study in the same population suggest that most of children diagnosed with autism are males with percentage of 81% (Giarelli et al., 2010). In Israel, also most of children diagnosed with autism are males with percentage of 84.4 %(Gal, Abiri, Reichenberg, Gabis & Gross, 2011). In Palestine, most diagnosed patients with autism are males reaching about 90 % (unpublished data).

1.5.3 Prenatal and neonatal environment

A meta-analysis study discussing the relationship between prenatal and neonatal environment and chances of having autism. In which 60 prenatal or neonatal factor were investigated it was found that the factors most linked to autism risk were abnormal representation, birth injury or trauma, fetal stress, umbilical cord complication, multiple birth, maternal hemorrhage, low birth weight, summer birth, small gestational birth, congenital malformation, feeding difficulties, neonatal anemia ABO or RH incapability and hyperbilirubinemia. However, factors that are not associated with autism include anesthesia, post term birth, high birth weight and assisted vaginal delivery

(Gardener, Spiegelman & Buka, 2011). Prenatal exposure to certain chemicals and medications such as valproic acid, ethanol, thalidomide and misoprostol proved to increase risk of autism (Dufour-Rainfray et al., 2011.Strömland, Nordin, Miller, Akerström & Gillberg, 2008). Regarding smoking in pregnancy, one study suggested, that there is increased risk for a child being diagnosed with autism by 1.4 times, if his mother was smoker during prenatal stage compared with nonsmoker mothers (Hultman, Sparén & Cnattingius, 2002).

1.5.6 Vitamins levels

Level of 25-hydroxy vitamin D has been found to be significantly lower in autistic children versus normal ones. In fact, 40% of these children whom were diagnosed with autism were vitamin D deficient (Mostafa & AL-Ayadhi, 2012). Regarding Folic acid intake during pregnancy, there is a clue that pregnant women who took folic acid during pregnancy had lower risk of having autistic child compared with women who did not took folic acid during pregnancy (Wang, Li, Zhao & Li, 2017). However, one study reported that highe intake of folic acid and B12 during pregnancy are associated with increased chance of having child with autism. Moreover, the high level of B12 and folic acid at birth had an increased the risk by 2.5 times (Raghavan et al., 2017).

1.6. Morphological features and minor anomalies

Children diagnosed with autism have distinguishable morphological features such as protruding forehead, lack of symmetrical feature in the face, and hair whorls that have been found significantly associated with autistic children compared with health individuals (Ozgen, Hellemann, de Jonge, Beemer & van Engeland, 2012). Minor physical anomalies, such as abnormal physical characteristic variation that themselves are not dangerous, those anomalies were found to strongly associate with autistic children. Examples on those anomalies include low set ears and high palate (Ozgen, Hop, Hox, Beemer & van Engeland, 2008). In fact, those distinguishable physical features of autistic children could be used as diagnosed tool; one study was able to construct a predictive model based on such features (Ozgen, Hellemann, de Jonge, Beemer & van Engeland, 2012). Regarding brain anatomy, brain and head overgrowth especially in the prefrontal cortex has been noticed in autistic children aged from 9 -18 months.

In fact, autism patients had 67% higher number of neurons in prefrontal cortex than normal individuals and 79% increase in neurons in the dorsolateral prefrontal cortex, and 29% more neurons in the mesial prefrontal cortex. It was found that brain weight was significantly higher in autism patients than normal individual was. It is worth to mention that the prefrontal cortex is the center for emotional, communication and cognitive functions (Courchesne et al., 2011). Figure 1.2 illustrates parts of the brain being affected with autism.

Parts of the Brain Affected by Autism

Cerebral Cortex:
A thin layer of gray matter on the surface of the cerebral hemispheres. Two thirds of this area is deep in the tissues and folds. This area of the brain is responsible for higher mental functions, general movement, perception and behavioral reactions.

Amygdala:
This is responsible for all emotional responses including aggressive behavior.

Basal Ganglia.
This is gray masses deep witin the cerebral hemisphere that connectes the cerebrum and the cerebellum. It helps regulate automatic movement.

Corpus Callosum:
This consists of closely packed bundles of fibers that connect the right and left hemispheres of the brain and allows them to communicate with one another.

Hippocampus:
This makes it possible to remember new information and recent events.

Brain Stem:
The Brain Stem is located in front of the cerebellum and serves as a relay station, passing messages between various parts of the body and the cerebral cortex. It controls the primitive funtions of the body essential to survival including breathing and heartt rate.

Cerebellum:
This is located at the back of the brain. It fine tunes motor activity, regulates balance, body movements, coordination and the muscles used for speaking.
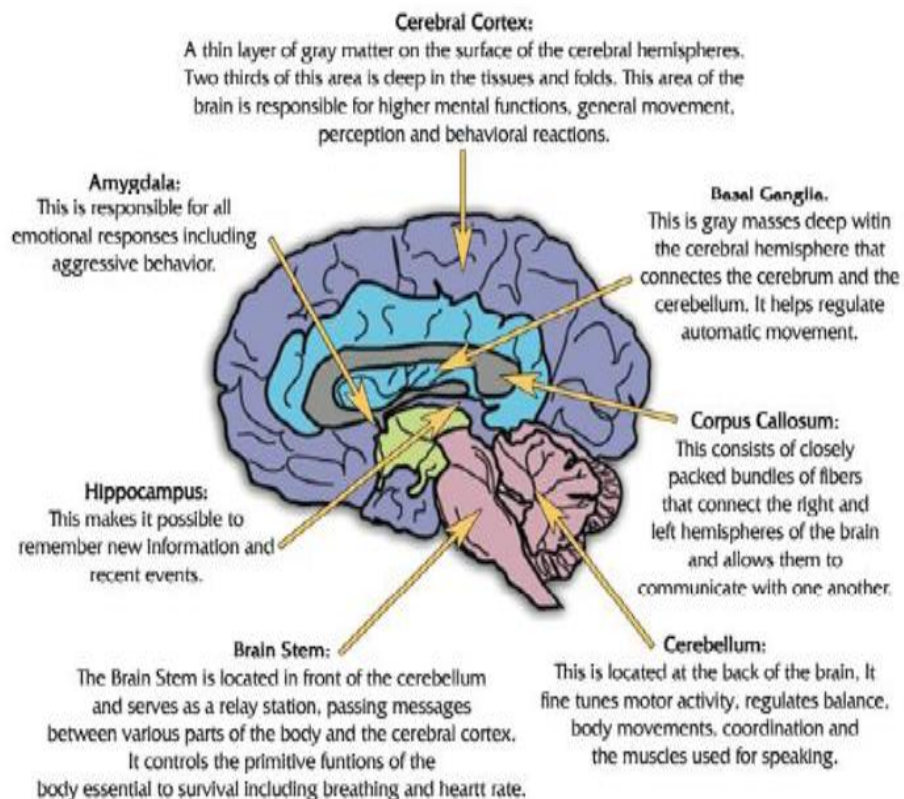
Figure 1.2: Autisms effect on brain (Islam et al., 2015)

1.7. Role of Genetics

There is no definitive cause of autism, and since its discovery, speculation of its cause has overlapped between biological and psychological factors but not until the twin studies in 1970s and 1980s, which suggested some evidence for a genetic cause. The first twin study in 1977 recruited 21 twin pairs with at least on twin showed signs of autism. 11 were monozygotic and 10 dizygotic. It was revealed that the monozygotic pairs showed significantly higher concordance for autism than the dizygotic ones. This study represented the first core of genetic evidence for autism although the mode of inheritance was not clear (Folstein & Rutter, 1977).

Another study done in population of Denmark, Finland, Iceland, Norway and Sweden which recruited 21 twin pairs (11 monozygotic, and 10 dizygotic) with same sex under age of 25. Concordance rate for autism was 91% in the monozygotic twin versus 0% dizygotic twin pair (Steffenburg et al., 1989). A Later study on autistic twins done in the British population has showed that 60% of the monozygotic pairs had concordance for autism while no concordance was evident in dizygotic pairs (Bailey et al., 1995). An expanded twin study which recruited the largest sample with 277 twin pairs (210 dizygotic and 67 monozygotic) aged 18 years of old or younger with at least one twin diagnosed with autism. It was revealed that monozygotic twins have significantly higher concordance rate with 88%, compared with dizygotic twins that have 31 % concordance rate (Rosenberg et al., 2009). Regarding family history and sibling genetics, one study has confirmed that the risk of developing autism increases by 22 times in children who has sibling affected with autism, and the risk of children being diagnosed with autism increased twice as high if their mother have a psychiatric disorder (Lauritsen, Pedersen & Mortensen, 2005). In fact, there is support if two autism patients are diagnosed in a family then the chances of having another child with autism could reach 25%. Moreover, the odds for having one or more traits of autism is 30% in adult sibling (Folstein & Rosen-Sheidley, 2001).

Many Minor cases of autism are common related to underlying medical disorders like fragile x syndrome or tuberous sclerosis complex. In fact, cytogenetic disorders and single gene abnormalities together with other rare diseases represent <10% of autism cases, making the remaining 90% known as idiopathic autism (Muhle, Trentacoste & Rapin, 2004). All the discussed studies confirmed that there is a hereditary component that participate in Autism risk. However, as single gene disorders such as, fragile x syndrome are ignored in major cases of autism, it would be more reasonable to study suspected single gene polymorphisms (Duchan and Patel, 2012).

1.8. Role of Epigenetic

Epigenetic is the study of changes and alteration to DNA that do not affect its structure, such as methylation or acetylation or post translational modification segments of the DNA that eventually lead to enhanced or reduced gene expression. (Dupont, Armant & Brenner, 2009). DNA methylation has been noticed in neurological disease such as Alzheimer and schizophrenia. in a cohort study for those two diseases targeting the temporal neocortex of 125 individuals whose age ranges from 17 weeks to 104 years old. A pronounced rise was shown in methylation state in 5'CpG Island of many genes, which have role in CNS growth and development such as GABRA2, HOXA1, and NEUROD2. (Siegmund et al., 2007).

Another example related to autism is Rett syndrome it is known that this syndrome is due to a mutation in methyl-CpG-binding protein2 (MeCP2), which act as inhibitor for gene expression at the level of transcription that bind and inhibit specifically methylated genes. Epigenetic alterations ware also noticed in fragile X syndrome and Huntington's disease (Ptak & Petronis 2010). Another study on 394 patients and 500 controls found that hypo-methylated state of ERMN gene and other different loci was significantly associated with autism (Homs et al., 2016).

An additional study investigated the impact of methylation pattern of the oxytocin receptor gene (OXTR) promoter on autism. The results revealed that hypomethylated state of the OXTR promoter at specific region (MT1 and MT3) has significantly associated with autism. (Elagoz Yuksel, Yuceturk, Karatas, Ozen & Dogangun, 2016).

1.9. Mortalities

One cohort study in Denmark calculated the mortality risk of autism and found that individuals diagnosed with autism has twice chance of death versus normal individuals with mortality ratio higher in female and the trend remained the same between 1993 to 2008 . In this study 314 autistic patients were recruited, where 26 died, and 12 of them their death was related to epilepsy (Mouridsen, Brønnum-Hansen, Rich & Isager, 2008). Another study in Sweden has calculated the death rate in 120 autistic patient and found it was 5.6 times higher than expected; also, the rate was higher in females (Gillberg, Billstedt, Sundh & Gillberg, 2009). It is noteworthy to mention that

the patients recruited in both studies had other medical disorders like epilepsy. Therefore, it is hard to conclude that autism alone was the main responsible cause for the increased death rate (Gillberg, Billstedt, Sundh & Gillberg, 2009).

