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Chapter VII

Genetics of Autism with Emphasis on Affected Females

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Abstract

Classical autism is a common early onset neurodevelopmental disorder with developmental difficulties noted by 3 years of age and belongs to a group of conditions known as autism spectrum disorders (ASD). Asperger syndrome and pervasive developmental disorder – not otherwise specified (PDD-NOS) are also included in this genetically heterogeneous group. Diagnostic features for classical autism include significant impairment in the development of social interactions, a marked and ongoing impairment of both verbal and non-verbal communication and restricted, repetitive or stereotyped behaviors and interests. The prevalence of autism is on the rise and ranges from 4 – 10 per 10,000. It ranks third among developmental disorders. It is unclear whether the prevalence of autism has really increased or if the increase is due to better awareness and identification with establishment of diagnostic criteria.

Genome-wide scans have shown several autism susceptibility loci using molecular markers in multiplex families and strong linkage reported for chromosomes 2, 3, 5, 7, 8, 13, 15, 16, 17, 19 and X as well as nominal evidence for linkage to chromosomes 1, 4, 10, 11, 12, 18, 20 and 22. Therefore, the inheritance is complex with involvement of over 20 genes. Autism is frequent in tuberous sclerosis and fragile X syndrome but these two conditions account for only a small proportion of affected individuals. Hence, diagnosable medical conditions, chromosome abnormalities and single gene conditions account for <10% of cases. The recurrence rate for siblings of affected children is

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between 2 to 8% which is higher than the general population but much less than the prevalence rate in single gene disorders. About 30% of children with autism have dysmorphic features, microcephaly and/or a structural brain malformation. In addition, macrocephaly is seen in about 20% of individuals with autism.
An excess of males with autism is reported in a proportion of about four to one which lends evidence for involvement of genes on the X chromosome. Additionally, X chromosome rearrangements have been reported in autism including translocations, duplications and deletions as well as mutations of single genes such as MECP2 (Rett syndrome), neuroligins (NLGN3 and NLGN4) and FMR1 (fragile X syndrome). Skewed X chromosome inactivation has been observed in X-linked mental retardation female carriers, and more recently, X chromosome skewness in females with autism. We review and summarize the current understanding of genetic causation and diagnosis of autism with special emphasis on affected females and discussion of X-linked candidate genes.

Introduction

Autism is not a single entity but consists of a complex, heterogeneous neuro-behavioral disorder with many causes and varying degrees of severity. Autism is defined by significant impairments in communication and social interaction accompanied by a pattern of repetitive or stereotypical behaviors and interests [1, 2]. Behavior problems in autism are identified prior to age three years. Infants with autism generally do not like to be held or cuddled and are often less consolable than other infants. They may fail to initiate eye contact or may stare and not focus on objects as expected in normally developing children. Repetitive behavior or movement may occur during the infancy period in those with developing features consistent with autism. Sleep disturbances are common. Children with autism frequently demonstrate tactile defensiveness. These presentations alone may not bring the patient to medical attention until speech is noticeably delayed.

The term autism was first used by Bleuler in 1911 to designate loss of contact with reality causing language difficulty or incapacity of communication [3]. In 1943, Dr. Kanner used the same term when describing 11 children with an innate inability to establish objective and interpersonal contact [4]. He assumed the 11 children he reported had a rare syndrome. Later in 1944, Dr. Asperger described individuals with several of the same characteristics, particularly the difficulty in social communication, but with normal intelligence [5]. In most children, the onset of autism is gradual but regression occurs in about 30% of those children who begin to develop speech but later lose language or develop other features consistent with autism such as poor eye contact [6].

About 25% of children will be diagnosed with autism by the age of two to three years. They may acquire speech and blend with varying degrees of success into a regular school setting by six to seven years of age. However, fewer than 5% of autistic children completely recover [7]. The remaining children with autism will continue to have a disability related to learning and behavior throughout life. Hence, 50 to 70 percent of children with autism fall within the mental retardation range using non-verbal intelligence testing, but the scores may change over time with intensive therapy [6, 8-12].

Cognitive development tends to be uneven. However, mental retardation can be a characteristic in subjects with autism. Those with a high intelligence level may have difficulty with concept formation, reasoning and abstract thoughts. They generally have better non-verbal than verbal skills and are better with visual than auditory learning. Some autistic persons have shown superior skills for a narrow range of abilities, such as calendars, calculations, or music, despite impaired cognition. During early childhood, the majority of autistic children demonstrate significantly strong deficits in imaginary or symbolic play. Along with language and cognitive impairments, social deficits may be striking. During infancy, autistic children may be extremely passive and require little attention but may be irritable, difficult to feed, or have irregular sleep patterns. These children should be followed by developmental pediatricians, child psychologists and other health related care providers with experience in autism to assist in medical management and follow up. Genetic and neurology evaluations should be undertaken to rule out an organic basis or a syndrome in the subject presenting with this condition.

In one recent review of the prevalence of neurological disorders, autism (130 per 100,000) was followed by epilepsy (650 per 100,000), brain paralysis (250 per 100,000), dementia (250 per 100,000), and Parkinson's disease (200 per 100,000). Autism was also recorded at a higher prevalence than congenital malformations of the brain (70 per 100,000) or for Down syndrome (50 per 100,000) (13, 14). Additionally, seizures and dysmorphic features may develop in about 25% of children with autism [15, 16]. The concordance for the diagnosis of autism and monozygotic twins is at least 60% [17].

Microcephaly occurs in about 10% of children with autism (18, 19) and may be associated with a poorer prognosis. Macrocephaly is reported in 20-40% of autistic children (20, 21). The frontal lobes show a larger brain volume while occipital lobes are smaller in volume [22, 23]. Interestingly, mutations of the PTEN tumor suppressor gene have been reported recently in subjects with extreme macrocephaly and autism [24].

Clinical Description

Classical autism is a complex neuro-behavioral disorder with developmental difficulties noted by three years of age and more commonly identified in males (4:1 male to female ratio). It belongs to a group of conditions referred to as autism spectrum disorders (ASD). Pervasive developmental disorder-not otherwise specified (PDD-NOS) and Asperger syndrome are included into this genetically heterogeneous group [2]. The behavioral impairments observed in ASD are grouped into three areas: significant impairment in the development of social interaction; a marked and ongoing impairment of both verbal and non-verbal communication; and restricted, repetitive or stereotyped behaviors and interests [1, 25].

Asperger syndrome is characterized by relatively normal language development [5, 26]. Asperger syndrome occurs more in males than females (8:1 ratio) and may represent the upper end of the autism spectrum. Intelligence is usually normal but poor coordination is common. Social and behavioral problems are usually present in this disorder while a correlation with possible birth trauma or prolonged labor and delivery has been reported. In

addition, pervasive developmental disorder-not otherwise specified (PDD-NOS) is also included in the autism spectrum group. PDD-NOS is sometimes used as a tentative diagnosis in young children or those with milder symptoms [6].

Children with ASD are unable to relate normally to other people and will ignore others by avoiding eye contact. They do not share interests with others, and typically do not comfort or seek comfort from others. The autistic child usually prefers to be by himself in the home environment, engaging in his own, often repetitive, activities. Toys are not used as other children for imaginative games and normal spontaneous play efforts seldom occur. Autistic children generally fail to establish friendships at home or in the school setting. They often watch other children from a distance and do not willing participate in play or other activities. Autistic children fail to develop typical reciprocal communication either by speech, gestures, or facial expressions. They fail to use eye contact in a communicative way. They are unable to grasp the concept of the use of speech to name objects, request items, or to engage with others [27].

The child with autism has impaired receptive language as well as expressive. When children with autism do learn to talk, they display a stereotypic speech pattern including echolalia or unusual inflections and intonations. Many autistic children appear to have difficulty in controlling or modulating sensory integration. Some individuals are particularly sensitive to sounds, voice or light touch such as from clothing seams or tags. They also may have altered pain sensation. Occasionally, autistic children appear to be excessively sensitive to odors and food texture. There is difficulty with regulation of attention and they are easily distracted. Transition from one task to another is also a challenge for most autistic children. Transitioning from one activity to another may cause disruptive behavior. They tend to be rigid and it is not easy for them to learn socially appropriate behavior. However, basic limited social skills can be taught but rarely becomes automatic for the autistic person [28, 29].

Repetitive and stereotypic behaviors are common in children with autism [30, 31]. They may stare or rock as infants while toddlers may develop motor stereotypic behavior such as twirling strings, turning book pages or licking. They may develop whole body movement such as rocking or swaying, hand flapping and complex physical activity with individualized sequence of patting, rubbing, and twirling which may last for hours. The causes of these repetitive movements are unclear but could have a calming effect on the child particularly during times of stress. However, they may develop elaborate, structured rituals in which the order of events, words or phrases and object arrangements must be followed precisely. If not done in an orderly fashion, inconsolable outbursts or disruptive behavior may occur. Tantrums, self-injurious behavior and aggression may also be brought on by changes in routine, touching, or for no apparent reason. In addition, they may demonstrate generalized hypotonia and joint hyperextensibility. Posture tends to be poor in these children. A significant proportion of autistic children walk later than other children and exhibit toe walking and abnormal gait patterns. Refined fine motor skills such as tying shoe laces, buttoning and handling a pencil can be difficult for children with autism. Additionally, poor oral motor movements may lead to difficulty chewing, drooling and articulation problems. Total disregard for danger may result in a high risk of injury or early death, most commonly from drowning. Young children with autism may appear socially aloof and not aware of

feelings of others or their interactions. They can be overly affectionate at times even with strangers, become attached excessively to a parent or may have difficulty in separation.

Diagnosis, Prevalence and Recurrence Risk

The Diagnostic and Statistical Manual of the American Psychiatric Association (DSM-IV) describes the current criteria for diagnosing autism but requires the precise identification and description of autistic symptoms and the age of occurrence [1, 25]. The DSM-IV diagnostic criteria are grouped into three major categories (**I, II, III**) and 12 sub-categories listed in **category I** including: **IA.** *qualitative impairments in social interaction* [***1.*** marked impairment in the use of multiple non-verbal behaviors, ***2.*** failure to develop peer relationships appropriate to developmental level, ***3.*** a lack of spontaneous seeking to share enjoyment, interests, or achievements with other people, ***4.*** lack of social or emotional reciprocity]; **IB.** *qualitative impairments in communication* [***1.*** delay in, or total lack of, the development of spoken language (not accompanied by an attempt to compensate though alternative modes of communication such as gesture or mime), ***2.*** in individuals with adequate speech, marked impairment in the ability to initiate or sustain a conversation with others, ***3.*** stereotyped and repetitive use of language or idiosyncratic language, ***4.*** lack of varied, spontaneous make-believe play or social imitative play appropriate to developmental level]; and **IC.** *restricted, repetitive and stereotyped patterns of behavior, interests and activities* [***1.*** encompassing preoccupation with one or more stereotyped and restricted patterns of interest that is abnormal either in intensity or focus, ***2.*** apparently inflexible adherence to specific, nonfunctional routines or rituals, ***3.*** stereotyped and repetitive motor mannerisms, ***4.*** persistent preoccupation with parts of objects (at least 6 items are needed from the **category I** sub-category list per subject)]; **category II.** *delays or abnormal functioning in specific areas with onset prior to age three years*; and **category III.** *the disturbance is not better accounted for by Rett syndrome or childhood disintegrative disorder.*

A number of checklists besides the DSM-IV are used as tools for diagnosis including CARS (Childhood Autism Rating Scale) [32], GARS (Gilliam Autism Rating Scale) [33] and ABC (Autism Behavior Checklist) [34]. These checklists have been verified and are relatively easy to use in a short evaluation period. In North America, another research diagnostic criteria is the ADI-R (Autism Diagnostic Interview-Revised) developed by Lord et al. [2] and based primarily on a detailed parent interview. A shorter diagnostic tool for autism is ADOS (Autism Diagnostic Observation Schedule) and also developed by Lord et al. [35]. These scales are based on the DSM-IV criteria but are not widely used in the clinical setting due to time and expense to administer.