Moreover, one systemic review study has calculated the percentage of death among autism associated with epilepsy to be 7-30 % in all the relevant studies, while other causes of death ranged from circulatory, respiratory, and malignancy complications. (Woolfenden et al., 2012)

1.10. Treatment

Until now, no cure for autism is currently available. Because the exact cause is not definite, but treatment approaches are followed to help manage symptoms. These include:

1.10.1. Psychosocial treatment

This treatment, which involves therapies, that are linked to behavior changes based on learning system such as applied behavioral analysis (ABA). ABA system depends on methods and principles which aim to instruct, guide, motivate, and train the patient toward certain behavior in order to induce alteration in the patient interest toward certain activities or skills weather, in speech or in communication and social behavior (Vismara & Rogers, 2010). Such treatment methods proved its efficacy, and was possible to regain in large percentage of autistic patients normal intellectual, and learning skills and consequently able to fit in normal schools (Lovaas, 1987). Another meta-analysis study aimed to validate the effectiveness of ABA method concluded that prolonged and intensive ABA is effective in regaining normal behavior, speech improvement and social communication rather than improvement in IQ (Virués-Ortega, 2010).

ABA method has many limitations such as the prolonged period it takes to notice its positive outcome, in addition to being expensive (Melissa & Dineen, 2016)

1.10.2 Dietary treatment

Nutritional restriction food proved its efficacy in improvement of autism symptoms. Studies had proved the gluten and casein free diet are effective in autism patients, and positive results are seen within 3 months (Kawicka and Regulska-Ilow, 2013). Another dietary treating strategy called ketogenic diet, which is rely mainly on low carbohydrates and high fat foods. In a pilot study which

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included 30 children, this diet produced positive outcome in improvement of autism symptoms when applied for six months (Evangeliou et al., 2003).

Another strategy is to limit oxalate from diet. It was observed that autistic patients have 3 times increase in oxalate plasma level compared with normal levels, which raises question whether the oxalate is crucial in pathogenesis of autism (Konstantynowicz et al., 2012). It is worth to note that dietary supplements such as omega-3 fatty acid, vitamin A, vitamin B6 and magnesium, have produced a positive effect on autism symptoms improvement (Kawicka and Regulska-Ilow, 2013).

1.10.3 Pharmacological treatment

Risperidone, which is atypical antipsychotic that work by blocking dopamine and serotonin receptors.in a randomized double blind study efficacy and safety of risperidone in treating autism symptoms had been studied compared with placebo , it has resulted in significant decrease in irritability compared with placebo. Moreover, it's the only FDA proved drug for treating symptoms in autism. However, it was noticed many side effects such, increased appetite, fatigue, dizziness, drowsiness and drooling. Risperidone was also safe and showed positive outcome in treating aggression and self-harm behavior (McCracken et al., 2002).

Another pharmacological agent called Aripiprazole, which is a partial dopamine agonist,

has been studied in term of efficacy and safety in autistic patients, and it has been found in double blind randomized study that is safe and effective in treating irritability in both adult and autistic children (Owen et al.,2009).

Another drug called Olanzapine, which is dopamine receptor antagonist; its efficacy has been studied on autism symptoms in an open-label trial recruited 40 children with a dose of 5-10 mg daily for 12 weeks. It was found that the drug has significantly lowered many symptoms associated with autism, such as irritability, hyperactivity, and inappropriate speech, and stereotyped behavior (Fido & Al-Saad, 2008).

1.11 Literature review

Many methods have been used to identify genes linked to autism including, linkage analyses studies, low scale genetic association studies, genome wide association studies (GWAS), expression profiling, experimental studies and genome wide copy number variant. Meta-analysis

reported over 2193 genes, 2806 SNPs/VNTRs, 4544 copy number variation and 158 linkage regions that have been linked to autism. Those genes were strongly involved in pathways such as, synapse transmission, Neuroactive ligand receptor interaction, axon guidance, transmission of nerve impulse and neuron differentiation. Those efforts lead to establish a database called AutismKB (Xu et al., 2011). Among those autism candidate genes, this thesis focuses on selected *CNTNAP2* gene polymorphisms and its correlation with autism and the various biochemical complications of autism.

1.11.1 *CNTANP2* gene

The Contactin associated protein gene (*CNTNAP2*) encodes a neuronal Trans membrane protein (Caspr2) which is a member of the neurexin super family involved in neural-glia interactions and clustering of potassium channels in myelinated axons (Poliak et al., 1999). The *CNTNAP2* is the largest gene in the human genome containing 25 exons and represent 1.5% of chromosome 7 at 7q35 (Nakabayashi & Scherer, 2001). (Figure 1.3)

CASPR2 was first discovered in 1999 as a mammalian homolog of drosophila neurexin IV, and as new member of the neurexin super family, localized at the juxtaparanodes of the myelinated axons and associated with potassium channels (Poliak et al., 1999).as illustrated in figure 1.4
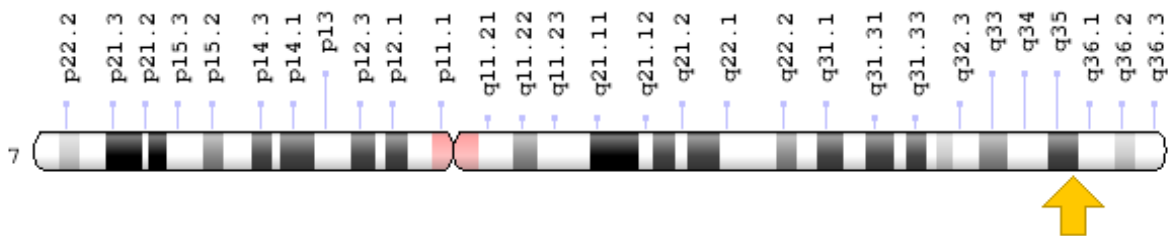
Figure 1.3: location of the *CNTNAP2* gene (https://ghr.nlm.nih.gov/gene/CNTNAP2#location).
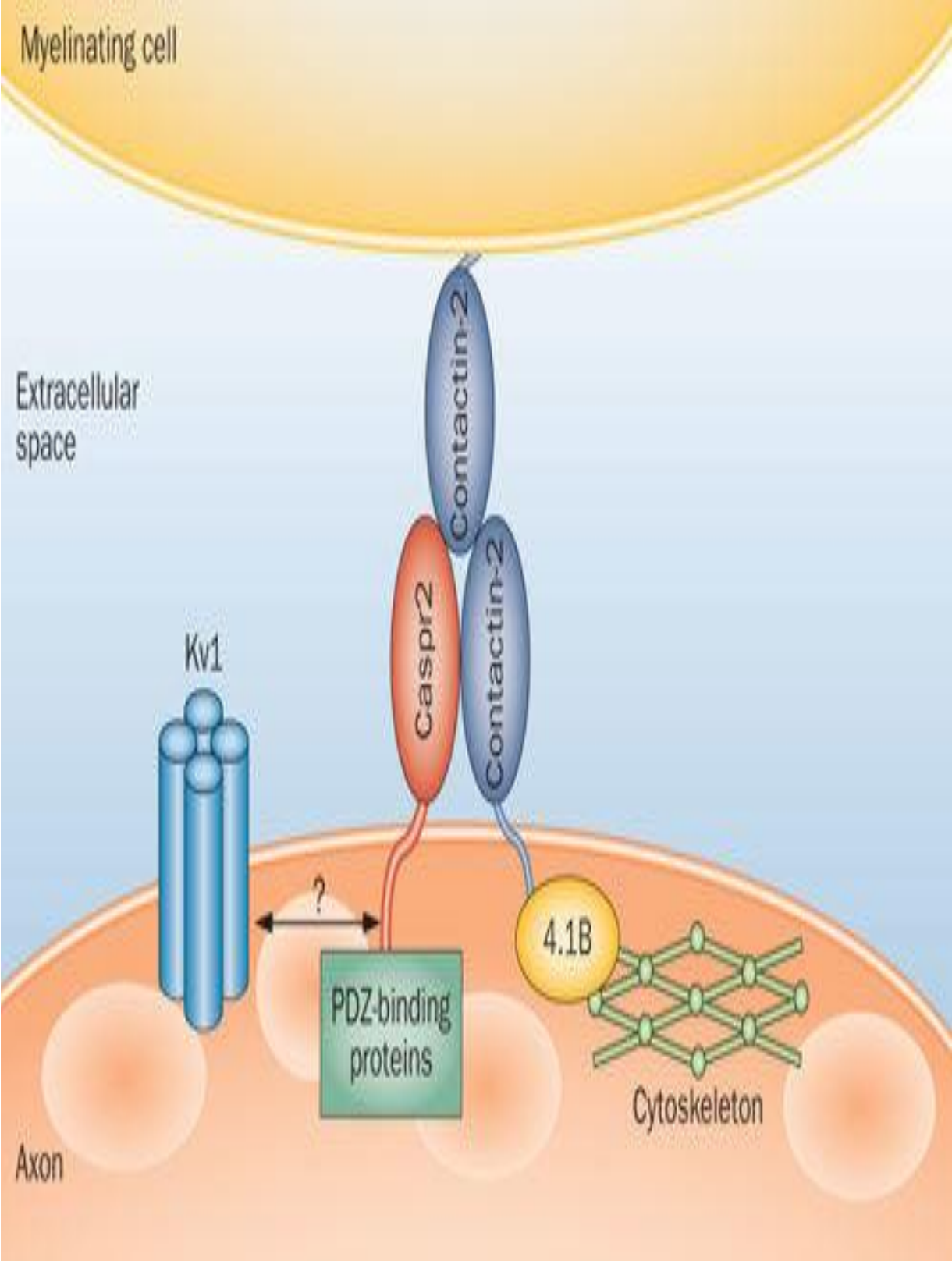
Figure 1.4: caspr2 protein localization (Lancaster & Dalmau, 2012).

CASPR2 contain seven domains, discoidin homology domain, laminin G domain, E= EGF like domain, FBG-like = fibrinogen –like region, TM = transmembrane protein, 4.1= protein 4.1 binding domain and PDZ interaction domain (figure 1.4 and figure 1.5). The discoidin and fibrinogen domains function in cell-cell adhesion and extracellular matrix interaction, the four-lamimin domains, and the epidermal growth factor domains are believed to function in receptor ligand interaction, migration, cell adhesion and differentiation while the rest of the protein are involved in protein- protein interaction. Interestingly caspr2 protein is highly conserved among other mammals with 94% and 99.5% similarity in mouse and chimpanzee, respectively (Rodenas-Cuadrado, Ho & Vernes, 2013).

The *CNTNAP2* gene encodes five transcripts, Transcript CNTNAP2-001, CNTNAP2-201, and CNTNAP2-002, which are translated into protein. CNTNAP2 gene contain four non-coding exons (Poot, 2015).