The causes of autism can be divided into *idiopathic* which comprises the majority of cases and *secondary* which may be due to environmental causes, a chromosome abnormality or a recognized genetic disorder. About 5-10% of individuals with autism can be diagnosed as having secondary autism while the remaining 90-95% of affected individuals have idiopathic autism. However, about 30% of children with idiopathic autism are considered complex with dysmorphic features, microcephaly or with a structural brain malformation [6].

including the hippocampus, amygdala, mamillary bodies, cingulate gyrus and septal nuclei were small in size, but with an increased cell density compared to controls. Therefore, one hypothesis for autism would be a delay in maturation of the limbic system circuits. In addition, they reported a low number of Purkinje cells in the posterior and inferior portions of the cerebellum but with no expected neuronal loss seen in the inferior olivary nucleus. These observations suggested that abnormal brain findings in autistic individuals occurred at about 30 weeks of gestation or before the anticipated time of connection between the olive and Purkinje cells. Neuropathological studies have also shown that most significant anatomical abnormalities are observed in the posterior and inferior portions of the cerebellar hemispheres and may involve cell loss. Recent data showing memory and procedural learning deficits in autistic subjects may relate to cerebellar dysfunction [51].

Neuroimaging

Neuroimaging studies of the brain in autism have supported clinical heterogeneity. Cortical abnormalities identified in autistic subjects include an enlarged volume of the left lateral ventricle or of both ventricles. Cortical malformations include polymicrogyria, macrogyria, and schizencephaly [52, 53]. These findings are not specific for autism. Abnormalities within the posterior fossa structures have been reported in autism including cerebellar vermis and brainstem hypoplasia [54]. Discrepancies between neuropathological and neuroimaging studies in autism indicate additional studies are needed.

Magnetic resonance brain imaging and morphometric analysis have shown a relationship between head circumference and brain volume in autistic subjects. The head size in autistic subjects appears similar to that of healthy children at birth [23]. However, between the ages of two and four years, 90% of autistic children have a larger brain volume than the average seen in similarly-aged children. Macrocephaly is seen in 20-40% of autistic children [20, 21]. Frontal lobes show a larger volume in very young autistic children while occipital lobes may be smaller in size.

Functional magnetic resonance imaging (fMRI) of brain areas performing specific functions (e.g., social processing) in autism have been undertaken. Interestingly, an anticipated marked activation of the fusiform gyrus (i.e., facial fusiform area) in response to pictures of different faces did not occur in autistic subjects while activation of other brain regions (e.g., frontal and occipital areas) occurred. Additional research in this area is needed to further understand the brain's social circuitry (fusiform gyrus-recognition of faces; amygdala-meaning assignment/emotional value; superior and medial tempori gyri-distinction of facial expressions) as well as activation patterns in other areas of the brain such as the cortex, hypothalamus and cerebellum.

Abnormal EEGs have been reported in a wide range (13% to 83%) of autistic children [55-57]. Twenty-four hour video-EEGs in autistic children with regression and without a history of seizures have shown epileptiform activities in 46% of subjects [58]. Magnetoelectro-encephalography (MEG) studies in children with autism and those with regression of skills but with suspected seizures had epileptiform activity reported in 82% of the cases [59]. Seizures and epileptiform activities in children with autism may support the

role of the amygdala in autism since this brain region is known to be a highly epileptogenic region. However, auditory evoked potential studies in autistic subjects without mental retardation did not show consistent findings even though hearing problems may coexist [60].

Neurochemistry

Neurochemical changes have also been identified including amino acid disturbances but the most consistent finding in autistic subjects is an elevation of the serotonin level in platelets [61]. Serotonin, its metabolites, receptors, and transporter protein and their roles in neurodevelopment and function, particularly in autistic subjects are under investigation. In addition, GABA levels are reportedly abnormal in autistic subjects [62].

Recently, several neuropeptide levels (e.g., vasoactive intestinal peptide, calcitonin gene-related peptide, neurotrophin nerve growth factor and pituitary adenylate cyclase – activating polypeptide) were found to be elevated in stored cord blood of children who were later diagnosed with autism or having mental retardation. The levels were normal in non-autistic children with cerebral palsy and in normally developing children [63]. Additional studies are needed for replication.

Modahl et al. [64] also reported significantly lower plasma oxytocin levels in children with autism versus age matched control subjects. Oxytocin is a neuromodulator potentially relevant to impaired social abilities noted in autistic subjects [65]. Endogenous opiates are also thought to play a role in autistic behavior and not surprisingly plasma beta endorphin levels were elevated in individuals with autism and those exhibiting self-injurious behavior [66].

Genetic Causes of Autism

Cytogenetics

Diagnosable medical conditions, chromosome abnormalities and single gene conditions account for less than 10% of cases. However, reported cytogenetic abnormalities involving the 15q11-q13 region have been found in up to 4% of individuals with autism, usually a supernumerary isodicentric chromosome with tetrasomy or trisomy of genes in this region [67, 68]. Several additional reports in autistic subjects have shown duplications, inversions and deletions of the 15q11-q13 region [38, 69, 70]. Inherited duplications of the 15q11-q13 region of maternal origin leads to autism but not present in those with duplications of paternal origin. Hence, the 15q11-q13 region contains an imprinting center which regulates gene expression depending on the parent of origin. A list of case reports of autism with chromosomal abnormalities are shown in Table 1.

The first conditions identified with genomic imprinting (i.e., differential expression of genetic information depending on parent of origin) were Prader-Willi and Angelman syndromes [98, 99]. A chromosome 15q11-q13 deletion of paternal origin is seen in about 70% of subjects with Prader-Willi syndrome (PWS). About 25% of PWS cases have maternal disomy 15 (both chromosome 15s from the mother) and the remaining subjects have a defect

of the imprinting center which controls gene expression in the 15q11-q13 region [99, 100]. PWS is characterized by infantile hypotonia, hypogonadism, hyperphagia with subsequent early onset of childhood obesity, mental deficiency and behavioral problems, short stature and small hands and feet. Angelman syndrome (AS) is generally due to a maternal deletion of the chromosome 15q11-q13 region while paternal disomy 15 or other defects involving this chromosome region are identified in affected individuals, including mutations of the UBE3A gene, a maternally expressed gene involved in brain development. AS is characterized by mental retardation, seizures, ataxia, lack of speech and a characteristic face. On occasion, PWS and AS subjects present with autism indicating the presence of causative genes for autism in the 15q11-q13 region [101, 102].

Table 1. Chromosomal abnormalities reported in individuals with autism.

Chromosomal abnormalities	Genes located at breakpoints or in regions	References
2q32.1-q32.2 or 2q32.2-q32.3 deletion		71
2q37.3 deletion		72
2q37 deletion		73
4q31.3-q33 deletion	AMPA 2, GLRA3, GLRB, NPY1R and NPY5R	74
13q13.2-q13 deletion	NBEA, MAB21L1, DCAMKL1, MADH9	75
13q14-q22 deletion		76
15q22-q23 deletion	PTPN9, SLP-1	77
22q13.3 deletion		78
Xp22.3 deletion		79
4p12-p13 duplication		80
15q11-q13 duplication (maternal)		81
17p11.2-p12 duplication	PMP22, HMSN1	82
3q tetrasomy		83
15q11-q13 tetrasomy		84
translocation (2;8) (q35;q21.2)	PAX3, MMP16	85
translocation (4;12) (q21.3;q15)		86
translocation (5;7) (q14;q32)	SSBP, T2R3	87
translocation (7;13) (q31.3;q21)	RAY1	88
translocation (7;20) (q11.2;p11.2)	AUTS2	89
translocation (20;22) (q13.3;q11.2)		90
translocation (X;8) (p22.13;q22.1)	GRPR, SDC2	91
7q22-q31.2 inversion		92
inverted duplication 7p11.2-p14.1	IMMP2L	93
inverted duplication 15pter-q13::q13-pter		94
mosaicism 46, XX/46, XX, inv(7)(p15q36)		95
isodicentric 15q11.2		96
ring 22p11.31-q13.31		97

An association with the 15q11-q13 region has been reported in a large group of autistic individuals [102] and specifically genetic polymorphisms in the gamma amino-butric acid (GABA) receptor subunit genes [103]. GABA is a major inhibitory neuro-transmitter with

gene expression in early development [104]. Therefore, abnormalities of the GABA receptor genes are good candidates for causing autism. Several of these receptors have been reported to have paternal bias in gene expression [105, 106]. For example, mice deficient in GABA receptor beta 3 gene (*Gabrb3*) are reported with epilepsy, as well as learning or memory deficits reminiscent of the findings seen in autism [107]. In addition, plasma GABA levels are abnormal in subjects with PWS compared with control subjects [108] and in autistic youngsters [62]. Therefore, GABA may become a biochemical marker for autism.

About 3 to 5% of individuals with autism have other chromosome abnormalities besides the chromosome 15q11-q13 region rearrangements. These include inversions, unbalanced translocations, deletions and rings [109]. Examples of chromosome regions involved include 2q, 7q, 8p, 18q, and Xp as well as sex chromosome aneuploidies [110]. In addition, segregation patterns of short tandem repeat polymorphic DNA markers from four chromosomes revealed null alleles at four marker sites in 12 families from a sample of 105 multiplex families with two or more affected siblings with autism [111]. The null alleles resulted from DNA deletions ranging in size from 5 to >260kb. Four families had DNA deletions involving chromosome 7 (3 families with deletion of D7S517 marker and 1 family with deletion of D7S630). Another eight families had DNA deletions involving chromosome 8 (3 families with deletion of D8S264 and 5 families with deletion of D8S272). However, a deletion allele at D8S272 was found in a sample of 299 unrelated controls but the other three deletions were not seen. Thus, it appears that these deletions are specific to autism kindreds and are potential autism susceptibility alleles. Further support for cytogenetic deletions causing autism is found in the recently applied molecular cytogenetic technology involving subtelomeric DNA probes and fluorescence in *in situ* hybridization (FISH). Approximately 10% of autistic subjects will show subtelomeric deletions using FISH [112].

Genetic Syndromes

Autism has been observed in several disorders besides Prader-Willi and Angelman syndromes. For example, children with Down syndrome have an increased chance of developing autism compared to the general population [113]. Approximately one-third to one-half of children with fragile X syndrome which generally affects males will have autistic features such as poor eye contact, speech delay, repetitive behavior, hand flapping, sleep disturbances, self-injurious behavior, hyperactivity and sensory integration problems [114]. Subjects with Rett syndrome due to mutations of an X-linked gene (*MECP2*) have autistic presentation. This condition usually affects females and is characterized by a period of normal development, followed by loss of language, hand wringing and decreased rate of head growth. Other syndromes associated with autism include those with cutaneous features such as tuberous sclerosis, neurofibromatosis type I and hypomelanous of Ito. Additional conditions include Duchene muscular dystrophy, Sotos syndrome, Williams syndrome, Joubert syndrome, de Lange syndrome, Cowden syndrome and Moebius sequence. Metabolic conditions are also associated with autism such as untreated phenylketonuria (PKU), adenylate succinase deficiency and mitochondrial dysfunction [6, 38].

observed on chromosomes 2, 3, 4, 8, 10, 11, 12, 15, 16, 18, and 20. Analysis of families sharing alleles produced an MLS of 3.56 for the DXS470-D19S174 marker combination region.