1.11.2 Molecular interaction in *CNTANP2* gene leading to different neurological disorders

CNTNAP2 expression is regulated by four transcription factors; storkhead box A1 (STOX1A), transcription factor 4 (TCF4), foorkhead box P1 (FOXP1), and foorkhead box P2 (FOXP2) (Rodenas-Cuadrado, Ho & Vernes, 2013). Interestingly de novo mutation in the transcription factor FOXP2 is linked to intellectual disability, autism and language impairment. In a case of two patients with de novo mutation in the FOXP2 had symptoms such as intellectual disability, autism behavior, mood liability, and physical aggressiveness and sever language impairment (Hamdan et al., 2010). This provide evidence on the potential importance of the CNTNAP2 gene regarding autism, as it is regulated by FOXP2
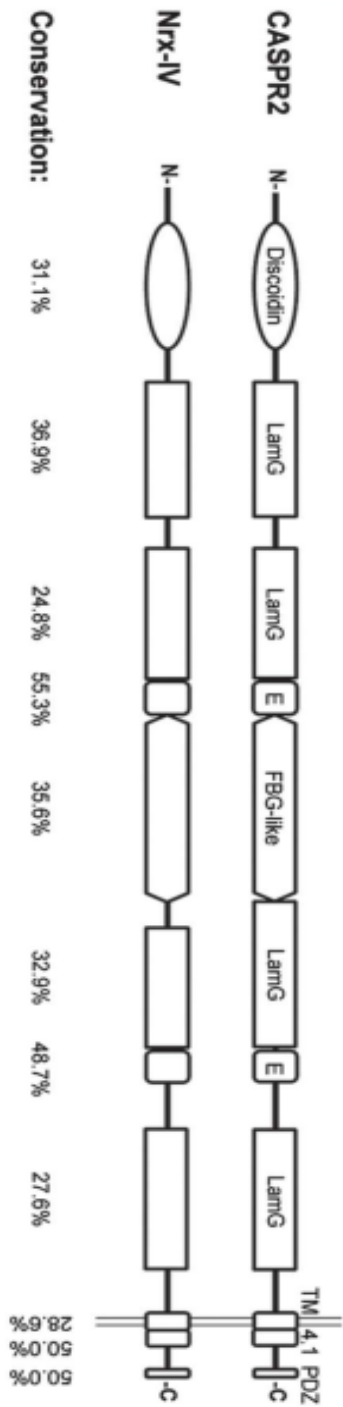
Figure 1.5: domains of CNTNAP2 gene product CASPR2 and their homology to Nrx-IV product. (Rodenas-Cuadrado, Ho & Vernes, 2013).

Another evidence suggested a role for CNTNAP2 gene in neurodegenerative diseases such as Alzheimer. Van Abel and colleagues found that CNTNAP2 gene expression is reduced in hippocampus of Alzheimer disease patients , and the mechanism is mediated by the transcription factor STOX1A which binds CNTNPA2 gene and down regulates its expression , while STOX1A expression is increased (Van Abel et al.,2012)

Moreover, it was found that malfunction in the transcription factor forkhead box P2 *(FOXP2)* that bind to intron 1 of the CNTANP2 gene, and consequently modulate its function, affect language and speech abilities, which represent another clue on the importance of CNTNAP2 gene in language function (Peñagarikano & Geschwind, 2012). Another study investigated the effect of polymorphisms in the FOXP2 gene on various speech and language impairments. It was found that variant rs7758412 in FOXP2 is significantly associated with non-word repetition in 188 family trio with dyslexia disorder (Peter et al., 2010). A further study identified variants in the promoter region of CNTNAP2 gene (rs150447075, rs34712024 and rs71781329) that altered the transcription factors binding site and thus reduced gene expression in differentiated and undifferentiated neuroblastoid cells due to reduced promoter efficacy, and were found to be associated with autism behavior (Chiocchetti et al., 2014).

1.11.3 Functional mutation in CNTNAP2 gene in different neurological disorders

The first link of CNTNAP2 gene to cognitive abnormalities was in 2003. It has been found the abnormal expression of the CNTNAP2 gene is associated with Tourette syndrome and obsessive-compulsive disorder, which was explained by disruption of repolarization of action potential as a result of defect in potassium channels that are related to CNTNAP2 function (Verkerk et al., 2003). In fact, it has been discovered that the CNTNAP2 gene contain 13 missense/nonsense mutations, and 282 single nucleotide variation (Poot, 2015).

Utilizing methods such as molecular karyotyping and mutational analysis, it was revealed that a mutation in the CNTNAP2 and NRXN1 cause mental retardation, seizures, autism behavior, and breathing defects resembling symptoms of Pitt-Hopkins syndrome Deletion of exon 2-9 in the CNTNAP2 gene was found in patients with Pitt-Hopkins syndrome (Zweier et al., 2009).

A study was done on a group of patients who belong to the Amish ethnic group. found that a single base mutation in the CNTNAP2 gene is associated with cortical dysplasia focal epilepsy (CDFE),

autistic behavior, hyperactivity language impairment, macrocephaly, weak or without tendon reflex, mental retardation rather than brain structural impairment such as unilateral dysplasia of the anterior temporal lope and malformation of the left striatum (Strauss et al., 2006). Moreover, neuropathology revealed abnormal organization of the neurons, increased neuron density in hippocampus and temporal neocortex, and decrease in the staining levels for CASPAR2. The patients were homozygous for the mutation and their parents were heterozygous which suggest a recessive effect for the mutation, which suggests Mendelian inheritance (Strauss et al., 2006).

In a case report which involve the same ethnic group, one girl previously found to have a homozygous mutation in CNTNAP2 gene rendered the gene inactive. Had physical abnormalities such as large head circumference and lowered deep tendon reflexes as well as clinical manifestation such as abnormal development, seizures, periventricular leukemia, mental retardation, hepatomegaly and autism characteristics like abnormal eye contact, detached personality and other related behaviors (Jackman, Horn, Molleston & Sokol, 2009). Another evidence presented by Penagarikano and his colleagues, showed that mice devoted from *CTNANP2*, had defects in vocal communication, repetitive and restrictive behavior and abnormal social interaction. This replicates the human autism behavior and its worth to note that treating knockout mice with the FDA approved drug Risperidone for treating autism symptoms has replicated its effect in human in which it has reduced symptoms like hyperactivity and other repetitive behavior. However, it did not reduce social symptoms as test in chamber social interaction test or sensory hypersensitivity (Peñagarikano et al., 2011).

One additional study reported microdeletion in a region that involves the first 3 exons of *CNTNAP2* gene in a Brazilian patient with stuttering and difficulties in learning and verbal communications. Remarkably, this deletion region of the *CNTNAP2* gene is target for binding the transcription factor *FOXP2*, which add greater emphasis on the role of molecular interaction on the *CNTNAP2* function (Petrin et al., 2010).

Another study reported a deletion in the *CNTNAP2* gene in a patient who suffer from schizophrenia, epilepsy and cognitive impairment rather than decline in communication and self-care capabilities. Since *CNTNAP2* function in organization of myelinated axons, and schizophrenia patients show myelin dysfunction, it might explain its role in schizophrenia (Friedman et al., 2007).

1.11.4 Effect of CNTNAP2 variants on brain structure in autism or related disorder

Using a neuroimaging technique, it was shown that the polymorphism rs7794745 in the CNTANP2 gene affect the changes in white and grey matter morphology in the brain, in which homozygous risk allele (T) significantly lowered the grey and white matter volume on parts in the brain related to autism such as cerebellum, fusiform gyrus, occipital and frontal cortices. (Tan, Doke, Ashburner, Wood & Frackowiak, 2010). In another study through utilizing functional magnetic resonance imaging found that certain parts of the brain are differently activated according to the CNTNAP2 variants (Whalley et al., 2011). They revealed that the homozygous risk allele (CC) of SNP rs2710102 was associated with increased activation of the right inferior frontal gyrus/frontal operculum and decreased activity of the left superior parietal lobule. Moreover, in a different study regarding SNP rs7794745, persons with the homozygous risk allele (TT) had significant increase activation of the right middle temporal gyrus (Whalley et al., 2011). Interestingly the frontal operculum and middle temporal gyrus are one of areas that also involved in language functions, which indicates a role for variants in *CNTNAP2* gene in language impairment by affecting brain structure (Whalley et al., 2011). Alarco´n and his colleagues documented one of the first linkages between autism and *CNTNAP2* gene. In this study, they genotyped 2758 SNPs covering almost 200 known genes. *CNTNAP2* and other genes were selected as previous literature emphasized its role in autism like traits and further neurodevelopmental disorders. Through in situ hybridization in human fetal brain, they found that the *CNTNAP2* gene was limited to the development of frontotemporal –subcortical circuits, striatum and dorsal thalamus noteworthy that these structures and circuitry of the brain an important for performing executive functions, joint attention, speech and language. They also found significant association between rs2710102 variant language malfunction in autism patients including age at first word (Alarcón et al., 2008).

1.11.5 *CNTNAP2* gene variants and neurological disorders

The Effect of *CNTNAP2* gene variants on schizophrenia and major depression have been investigated in Chinese Han population, specifically variant rs17236239 was found to significantly associate with schizophrenia Moreover, the variants rs2710102 and rs2710117 were found to be significantly associated with major depression in unrelated major depression patients comparing healthy control (Ji et al., 2013). It is noteworthy to mention that SNP rs2710102 in the CNTNAP2

gene was found to be associated with a form of language impairment like nonword repetition in dyslexia patients' (Peter et al., 2010). An Additional study found significant association between the CNTNAP2 gene variants, rs2710102, rs759178, rs17236239 and rs2538976 in exon 13-15, and the risk of language impairment or autism (Whitehouse, Bishop, Ang, Pennell & Fisher, 2012).

In a study on Chinese Han population, aimed to investigate the impact of different variants of the CNTNAP2 gene on speech sound disorder, which is related to communication capability. Using 300 patients and 200 healthy individuals, they found that SNPs rs2538976, rs2710102 rs17236239, rs 2538976 and rs 2710117A have significant association with speech sound disorder (Zhao et al., 2015).

In selective Mutism, a social anxiety disorder which is related to autism in having impaired communication and social skills, it was examined whether SNPs in the CNTNAP2 gene have any impact on the disorder. The data showed that rs2710102 variants was significantly associated with the disorder in US population with odd ratio of 1.33 (Stein et al., 2011).

1.11.6 Effect of *CNTNAP2* gene variants on Autism in different populations

Gene variants association studies regarding the *CNTNAP2* gene provided an important resource for further understanding of ASD disease mechanisms. Of which showed strong association the rs7794745 (A/T) and the rs2710102(C/T) Variants in the *CNTNAP2* gene with ASD, located between exon 2 and 3, and exon 13 and 14, respectively. As shown in figure 1.6 (Scott-Van Zeeland et al., 2010, Nascimento et al., 2016, Arking et al., 2008, Zare, Mashayekhi & Bidabadi, 2017, Li et al., 2010, Peñagarikano et al., 2011).