Yanon et al. [122] reported a follow-up genome-wide screen for autism using 345 families, a sample size of three times greater than previous linkage studies conducted by the same group [121]. Multipoint MLS generated from affected sib-pair analysis identified suggestive linkage evidence on chromosomes 17, 5, 11, 4, and 8. The most significant findings were on chromosome 17q (MLS=2.83), and on 5p (MLS=2.54).

Although several linkage studies have been performed to identify possible susceptibility genes for autism, only a limited concordance of linked loci was seen among different reports. The discordance of linkage results could be a reflection of either numerous genes with weak effects and/or sample heterogeneity. Therefore, in an attempt to increase the power for identifying susceptibility genes, more homogeneous group of subjects with autism have been examined in a number of more recent linkage studies. Buxbaum et al. [123] studied a sample of 49 affected-relative-pair families with autism and phrase speech delay (PSD). Analysis of this restricted subset increased their evidence for linkage to chromosome 2q. The authors concluded that using specific phenotypic criteria (i.e., PSD) can provide a more genetically homogeneous population, which could elevate the likelihood of positional mapping of susceptibility genes for autism. In an independent genome screen, Shao et al. [124] found a suggestive evidence for linkage to a similar region on chromosome 2q (MLS=1.12 for maker D2S116) by analyzing 99 families with ASD. Among their study group were 45 families identified with autism and PSD from a total of 82 sib pairs with autism. Interestingly, analysis of these 45 classified families provided evidence for stronger linkage to 2q (MLS=2.86 for marker D2S116).

Using similar methodology, evidence for a language quantitative trait (LQT) locus on chromosome 7q was reported in 152 multiplex autism families [125]. Additionally, Shao et al. [126] used fine mapping of autistic disorder to chromosome 15q by undertaking a phenotype subtype approach. They used a factor that represented insistence on sameness (IS) which was derived from the repetitive behaviors/stereotyped patterns domain in the Autism Diagnostic Interview-Revised (ADI-R) (2). After inclusion of the IS factor, linkage evidence for the 15q region increased significantly by changing the LOD score from 1.45 to 4.71. Therefore, several genome-wide scans have shown several autism susceptibility loci using molecular markers from multiplex families with more than one affected individual. Strong linkage has been reported for chromosomes 2, 3, 5, 7, 8, 13, 15, 16, 17, 19, and X as well as nominal evidence for linkage to chromosomes 1, 4, 10, 11, 12, 18, 20 and 22. Thus, the genetic cause is complex and inheritance is poorly understood although the involvement of over 20 genes is proposed. However, the most consistent linkage finding has been for chromosome 7q confirmed by several independent genome-wide scan studies.

Candidate Genes

To date, several candidate genes have been examined to evaluate their possible associations with autism. These candidate genes have been generally selected based on a

supportive linkage/cytogenetic evidence or the presence of certain findings at the clinical level in subjects affected with autism. The main technique in screening of these candidate genes has been direct sequencing of the gene exons using genomic DNA from subjects with autism compared with controls. As a result, a number of nucleotide changes have been reported in association with autism but have not led to replicated findings by other investigations. Given that individuals with ASD are clinically heterogeneous, it is not surprising that most of the observed DNA sequence changes in candidate genes have not been reproduced in other studies. A list of proposed candidate genes for autism with their chromosomal location and cited references is shown in Table 2.

Gene Expression and Protein Analysis

Purcell et al. (204) examined gene expression levels in the cerebellum from 10 subjects with autism and compared with 23 matched controls using cDNA microarray technology and Western blotting. Their results suggested that subjects with autism may have specific abnormalities in the AMPA (i.e., alpha-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid) type glutamate receptors and glutamate transporters in the cerebellum which may be involved in the pathogenesis of autism. In another study, a high-throughput quantitation of MeCP2 expression was examined on a tissue microarray containing frontal cortex samples from 28 different patients with neurodevelopmental disorders including autism and controls [205]. Analysis of these expression data indicated abnormalities in multiple pathways that regulate the expression of MeCP2 in autism spectrum disorders in addition to Rett syndrome. In addition, Martin-Ruiz et al. [206] studied nicotinic receptor gene expression in autism by analyzing nicotinic gene receptor subunit mRNA in conjunction with protein levels and receptor binding in cerebral cortex and cerebellum. The data obtained from this molecular analysis, indicated a reduced gene expression of the alpha4-beta2 nicotinic receptor in cerebral cortex as a major feature of the neuro-pathology of autism. Furthermore, post-transcriptional abnormalities for the alpha7 subtype were seen in the cerebellum.

Females with Autism

As reported previously, an excess of males with autism compared with females occurs at a proportion of about four to one [207]. Sex differences in the prevalence of pervasive developmental disorders including autism have also been described in both singleton and multiplex families [2, 207]. The reason for this gender discrepancy in ASD has not yet been explained. However, the role of epigenetic factors such as imprinting was proposed by Skuse [36] as a possible mechanism involved in sex differences in the liability of autism. In this model, it was hypothesized that the threshold for phenotypic expression of many autistic characteristics is influenced by an imprinted X-linked gene(s) that is protective in nature. If predisposing X-linked gene(s) for autism exists that are maternally imprinted or silenced when transmitted from the mother, these genes can only be expressed when inherited from the father. Because only females have a paternal X chromosome, Skuse [36] concluded that the threshold for phenotypic expression is higher in females than in males.