An evidence has been found in the Han Chinese population where SNPs in the *CNTNAP2* gene were examined in 185 autism families using restriction fragment length polymorphism (RFLP). The authors found that patients with rs10500171 variant have increased risk for autism (Li et al., 2010).
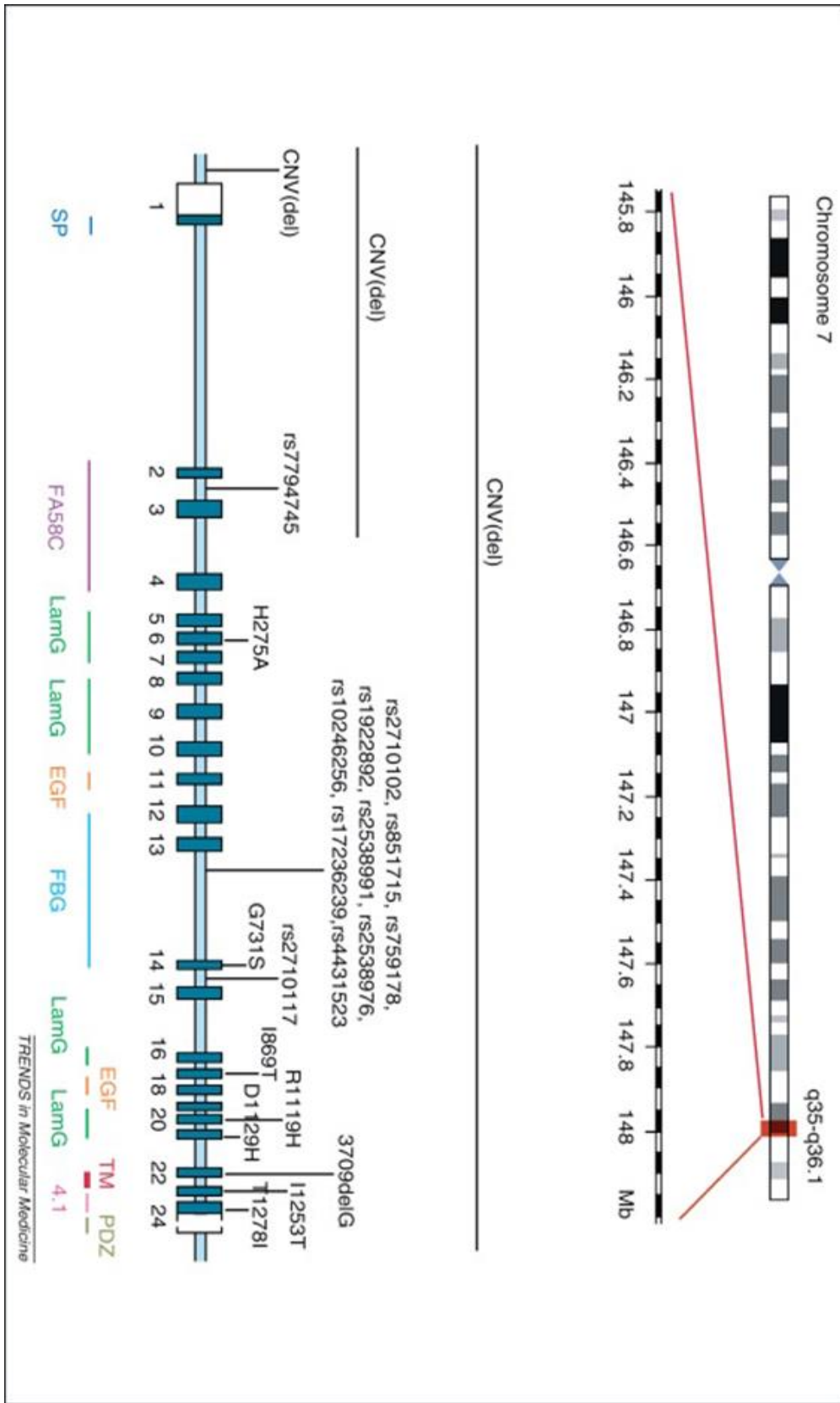
Figure 1.6. The location of variants within CNTNAP2 gene (Peñagarikano et al., 2011)

A recent study done on Iranian population, in which 200 autism patients and 260 healthy control were studied for the association of rs7794745 polymorphism in the CNTNAP2 gene, through PCR-RFLP. The data showed significant association between rs7794745 and autism (Zare, Mashayekhi & Bidabadi, 2017).Another study in the Brazilian population, involving 210 autism patients and 200 healthy individuals, found that the homozygous allele TT in rs 7794745 variant is associated with Autism. However. They showed no significant association between rs2710102 variant and Autism (Nascimento et al., 2016)

1.12 Study objectives

Due to the evidence that genetics play an important role in the development of autism. The *CNTNAP*2 gene emerge as a strong candidate for this role. It was decided to study the association of rs7794745 and rs2710102 Variants in the CNTNAP2 gene with Autism among Palestinian patients. This will potentially provide a predicting genomic marker for the disorder among suspected children and will allow early diagnosis and starting intervention at an early stage, which might increase the healing process and prevent increasing symptoms severity and consequently reducing therapy period and cost. This study is considered a pioneer trial regarding autism in the Palestinian population.

Specific Objective

To investigate the association between rs7794745 (A/T) and the rs2710102 (C/T) variants in the CNTNAP2 gene with Autism among Palestinian patients.

Chapter Two

Methodology

2.1 Study design

This present study is a case control-based study conducted from November 2016 until March 2018. Subject recruitment with clinical and behaviors representations that rule their Autism diagnosis were based on the DSM criteria. In addition, the subjects were well diagnosed by pediatric neurologist or psychiatric in special clinics or different Autism school and societies.

Demographic data like gender in addition to Medical records were obtained from patients records

2.2. Study Area

Our study areas included the various districts or governorate in west bank or east Jerusalem targeting Palestinian autism patients that are registered and cared by special societies in the region who provide mental, medical and social care for these patients.

2.3. Study Subjects and blood sampling

Many societies in the west bank and east Jerusalem were targeted with the aim to get about 100 autism subjects, however because the number of authentically diagnosed patients were limited and the process of recruiting patients through direct family contact was very tedious and challenging, table 3.1 shows the distribution of patients for the various societies in the west bank. Palestinian patients residing in east Jerusalem could not be reached even after several attempts and promises to reach with no link at the end. The study subjects comprised of 45 patients firmly diagnosed with Autism by Neuro-specialists in private clinics and specialized societies who suffer from the various clinical complications of the disease and subjects that suffer mainly from other neurological disorder were excluded. The control subjects included 145 healthy individual. Blood samples were

collected from all subjects and transferred on ice to the Medical Research Center at Al-Quds University for genomic DNA purification and analysis.

2.4. Methods of the study

2.4.1 DNA extraction

Genomic DNA from each sample extracted using the Epicenter DNA purification Kit (Master Pure TM Genomic DNA Purification Kit) according to the protocol described by the manufacturer as follows:

1- Five ml of blood drawn into 5 ml K EDTA tube and centrifuged at room temperature for 15 minutes at 1500 X g. The buffy coat (white blood cells) was withdrawn in 150 µl volume and transferred into 1.5 ml Eppendorf tubes.

2- 0.6 ml Lysis buffer 1 (10mMTris-HCl, 400 mM NACL and 2mM NA2EDTA, pH 8.2) added and tubes gently inverted 6-8 times to mix, and the bottom of the tubes flicked to suspend any remaining material.

3 -Tubes were incubated at room temperature for 5 minutes, inverted 6-8 times to mix and flicked at the bottom then incubated for an additional 5 minutes at Room temperature. At the end of the second incubation period, tubes were inverted 6-8 times and the bottom flicked.

4- Tubes were centrifuged for 25 seconds at 10,000 x g.

5- The supernatant (lysed RBCs) was discarded leaving approximately 25 ul with the pellet and then vortexed to re suspend the white blood cells.

6- 300µl of Lysis buffer 2 (10%SDS, protease K solution,1mg protease K in 1 % SDS and mM Na2EDTA] were added, then the cells were pipetted up and down 5-7 times for complete suspension and lysis. Then, 250 ul of the Precipitation Solution (6M NaCl) was added to the mixture, mixed vigorously by vortex, followed by centrifugation for 10 minutes at 10,000 x g.

7- The supernatants was transferred into a clean microfuge tube, 700 ul of ice-cold isopropanol was added to precipitate the DNA after gentle mixing

8- DNA was pelleted after following centrifugation at 4° C for 10 minutes at 10,000 x g.

9- The supernatants were carefully discarded without dislodging the pellets, washed by absolute ethanol, allowed to dry in the air and the DNA is resuspended in 100 µl sterile distilled water.

10- Purified suspended DNA was stored at -20° C until further use for genomic analysis.

DNA quality was tested by gel electrophoresis prepared by dissolving 1 gm of agarose in 100 ml TAE buffer to obtain 1% agarose. The purity and concentration of DNA sample was evaluated through measurements with nanodrop (Nanodrop 2000 C) and all samples had A260/A280 ratio above 1.7.Table 2.1 shows some of the obtained data regarding DNA purity and concentration.

Table 2.1: Concentration and purity of representative DNA samples.

DNA sample	DNA conc. ng/µl	A260	A280	260/280	260/230
1	228.5	4.569	2.360	1.94	2.09
2	175.1	3.502	1.867	1.88	1.82
3	161.1	3.223	1.687	1.91	1.85
4	235.8	4.717	2.446	1.93	1.93
5	874.7	17.493	9.342	1.87	1.77

2.4.2 PCR Amplification

We performed conventional polymerase chain reaction (PCR) to amplify the targeted area of the gene contained the indicated SNP. We used a ready-mix PCR tubes (Syntezza) that contain lyophilized mixture of Taq polymerase, Mgcl2, dNTP and buffer. the PCR reaction was run in 25-µl reaction volume containing 150 ng reverse primer, 150 ng forward primer, 22 µl distilled water and 1 µl DNA template.

The sequence of the primer and the PCR program used in the amplification was according to (Nascimento et al., 2016). Table 2.2 illustrate the primer sequences and the size of each amplification product containing every sequence of the variant in the gene

Table 2.2: Sequence of the primers needed to amplify the DNA fragment containing the two indicated SNP

SNP	Primer sequence (5'-3')	Product (bp)
rs 2710102	ATGGATGGACTGACCGATTG TTGTGTTGTTTGGCCATGAT	198
rs 7794745	CAACATTGATCCCTTCAGCCAT CTCACCAGTGTGCTTCAGACCA	311

The PCR program for each variant was as following:

1- Initial denaturation at 95° (5 min)

2- 35 cycles of denaturation at 95°C for1 min, annealing at 59°C for 30 second, and extension at 72°C for 30 second

3- Final extension at 72°C for 5 min.

After running the PCR program, the amplified DNA fragment were visualized using 10 µl PCR mix and utilizing 2% agarose gel and 7-µl 50 bp DNA ladder.

2.4.3 Restriction Fragment Length Polymorphism (RFLP)

RFLP analysis of the PCR product was performed utilizing restriction enzymes described by Nascimento et al., 2016 as outlined in table 2.3

Table 2.3: the indicated restriction enzymes for every studied variant and the cut position.

SNP	Restriction enzyme	Allele (bp)	
rs 2710102	AvaI (Eco881)	C (43/155)	T (198)
rs 7794745	Tsp509I	T (78/223)	A (311)

The enzyme digestion protocol for each PCR amplified fragment was performed in 15 µl reaction volume using the following reaction:

10 µl PCR mix

1.5 µl 10 x enzyme buffer

0.2 ml (54 units of enzyme)

3.3 µl distilled water.

The mix was incubated at 37 °C for 1 hour followed by running the mix in 3% agarose by dissolving 3 gm agarose gel in 100 ml of TAE buffer, and using 50 bp DNA marker ladder to analyze the obtained fragment

2.4.4 Sequencing Analysis

Several random samples (four samples) were subjected to direct DNA sequencing to validate the genotyping result. After PCR Purified DNA fragments were sequenced, using API 3500 automated sequencer in the molecular genetic center at the Arab American University .and the DNA sequence was compared to the corresponding sequence of the CNTNAP2 gene using BLAST analysis in the NCBI

2.4.5 Statistical Analysis

The significance of the association between the indicated haplotypes and the disease and its various clinical impacts regarding other related information were analyzed using SPSS version 20. The Hardy- Weinberg equilibrium was calculated through the http://.oege.org/software/hwe-mr-calc.shtml website.

2.4.6 Ethical Consideration

All participating subject's parents were i.nterviewed to fully explain for them or their parents the purpose of the intended project, its significance and potential impact. At the end of the interview, the parent's subjects were required to sign a consent form declaring their acceptance to participate in the study. All private information regarding patient's identity and medical information was firmly kept confidential and all participants retain the full right to withdraw their participation in the study at any time during the process.