Table 2. Proposed candidate genes for autism

Gene	Gene Symbol	Location	References
cAMP binding protein	cAMP-GEFII	2q21-q33	127
Solute carrier family 25	SLC25A12	2q24	128
Voltage-gated sodium channel, type I, aplpha	SCN1A	2q24.3	129
Voltage-gated sodium channel, type III, alpha	SCN3A	2q24.3	129
Oxytocin receptor	OXTR	3p25-p26	65, 131
D5 dopamine receptor	DRD5	4p16.1	132
Major histocompatibility complex, class II, DR beta 1	HLA-DRB1	6p21.3	133
Glutamate receptor 6	GluR6	6q16.3-q21	134, 135
Homeobox A1	HOXA1	7p15-p14.2	136-143
Plasminogen activator inhibitor-1	PAI-1	7q21.3-q22	144
Reelin	RELN	7q22	145-148
Transcription factor containing a polyglutamine tract	FOXP2	7q31	149-152
Cortactin binding protein 2	CORTBP2	7q31	153
FAM4A1 splice variant a	Ray1 (FAM4A1)	7q31.1-q31.3	154
Ca^{2+} dependent activator for secretion protein family	CADPS2	7q31.3	155
Wingless-type MMTV integration site family member 2	WNT2	7q31-33	148, 156, 157
Homeobox transcription factor Engrailed 2	EN2	7q36.2	158-160
Vasoactive intestinal peptide receptor type 2	VIPR2	7q36.3	161
Exostoses (multiple) 1	EXT1	8q24.11-q24.13	162
PTEN Tumour suppressor gene	PTEN	10q23	24
c-Harvey-ras oncogene	HRAS	11p15.5	163-166
Secretin	SCT	11p15.5	167
Arginine-vasopressin receptor 1a	AVPR1A	12q14-q15	168
Serotonin 2A receptor	HTR2A	13q14-q21	169
Ubiquitin protein ligase E3A	UBE3A	15q11-q13	170
Aminophospholipid translocase	ATP10C	15q11.2	171
Gamma-aminobutyric acid receptor subunit beta3	GABRB3	15q11.2-q12	172-174
Serotonin transporter (5-HTT)	SLC6A4	17q11.1-q12	175-177
Neurofibromatosis type 1	NF1	17q11.2	178-180
Homeobox B1	HOXB1	17q21-q22	136, 137, 139, 140, 142
Adenosine deaminase	ADA	20q13.12	181-183
Adenylosuccinate lyase	ADSL	22q13.1	184
Monoamine oxidase A	MAOA	Xp11.4-p11.3	185
Aristaless related homeobox	ARX	Xp22.12-p21.3	186
Gastrin-releasing peptide receptor	GRPR	Xp22.2-p22.13	91, 187
Neuroligin 4	NLGN4	Xp22.32-p22.31	188-192
Human OPA-containing protein	HOPA	Xq13	193, 194
Neuroligin 3	NLGN3	Xq13.1	189-191
Fragile X mental retardation 1	FMR1	Xq27.3	195-197
Methyl-CpG-binding protein 2	MECP2	Xq28	198-203

Because an unequal sex ratio in autism could be explained by X chromosome gene involvement, we proposed that X chromosome inactivation patterns may play a role in a subset of affected females [37]. Therefore, we compared X chromosome inactivation patterns in 77 autistic and control female siblings from the Autism Genetics Resource Exchange (AGRE) to determine if skewness of the X chromosome inactivation pattern exists in females with autism which may allow for expression of genes for autism on the X chromosome. Plenge et al. [208] reported that skewed X chromosome inactivation (i.e., one X chromosome may be more or less active compared with the second X in somatic cells at an arbitrary assigned ratio of, for example, > 80:20%) was a common finding among X-linked mental retardation female carriers using the polymorphic human androgen receptor (AR) gene located at Xq11.2 that becomes inactivated on one of the X chromosomes.

X Chromosome Inactivation

X chromosome inactivation occurs early in embryonic development of somatic cells in human females to achieve gene dosage compensation with males (209). Therefore, one of the two X chromosomes is inactivated in each female cell at random which then results in the same number of active X chromosome genes in both male and female cells. The X inactivation is a complex process and requires three main steps: initiation, spreading, and maintenance [210, 211]. During the initiation step, one of the two X chromosomes is selected to be inactivated and requires the presence in *cis* of the X inactivation center (XIC) [212, 213]. In humans, the candidate region for XIC is located on the proximal long arm of the X chromosome (at band Xq13).

In 1991, the XIST gene (X-inactive specific transcript) was discovered in the X chromosome inactivation center (XIC) and became a candidate gene for initiation of inactivation [213]. The XIST gene is constitutively expressed from the inactive X chromosome and encodes a transcript but does not code for a protein [214]. The untranslated RNA product of the XIST gene "coats" the presumptive inactive X chromosome which results in spreading of inactivation from the XIC region. However, certain X linked genes have been found to escape inactivation and are expressed from both the active and the inactive X chromosome [215]. Genes that are not subject to X inactivation are distributed non-randomly along the X chromosome with the majority clustered on the short arm of the human X [216].

The human androgen receptor (AR) gene located at Xq11.2 contains a highly polymorphic in-frame CAG gene codon repeat encoding 11-31 glycine residues in exon 1. X inactivation patterns can be assessed using the AR gene in females informative at the CAG repeat following PCR amplification and digestion with methyl sensitive restriction enzymes (e.g., *Hpa*II). X chromosome skewness is classified into three groups: randomly skewed (50:50%-64:36%), moderately skewed (65:35%-80:20%) and highly skewed or significant (> 80:20%) [217, 218].

Autism and X Chromosome

In addition to genetic linkage data, several lines of evidence such as X chromosome rearrangements, indicate that the X chromosome should be further studied in autism. For example, autistic patients have been reported with Xp22 duplications and/or deletions [219,

220] and autistic females have been described with translocations involving the X chromosome [91]. In particular, a female showed a breakpoint at Xp22.13 which occurred in the first intron of the GRPR gene (gastrin-releasing peptide receptor). In addition, mutations in three X-linked genes (i.e., NLGN3, NLGN4 and MECP2) have been reported in subjects (both males and females) with autism.

Neuroligin Genes (NLGN3 and NLGN4)

Recently, Jamain et al. [188] reported mutations in two X-linked neuroligin genes, NLGN3 and NLGN4, in male individuals with autism spectrum disorders (ASD). The neuroligins are encoded by a family of five genes producing cell-adhesion molecules essential for the formation of functional neural synapses [188]. These authors hypothesized that the identified neuroligin mutations may abolish formation, stabilization or recognition of specific synapses essential for the communication processes which is implicated in patients with autism.

Jamain et al. [188] screened for mutations in NLGN3 (localized at Xq13), NLGN4 (Xp22.3) and NLGN4Y (Yq11.2) in 158 subjects (140 males and 18 females), including 36 pairs of affected siblings and 122 trios with an affected ASD individual. The NLGN3 mutation was a C→T transition in exon 6 which changed a highly conserved arginine residue to cytosine (R451C) in a Swedish family with two affected brothers, one with typical autism and the other with Asperger syndrome. The mother was heterozygous for this substitution but otherwise healthy. The NLGN3 mutation was not found in 200 unaffected controls (100 females and 100 males).

In another Swedish family, the authors reported a 1-bp insertion (1186insT) in exon 5 of the NLGN4 gene in two affected sons (autism and Asperger) resulting in a frameshift mutation causing a premature termination. It was concluded that the mutation was a *de novo* mutation originating in the mother. This mutation was not seen in 350 unrelated and unaffected controls (250 females and 100 males). Later, Laumonnier et al. (191) reported a 2-base pair deletion in the NLGN4 gene in a large French family including several male members affected by nonspecific X-linked mental retardation, with or without autism or pervasive developmental disorder. Two transcriptional isoforms have been identified for NLGN4 [188]. Functional analysis revealed that point mutations at arginine 451 (NLGN3 gene) and nonsense mutations at aspartate 396 of the NLGN4 gene results in intracellular retention of mutant proteins and loss of the synaptic function [221, 222]. These functional findings further support that reported mutations in these two neuroligin genes are likely to be relevant for the neurodevelopmental defects seen in ASD and with mental retardation by impairing the function of a synaptic cell adhesion molecule [222].

Despite these positive findings, no mutations were found in 96 individuals affected with autism in a Quebec population [190] studied and 196 autistic probands screened by Vincent et al. [189]. To determine whether reported mutations in NLGN3 or NLGN4 occur in both males and females with ASD in the American population, we screened 67 autistic subjects (64 with classical autism and 3 with Asperger syndrome) [192]. The reported mutations in the two neuroligin genes were not detected in our group of individuals with ASD. Together, these negative findings suggest that mutations in the NLGN3 and NLGN4 genes are not common or occur at a low frequency in the autism population.

MECP2 Gene (Rett Syndrome)

Mutations in the X-linked methyl-CpG-binding protein 2 gene (MECP2) have been reported in approximately 80% of patients with classical Rett syndrome (RTT). RTT is a childhood neurodevelopmental disorder that occurs almost exclusively in females. Subjects with RTT may also manifest autistic features. The phenotypic overlap between autistic disorder and RTT suggests that the MECP2 gene should be screened for subjects with autism. Carney et al. [202] analyzed 289 autistic patients (including 220 males and 69 females) for the presence of mutations in the MECP2 gene. Two autistic females were found to have *de novo* mutations in this gene. Furthermore, X chromosome inactivation studies revealed a borderline X chromosome skewness in these two autistic females [202].

Description of X Chromosome Inactivation Assay

In order to evaluate X inactivation, genomic DNA is extracted from peripheral blood and amplified with polymerase chain reaction (PCR) in the presence of forward and reverse primers for the polymorphic AR gene. The polymorphic CAG repeat size is determined by capillary electrophoresis using an ABI 310 DNA sequencer (Foster City, CA). Subsequently, 200 nanograms of genomic DNA is digested with the methyl sensitive *Hpa*II restriction enzyme as described elsewhere [223]. The 5' end of the reverse primer was fluorescently labeled with 6-FAM (6-carboxyfluorescein) and the resulting PCR fragments analyzed by capillary electrophoresis following established protocols [224, 225]. X chromosome inactivation is calculated as the ratio of the height of the shorter peak to the sum of the two peaks using genotyping software after digestion (see **Figure 1**).

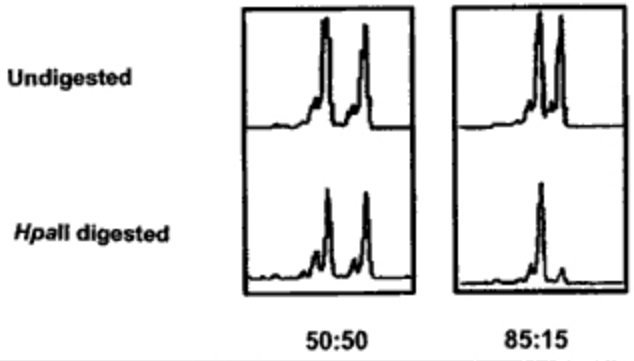

Figure 1. X inactivation analysis by genotyping of the CAG repeat in the human androgen receptor (AR) gene after digestion. An example of the random and skewed X inactivation is shown. The peak representing the active (unmethylated) allele would be digested and reduced in size. If skewness is present the peak height would differ between the two peaks representing each X chromosome [37].