Chapter Three

Results

3.1 patient's record

Forty-five patients were recruited from several societies located in different cities in the west bank including Ramallah, Nablus, and Jenin. However, five of the recruited patients did not have their detailed clinical data available. Table 3.1 shows detailed description of the forty patients with their clinical data included in the study. The age of the patient is ranged from 4 -16 years of age. Five of the recruited patient's did not have their detailed clinical data available. During the collection of patient's samples, the number of autism patients provided by the various societies was much higher than expected based on authentic clinical diagnosis of autism by the specialized physicians who followed these patients in those societies. Many of the indicated patients suffered from other neurological disorders. In addition, several of the patients guardians refused to include the affected children. We found only one patient with sibling affected with the disease. Concerning consanguinity, 25% of the patients (10/40) parents are directly related. In addition, 60.3% (25/40) of the patients showed normal self-behavior while 15 % (6/40) have aggressive behavior and 20% (9/40) showed moderate aggressive behavior. In relation to their language skills 50% (20/40) of the patients have significant difficulty in theirs ability to express their needs directly using their language ability, while 50% (20/40) were able to communicate clearly by verbal language to express their needs. Moreover, about 35% (14/40) of the patients showed normal self-expression in response to their environment, about 10% of the patients (4/40) showed moderate self-expression, and about 15% (6/40) showed minor expression and about 44% (16/40) failed to express self-expression. The patients' behavioral data was determined by social worker employee in the indicated societies and extracted from their medical records. This disease related complications were later correlated with the various haplotypes obtained from the two indicated SNP locations rs 2710102 and rs 7794745 in the gene.

Table 3.1: Patients medical records involved in the study

Patient number	Society	Code	Age	Sibling	Consiguinty	Self Behavior	language Skils	Self Expression	rs 2710102	rs 7794745
1	Farah	M.A	6.9	N	N	Normal	Y	Normal	CT	AT
2	Farah	M.D	8	N	N	Aggressive	Y	Moderate	TT	AA
3	Farah	KH.H	9.4	N	Y	Normal	N	Normal	CT	AT
4	Farah	A.K	7.2	N	N	Normal	N	Moderate	TT	AT
5	Farah	M.H	8.9	N	N	Normal	N	Normal	CT	AT
6	Farah	A.KH	8.8	N	Y	Normal	N	Moderate	CC	AT
7	Farah	A.S	5.4	N	N	Normal	N	Normal	CC	AA
8	Farah	S.A	7.3	N	N	Normal	Y	Normal	CT	AT
9	Farah	Y.S	4.8	N	Y	Normal	Y	Normal	CT	AT
10	Yasmine	M	9	N	Y	Aggressive	N	None	CT	AT
11	Yasmine	R	5	N	N	Normal	N	Minor	CC	AA
12	Yasmine	M.GH	6	N	N	Normal	Y	Moderate	CT	AA
13	Yasmine	S	9	N	N	Normal	Y	Normal	TT	AT
14	Yasmine	A	5	N	N	Aggressive	N	None	CC	AA
15	Yasmine	AS	7	N	N	Normal	Y	Normal	CT	AT
16	Yasmine	H	11	N	N	Moderate	Y	Normal	CC	AT
17	Jod	A.A	6	N	N	Normal	Y	Minor	CT	AA
18	Jod	A.M	7	N	N	Moderate	Y	Minor	TT	AT
19	Jod	M	4	N	Y	Aggressive	Y	Minor	CT	AT
20	Jod	M2	8	N	Y	Moderate	Y	None	CT	AT
21	Jod	Y	4	N	Y	Moderate	N	Minor	TT	AT
22	Ghad	S.R	12	N	N	Normal	Y	None	TT	AA
23	Ghad	M.R	8	N	Y	Normal	N	None	TT	AT
24	Ghad	A.Z	12	N	Y	Normal	y	None	TT	AT
25	Ghad	M	11	N	N	Normal	N	None	CT	AT
26	Ghad	M.A1	14	N	N	Moderate	N	Normal	CC	AT
27	Ghad	A.B	9	N	N	Normal	N	None	TT	TT
28	Ghad	W	9	N	N	Normal	N	None	CT	AT
29	Ghad	A.B	10	N	N	Normal	Y	Minor	CC	AT
30	Ghad	Y	8	N	N	Normal	Y	Normal	CC	AT
31	Ghad	Y.Z	16	Y	N	Moderate	Y	Normal	CT	AT
32	Ghad	A11	N/A	N/A	N/A	N/A	N/A	N/A	TT	TT
33	Ghad	M.M	10	N	N	Moderate	N	None	CT	AT
34	Rawan	B.S	6	N	N	Moderate	Y	Normal	CC	AA
35	Rawan	B	9	N	N	Normal	N	None	TT	AA
36	Rawan	N.A	5	N	N	Normal	N	None	TT	AA
37	Rawan	A7	N/A	N/A	N/A	N/A	N/A	N/A	TT	AA
38	Rawan	A8	N/A	N/A	N/A	N/A	N/A	N/A	TT	AA
39	Amal	H.D	12	N	N	Normal	N	None	TT	AT
40	Amal	A3	12	N	N	Normal	N	None	TT	AA
41	Amal	M3	9	N	Y	Moderate	N	Normal	CC	TT
42	Amal	A5	9	N	N	Aggressive	Y	None	TT	AA
43	Amal	A6	8	N	N	Aggressive	Y	None	TT	AT
44	Amal	A9	N/A	N/A	N/A	N/A	N/A	N/A	CT	AT
45	Amal	A10	N/A	N/A	N/A	N/A	N/A	N/A	CC	AT

In this study 145 control subjects, where used including adult university students who are not affected from neurological disorder and nor any of their siblings.

3.2 DNA quality

Figure 3.1 shows representative gel for the purified genomic DNA from 12 participants in the study, the gel shows intact high molecular weight DNA bands without any sign of damage and Or additional bands in the gel.
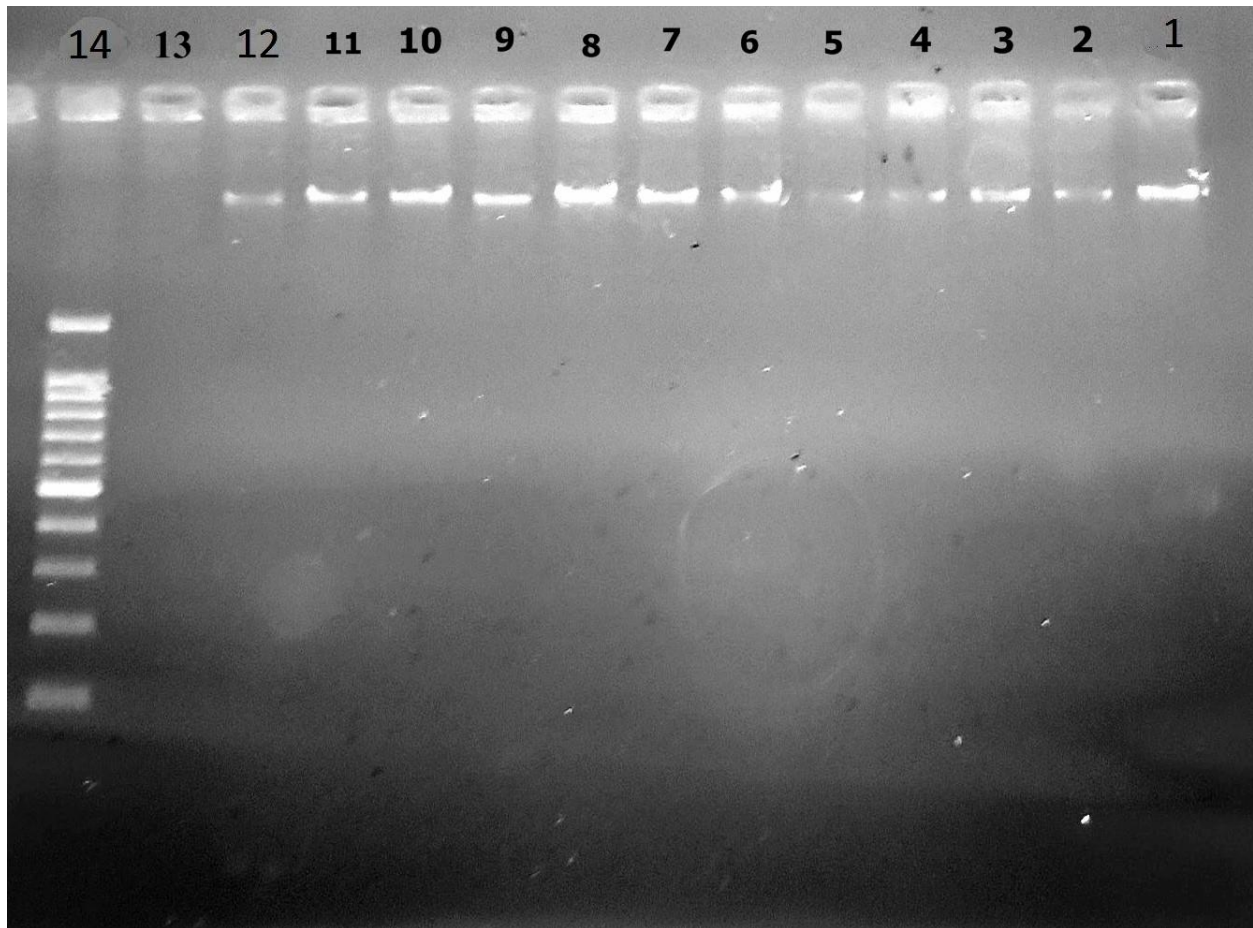

Figure 3.1: Quality of genomic DNA prepared from patients and control. Lane 1-12: DNA samples, Lane 13: negative control. Lane 14: 100 bp DNA ladder

3.3 PCR amplified gene fragments.

After performing PCR amplification of the DNA fragments containing the indicated haplotypes, fraction of the PCR mix was tested to validate the size of the amplified product and compare it to the reference size for each DNA fragments containing the indicated SNPs. Figure 3.2. Shows representative gel for eight samples of each amplified DNA fragment .The result here showed a single 311bp DNA fragment with the rs 2710102 SNPs and a 198 bp fragment with rs 7794745 haplotypes. The size of both DNA product are in exact agreement with expected fragment size.
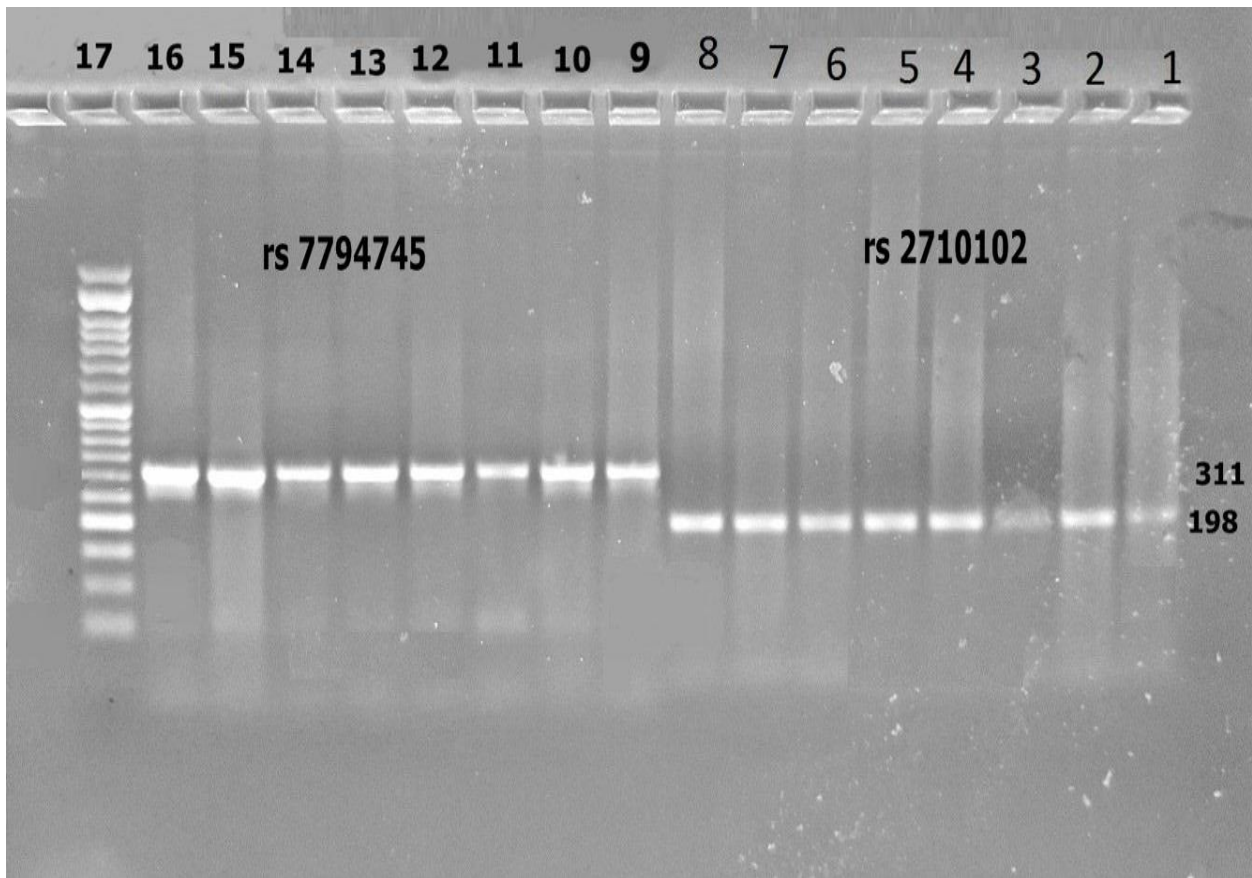

Figure 3.2: Gel electrophoreses showing PCR amplified DNA fragments containing the rs7794745 and rs 2710102 SNPs. Lanes1-8 on the right side of the figure shows 198 bp fragment for the rs 2710102 SNPs and lanes 9-16 on the left side of the figure shows 311 bp amplified fragments the rs7794745. Lane 17 shows 50 bp DNA ladder.

3.4 PCR-RFLP analysis

Figure 3.3 shows representative RFLP analysis of the two indicated SNPs variants rs 2710102 and rs 7794745. The upper part of the Figure shows digestion pattern of 19 control samples (lanes 1-19) with ava1 restriction enzyme for the rs 2710102 haplotypes and the lower part of the gel shows the digestion pattern of 19 control samples digested with Tsp restriction enzyme for the rs7794745 haplotypes as indicated on the figure. Lane 12 shows CC genotype, lanes 1,3,4,5,7,8,9,11,14,15,18,19 shows CT haplotypes and the remaining samples shows TT haplotypes. The lower panel of the figure showing the haplotypes rs 7794745. Lane 2 haplotype is TT, Lanes 5,13,14,16 haplotypes are AA and the rest of samples are AT.

Figure 3.3. Gel electrophoresis showing PCR-RFLP analysis for selected control samples. The upper part of the figure shows the results of aval digestion (Lanes 1-19). The lower part of the gel shows the result of the Tsp digestion (1-19). Lane 20: 50 bp DNA ladder

Regarding samples in the upper panel in figure 3.4, Lanes 1, 3,5,8,9 shows haplotype CT. Lanes 2, 4: haplotype TT. Lane 6, 7 haplotype CC. regarding samples in the lower panel. Lanes 1,3,4,5, 6, 8, showing AT haplotypes .lanes 2, 7 shows AA haplotype and lane 9 shows TT.
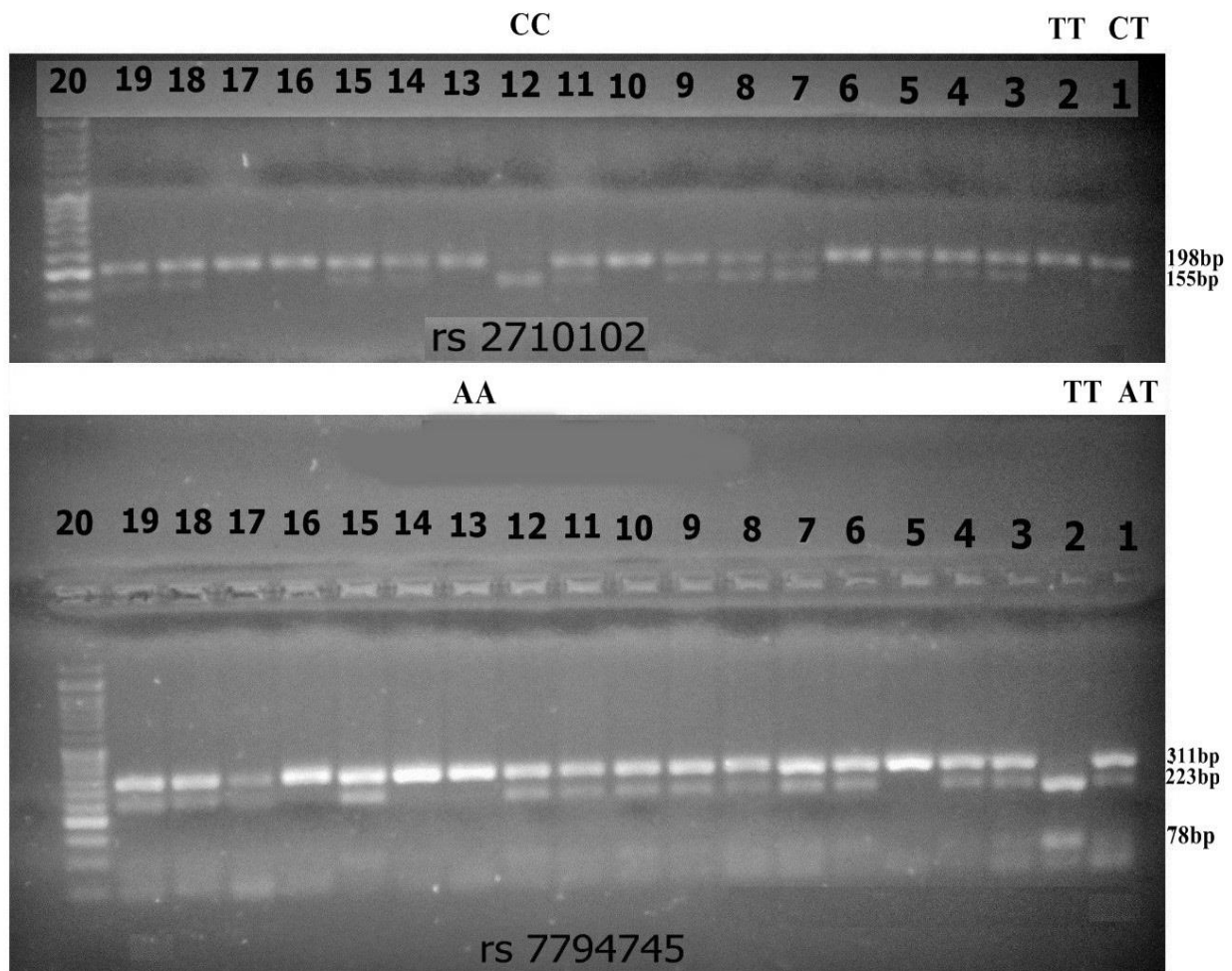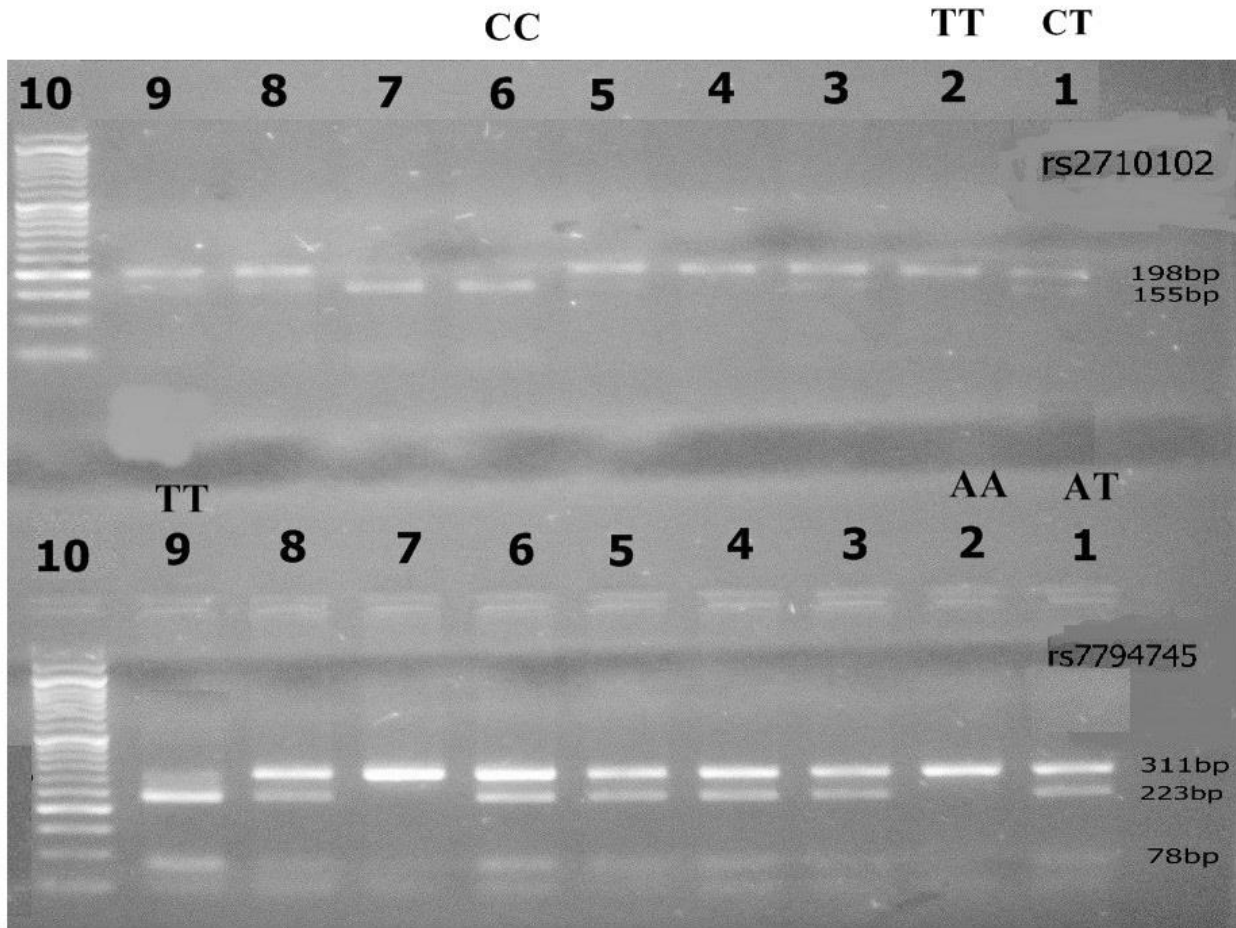

Figure 3.4 Gel electrophoresis showing PCR-RFLP analysis for selected patient's samples. The upper part of the figure shows the results of aval digestion (Lanes 1-9). The lower part of the gel shows the result of the Tsp digestion (1-9). Lane 10: 50 bp DNA ladder

3.5 Direct DNA Sequencing results.

Four selected amplified DNA fragments were subjected to direct DNA sequencing analysis to confirm the genotype of these amplified fragments. Four representative sequences showing two rs 2710102 genotype (homozygous CC and heterozygous CT) in panel A of figure 3.5 and two rs 7794745 genotype (homozygous AA and heterozygous AT) in panel B of figure 3.5 as indicated by the arrows .These genotypes exactly matches the relevant genotypes of the same samples obtained by RFLP analysis.