In our study, X chromosome skewness was classified into three groups: randomly skewed (50:50%-64:36%), moderately skewed (65:35%-80:20%) and highly skewed or significant (> 80:20%) as similarly reported by others [217, 218]. The selection of a ratio of >

80:20% for demonstrating X chromosome skewnees is arbitrary but used consistently in other published studies [208, 217, 218].

Three sets of control experiments were performed in our studies to confirm complete digestion of genomic DNA and to ensure correct assessment of the ratio of an active (unmethylated) versus an inactive (methylated) X chromosome: 1) a serial dilution of DNA from two males carrying a different AR allele as a control experiment, 2) genomic DNA from a male with a different CAG repeat size was added to the digestion reaction as an internal control, and 3) the digestion, PCR and genotyping were repeated up to three times in several samples to ensure reproducibility and equal amplification of both alleles.

The presence of a known mutation in the promoter region of the XIST gene was examined in all females with X inactivation skewness greater than 80%. A 276-bp fragment comprising the mutation was amplified and subsequently digested with *Hpa*II enzyme following the protocol reported by Plenge et al. [226]. After digestion, a normal promoter region would produce two fragments while the mutation produces three DNA bands on an agarose gel.

X Chromosome Inactivation Studies in Our Females with Autism

Using the X inactivation assay described above, we studied X chromosome skewness in 35 females with classical autism and similarly aged female siblings as controls with an age range of 5 to 12 years obtained from AGRE using the polymorphic androgen receptor (AR) gene. Thirty of the 35 females with classical autism were informative for the AR gene polymorphism (i.e., demonstrating two different allele sizes) while 35 of the 42 unaffected female controls were informative. A greater percentage of X inactivation skewness (highly skewed > 80:20%) was seen in the autism group (10 of 30 or 33%) compared with the control group (4 of 35 or 11%) ($p=0.04$; Fisher's exact test, 2-sided). Examination of the promoter region of the XIST gene reported to be involved in X chromosome inactivation in our subjects did not demonstrate any mutations. The subjects were classified into three subgroups representing highly, moderately, and randomly skewed X inactivation patterns as previously described (see **Figure 2**). When X inactivation patterns were compared using this subgroup classification, as reported in other studies, differences were observed between the autism and control groups ($p= 0.03$; Exact test of Wilcoxon-Mann-Whitney, 1-sided). In healthy females, X chromosome inactivation is considered to follow a Gaussian or bell-shaped distribution with highly skewed patterns being uncommon events [227].

Although no data were previously available for females with autism, X inactivation studies have been performed in other neurological conditions. For example, a significantly higher percentage of X inactivation skewness was observed in X-linked mental retardation carriers [208] and a high concordance of skewing of X inactivation was reported between mothers and daughters in families with dystrophinopathies [228, 229]. In addition, Villard et al. [225] described a totally skewed pattern of X inactivation in four familial cases of Rett syndrome without the MECP2 gene mutation. Female carriers of X-linked adrenoleukodystrophy (X-ALD) were also more susceptible to X chromosome inactivation skewness [218] with an equal proportion of moderate and highly skewed findings observed in

X-ALD female carriers as seen in our study of females with autism. Furthermore, extreme skewing of X-inactivation was observed in fetuses and newborns associated with confined placental mosaicism of an autosomal trisomy and in Prader-Willi syndrome females with uniparental disomy of chromosome 15 [230]. Skewed X-inactivation was also seen in female carriers of dyskeratosis congenita [231] and in females with recurrent spontaneous abortions [232]. Non-random X inactivation was also suggested to explain reduced penetrance in carrier females with the fragile X gene mutation [233].

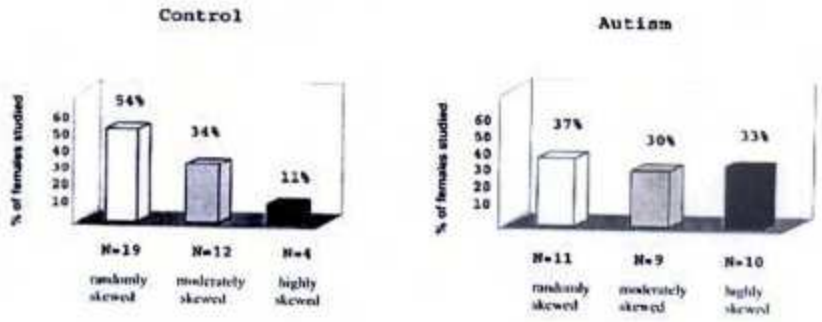

Figure 2. Distribution of patterns of X inactivation in 30 autistic females and 35 unaffected female siblings. X inactivation patterns were divided into random (50:50%-64:36%), moderately skewed (65:35%-80:20%), and highly skewed (80:20%). A significantly greater percentage of highly skewed X inactivation (>80:20%) was detected in the autism group compared with the unaffected female sibling group ($p=0.04$; Fisher exact test, 2 sided). When comparing X inactivation patterns classified into three subgroups (random, moderately skewed, highly skewed), significantly differences were found in the two subject groups ($p=0.03$; Exact test of Wilcoxon – Mann- Whitney, 1 – sided) [37].

Possible explanations for the observed X chromosome skewness in our study includes selective cell death after initial random X inactivation (e.g., carriers of X-autosome translocations, lymphocytes of carriers of X-linked immunodeficiency disease) but probably unlikely in the peripheral blood of our females with autism. A second possibility for the X chromosome skewness may be selective ascertainment of individuals from the tail of a random distribution of inactivation because of an unusual or unexpected phenotype. Examples of this phenomenon would include female carriers of Duchenne muscular dystrophy manifesting the disease state. If there is an X-linked recessive susceptibility gene for autism, this could explain the observation of X chromosome skewness in our females with autism. X chromosome inactivation is a complex, multi-process phenomenon which involves several epigenetic factors such as: DNA methylation, X chromosome reactivation, genes escaping inactivation, parental origin effect (imprinting) and possible elements influencing X inactivation skewness.

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