A
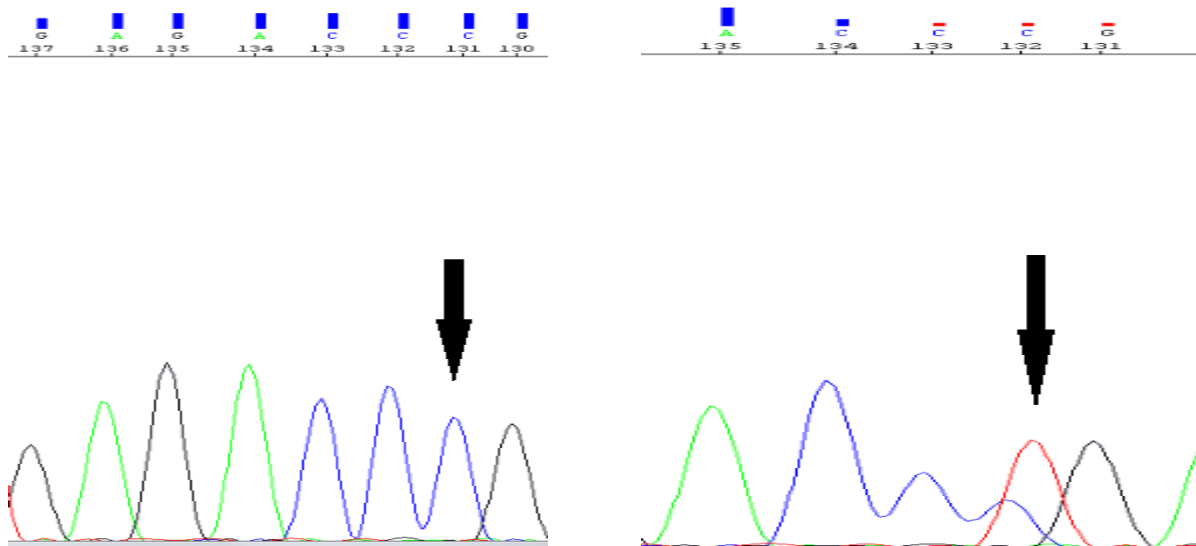

```
Query    1   TTTATGGATGGACTGACCGATTGGTTAACATTTACTCTGAGACCCGAGAAAGAAGGAAAA   60
             ||||||||||||||||||||||||||||||||||||||||||||||| |||||||||||||
Subject  1   TTTATGGATGGACTGACCGATTGGTTAACATTTACTCTGAGACCTGAGAAAGAAGGAAAA   60
```

B
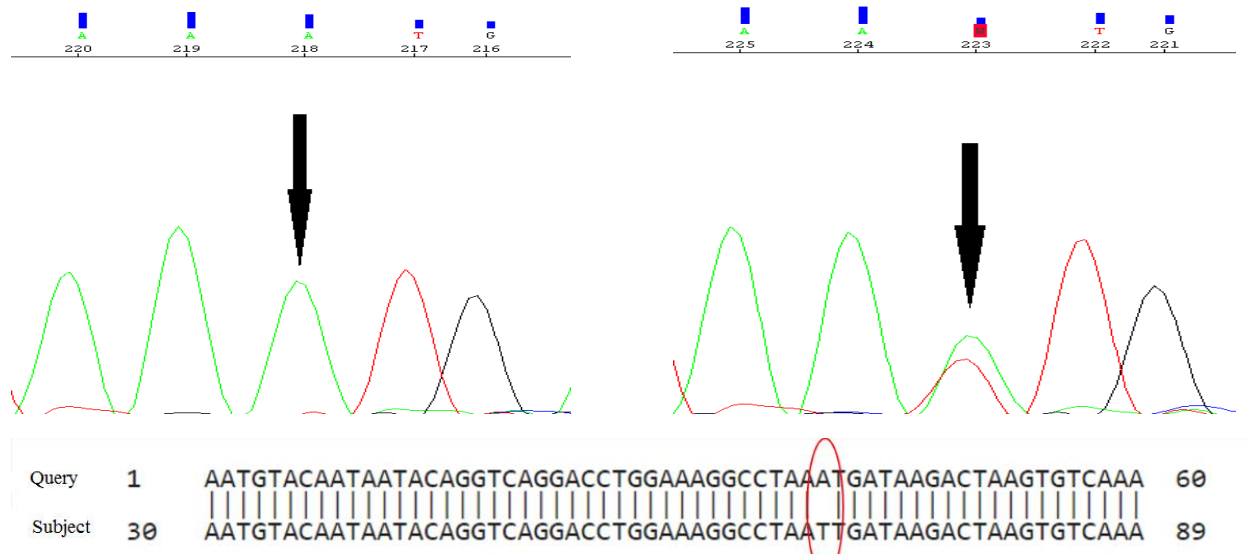
Figure 3.5: Panel A: representative sequencing result of selected DNA samples regarding the rs 2710102 variant. The Left side sequence shows CC homozygous genotype and right side sequence shows CT heterozygous genotype. The lower part of the panel shows exact sequencing alignment. Panel B: representative-sequencing data regarding rs 7794745 variant. Left panel shows AA genotype homozygous and right panel shows AT heterozygous genotype. The Lower part of the panel shows exact sequencing alignment

3.6 Statistical correlation of the indicated genotypes for rs 2710102 and rs 7794745 sites with the disease.

The genotype distribution in the two variants was tested for Hardy- Weinberg equilibrium and the p value was 0.51 for the control group and 0.56 for the case group regarding the rs 2710102 variant. Regarding the rs 779454 variant the p value was 0.54 for control group and 0.63 for the case group.

Among the three various genotypes of the rs 2710102 (CC, TT, CT) the CT genotypes showed significant correlation between patients and control subjects (p value 0.043) as shown in table 3.2 This result Indicates the CT genotypes is associated with healthy subjects, while the other two genotypes (CC, TT) showed no significant difference between the control and patients groups.

However, none of the three genotypes of the rs 779454 haplotype (AA, TT, AT) showed any significant difference between the two groups. These results indicate the two sites do not seem to represent susceptible predicting genetic marker for autism.

The observed comparison of indicated genotypes in the two sites are not expected to be different if more patient's subjects were included due to the fairly high number of control subject (145) in comparison to the patient's subjects (45).

Table 3.2: Correlation of the indicated genotype between control and case with the disease status.

Genotype (rs 7794745)	Healthy control (N=145)	ASD (N=45)	P value	Odd ratio (95%CI)
AA	38 (26%)	15 (33.3%)	0.352	0.710 (0.3-1.4)
TT	25 (17%)	3 (6.7%)	0.08	2.917 (0.83-10)
AT	82 (57%)	27 (60%)	0.683	0.868 (0.4-1.7)

Genotype (rs 2710102)	Healthy control (N=145)	ASD (N=45)	P value	Odd ratio (95%CI)
CC	29 (20%)	11 (24%)	0.532	0.773(0.3-1.7)
TT	33 (23%)	16 (36%)	0.086	0.532 (0.259-1.1)
CT	83 (57%)	18 (40%)	**0.043**	2.008 (1-3.9)

Next, the correlation between the individual genotypes of both SNPs sites between patients and control subjects was evaluated. As shown in table 3.3, none of the individual genotypes of both sites showed any significant difference between control and patients subjects. This is clearly indicated with subjects having the C genotype (CC) in comparisons with subjects having the T genotype (CT+TT) of the rs 271012. Similarly the same results were obtained after comparing subjects with the A genotype (AA) with those having the T genotype (AT+CT) of the rs 7794745. This result here more strongly support the notion that the various genotypes of the two indicated SNPs sites do not represent predicting genetic marker for autism as indicated above in table 3.1

Table 3.3: correlation of combined individual genotypes of the indicated haplotypes with the disease status.

GROUP	GENOTYPING FREQUENCY (rs 2710102)		P VALUE	ODDRATIO
	CC	CT+TT	0.523	1.294
CONTROL	29	116		
AUTISM	11	34		

GROUP	GENOTYPING FREQUENCY (rs 2710102)		P VALUE	ODDRATIO
	TT	CT+CC	0.086	0.534
CONTROL	33	112		
AUTISM	16	29		

GROUP	GENOTYPING FREQUENCY (rs 7794745)		P VALUE	ODDRATIO
	AA	AT+TT		
CONTROL	38	107	0.353	1.408
AUTISM	15	30		

GROUP	GENOTYPING FREQUENCY (rs 7794745)		P VALUE	ODDRATIO
	TT	AT+AA		
CONTROL	25	120	0.08	2.917
AUTISM	3	42		

Since Most of the affected subjects with autism are males while females represent only small fraction (4/45 , 9%) while the control subjects comprised of both males and females (44%) males 64/145 and (56%) females 81/145, the distribution of various genotypes between females and males in control subjects was evaluated. As shown in table 3.3, all genotypes of both rs 2710102 and rs 7794745 showed no significant differences between males and females subjects. Therefore, all control subjects were included in our correlation evaluation. However, the correlation between the various genotypes and gender between control and autism patients is of no significant since the number of female's patients is very limited.

Table 3.4: distribution of various genotypes rs 2710102 and rs 7794745 between males and females subjects.

Gender	CC	rs 2710102 P value	CT	P value	TT	P value
F (N=81)	15 (18.5%)	0.616	49 (60.5%)	0.373	17 (21%)	0.567
M (N=64)	14 (22%)		34 (53%)		16 (25%)	

Gender	AA	rs 7794745 P value	AT	P value	TT	P value
F (N=81)	23 (28.4%)	0.5	46 (56.8%)	0.948	12(14.8%)	0.384
M (N=64)	15 (23.4%)		36 (56.3%)		13 (20.3%)	

The interaction between the various genotypes of both sites were correlated between control and autism subjects as shown in table 3.5. Interestingly two combinations showed significant difference between the two groups. The TT genotype of the rs 271012 in combination with the TT genotype of the rs 7794745 showed significant difference, between the two groups. Furthermore, the CT genotype of the rs 270102 in combination with AA genotypes of the rs 7794745 showed very significant difference in comparison with the TT genotypes of the rs 270102 in combination with AA genotype of the rs 7794745 between the two groups (p value 0.003). These results here strongly suggest the three indicated genotypes combinations could represent strong predictors for autism. This could have important application in providing an early genetic marker for individuals affected by the disease, which could provide a tool for very early intervention and care for these patients.

Table 3.5: correlation between haplotypes combination rs 2710102 and rs 7794745 in control and autism patients.

Genotype combination*	P value
CC VS AA	0.701
CC VS TT	0.092
TT VS AA	0.550
TT VS TT	**0.04**
CT VS AA	0.132
CT VS TT	0.367
CT VS AT	0.220
CC VS AT	0.735
TT VS AT	0.303
CT+AA VS TT+AA	**0.003**
CT+AT VS CC+AA	0.335

*Genotype from rs 2710102 vs genotype from rs 7794745 polymorphisms

3.7 Correlation between the genotypes of the rs 2710102 and rs 7794745 haplotypes with various clinical expression of the diseases.

In order to evaluate the link between the various genotypes of the rs 2710102, rs7794745 sites, and the clinical expression of the disease. The correlation between the various individual genotypes and genotype combinations in the two indicated sites with the main observed clinical and behavioral complications expression of the disease including self-expression, aggressivity in behavior and use of language and consanguinity among patient's parent was investigated. Table

3.6 shows interestingly the presence of significant correlation between higher self-expression with the C (CC+CT) allele of the rs 2710102 site in comparisons with the homozygous TT genotype (p value 0.0170). No significant correlation could be detected between the all other individual various genotypes of either sites (rs 270102 and rs 7794745) and their allele combinations with the three indicated disease complications. It is not clear at this point the exact meaning or use of the single detected correlation with self-expression in these patients at this level. However, it may indicated a potential specific link between this genotype with the self-expression complication of the disease that could provide potential marker for early specific therapeutic intervention with these patients in relation with this complication.

Table 3.6: correlation between haplotypes and patients major altered behavior

Record	rs 2710102	rs 7794745
Self-expression	**CC+CT > TT, (p 0.017)**	Non-significant
Aggressivity in behavior	Non-significant	Non-significant
Language	Non-significant	Non-significant

Chapter Four

Discussion

The impact role of the *CNTNAP2* gene, a member of the expanded Neurexin gene superfamily, on the development of autism (ASD) and many related disorder such as dyslexia, language impairment, epilepsy and schizophrenia is clearly evident (Rodenas-Cuadrado, Ho & Vernes, 2013). Genomic variants in *CNTNAP2* gene has been positively and negatively linked to autism with varying results in different populations. (Scott-Van Zeeland et al., 2010, Nascimento et al., 2016, Arking et al., 2008, Zare, Mashayekhi & Bidabadi, 2017, Li et al., 2010, Peñagarikano et al., 2011). Since no genetic marker has been linked to ASD development among Palestinian patients, the present study was initiated to investigate the potential association between specific selected *CNTNAP2* gene variants as an early genetic marker that might be involved in the prediction of the risk of autism development and consequently provide a lead to initiate early medical intervention and subsequently better disease management. The present study represents a pioneer investigation towards understanding the molecular genetics of autism and the association of polymorphisms in the *CNTNAP2* gene with autism among these patients as a major step leading towards more extensive understanding of molecular mechanism of *CNTNAP2* gene role in the disease. Evidently, the association of two variants in the *CNTNAP2* gene on the risk of developing autism provides insight regarding the linkage of the gene variants with the ASD development in the Palestinian patients and provides the bases for subsequent extensive functional studies of the exact gene role in the process.

A significant difference was evident between the frequency of the heterozygous CT genotype in the rs2710102 variant between patients and control subjects indicating this genotype is significantly associated with healthy individuals and seems protective against ASD. Even though the CT genotype of the rs270102 showed a significant difference between the two groups, however it represents a weak marker for predicting the disease development. None of the previous studies reported a link between this genotype and autism. (Scott-Van Zeeland et al., 2010, Nascimento et al., 2016, Arking et al., 2008, Zare, Mashayekhi & Bidabadi, 2017, Li et al., 2010, Peñagarikano et al., 2011).

No significant difference between either the homozygous CC or homozygous TT of the rs2710102 variant site could be detected between ASD patients with healthy control subjects which is in agreement with many studies in different populations (Whalley et al., 2011; Nascimento et al., 2016; Sampath et al., 2013; Jonsson et al., 2014). However, previous studies found significant association between this variant in the *CNTNAP2* gene and autism complications including impaired language skills and social anxiety (Peñagarikano & Geschwind, 2012). Interestingly, in a study done in the US population on selective mutism, a disorder related to autism with deficits in social communication and interaction, a significant association was reported between this variant in rs2710102 of the *CNTNAP2* gene and this disease (Stein et al., 2011)

Regarding the rs7794745 variant in the *CNTNAP2* gene, no significant difference in the frequency of all tested genotypes (homozygous or heterozygous) and ASD was detected, similar to previous studies in different populations including Swedish populations (Jonsson et al., 2014), Korean (Yoo et al., 2017) and Spanish (Toma et al., 2013) populations. However, a study in the Brazilian population found a significant association between the TT genotype in this variant and autism (Nascimento et al., 2016). Regarding the rs7794745 variants, the AT genotype was found to be significantly associated with ASD in Iranian (Zare, Mashayekhi and Bidabadi, 2017) and AT in Chinese ASD patients (Li et al., 2010) also similar findings were reported in the US population (Arking et al., 2008). It should be mentioned here that the Palestinian population have diverse genetic background of a Caucasian origin different from the European, Chinese or US populations, which might have an impact on the overall results obtained in the present study and their link ASD development.

Interestingly, specific interaction between SNPs variants in the two indicated sites (rs2710102 and rs7797745) showed significant difference between the two subject groups. Specifically, the combination of TT genotype of the rs2710102 variant and the TT genotype of the rs7794745 variant (TT+TT) showed a significant higher association with the control subjects compared to the ASD group indicating its protective role against the disease. In addition, the combination of the CT genotype of the rs2710102 variant and the AA genotype of the rs 7794745 variant (CT+AA) versus the combination of TT genotype of the rs 2710102 variant and the AA genotype of the rs 7794745 variant (TT+AA) showed the CT+AA genotype combination is strongly associated with the control group while the TT+AA genotype combination was is strongly associated with ASD .

No previous studies showed similar correlation between the combined genotypes of the two indicated variant sites and the risk of ASD development. This could represent a strong genotype marker of the *CNTNAP2* gene role in the development of ASD.

In relation to the association between the various genotype of the two indicated haplotype sites with various clinical symptoms of autism, one significant difference was evident with the ability for self-expression where the C allele (CC+CT) was significantly higher than the T allele (TT) in the rs2710102 variant among the ASD group compared and therefore represents a risk genotype for this symptom. No significant association could be detected between the other reported clinical symptoms and all other genotypes in the two indicated sites. Only one previous study showed the rs2710102 variant had significant impact on language skills while rs7794745 variant had no significant impact on the same symptom (Whitehouse et al., 2012).

The functional impact of the various genotypes in the indicated variant sites, rs2710102 and rs7794745 and the development of ASD and its clinical symptoms is not clear at this stage. Both variants are located in intronic sequences (intron 2 for rs2710102 and intron13 for rs7794745) that could be involved in gene expression regulation of the CNTNAP2 gene through participation of potential transcription factors binding to the DNA sequence around these sites. A previous report regarding the expression of the CNTNAP2 gene and ASD showed a strong association between the reduced expression level of the gene during neuronal development evidently represents a risk for ASD development (Chiocchetti, A. et al 2014). In that study, four novel mutant variants were identified that altered the specific transcription factors binding sites in the promoter region which caused decreased level of the promoter activity and association with the risk of ASD. These activities were confirmed by reporter systems activities in various cell lines. These promoter variants seem to be involved in the upregulation of the gene during neuronal differentiation and therefore protect against ASD while mutant variants in these sites resulted in decreased expression of the gene associated of high liability of ASD (Chiocchetti, A. et al 2014). One of the indicated variants in the promoter (rs71781329) showed association with language development and delayed onset of speech. The reported mutant variants in the indicated sites were proposed to either decrease the affinity of the specific transcriptional factors to these sites or results in damaging mutations in their recognition sites both of which lead to decreased expression level of the gene. The exact functional role of the described variants in the two investigated sites needs further

exploration and investigation. It is evident that a DNA binding site for the FOXP2 transcription factor is located in intron 1 of the CNTNAP2 gene, which is linked to language development (Vernes SC. et al 2008). Therefore the present identified variants in the studied variant sites is very likely to affect the activity of specific transcription factors in these locations that are involved in the differential expression level of the gene resulting in its impact on the liability of ASD as evident with the related promoter variants. This potential effect and those of other related variants in the gene will definitely need more extensive investigations to understand the full impact of this gene variants on the liability on ASD.

4.1 Conclusion

The present data in combination with other previous data indicate the contribution of the *CNTNAP*2 gene polymorphisms and mutations on autism seems complex, and require further studies to determine its full spectrum of its contribution to the risk of autism development and the associated clinical complications as suggested before (Alarcón et al., 2008). No significant association was evident between the individual selected *CNTNAP2* gene variants (rs2710102 and rs7794745) and autism in the Palestinian ASD patients. However, three significant associations were evident between specific genotype interactions of the two sites and autism. Based on this data, these genotype interactions could represent a marker for development of autism among the new born. However, the functional impact of these genotypes at the cellular level needs further investigation at the level of gene expression and various potential transcription factors contribution in the process. Definitely, the development of autism most likely involves other genes in the genome that may provide stronger or weaker impact of the CNTNAP2 gene contribution to the development of ASD that require further studies with larger number of patients since this could have significant a impact on the various allele distribution (Murdoch & State, 2013).

4.2 Recommendations.

1. Investigate the functional impact of the identified variants on ASD liability and other gene variants that are linked to neuronal cognitive or depression disorders.

2. Expand the study to include a more patients and their families regarding the various CNTNAP2 gene variants.

3. Analysis of the association of other SNPs of CNTNAP2 gene with ASD.

4. Subject various patients genome for whole exome sequencing analysis.

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العلاقة بين المتغيرين الجينين (rs2710102 rs7794745) في جين ال *CNTNAP2* مع مرضى التوحد الفلسطينيين

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الملخض:

التوحد هو اضطراب النمو العصبي الذي ينطوي على عيوب مختلفة مع شدة متفاوتة مثل عدم التواصل والسلوك الاجتماعي أو تطوير الكلام. حتى الآن، ما زال سبب التوحد مجهولاً، ومع ذلك يعتقد أن الخلفية الوراثية للإصابة بالتوحد قد تلعب دوراً كبيراً في هذا الاضطراب

ارتبطت المتغيرات الجنية في جين CNTNAP2 مع الإصابة بالتوحد في العديد من الدراسات السابقة التي تناولت شعوب أخرى. وبالتالي كنا مهتمين للتحري عن مدى ارتباط المتغيرات الجنية (rs7794745) و(rs2710102) في جين CNTNAP2 مع مرض التوحد في المرضى الفلسطينيين.

أجريت الدراسة بين عامي 2016 و2018.شملت الدراسة 45 من مرضى التوحد و145 من الأفراد الأصحاء. تم استخدام تقنية(PCR-RFLP)لتحديد المتغيرات الجينية لدى المرض والاصحاء وقد تم جمع البيانات الطبية للمرض من الجمعيات والمراكز المختصة.

لمقارنة التوزيع الوراثي في المتغيرات الجينية في مجموعة التوحد والمجموعة صحية وأيضا لمقارنة البيانات الطبية للمرضى تم استخدام برنامج ال SPSS

لم يعثر على أي ارتباط مهم في المتغيرات 2710102 و7794745 والتوحد داخل المرضى الفلسطينيين لكن عن طريق التفاعل ما بين المغيرات فد نكون توصلنا الى ارتباط قوي مع مرض التوحد