CHAPTER 1

INTRODUCTION

Background and Rationale

Autism is a behavioral disorder characterized by severely abnormal development of social interaction and communication skills and repetitive or stereotypical behaviors. Its prevalence is about 4-5 cases per 10,000 children in the general population. The disorder normally becomes evident before the age of 3 years and is found in males more than females (4:1). A subgroup of less than 10% of autistic children are categorized as having secondary autism, which results from chromosomal abnormalities, single gene disorders and/or environmental factors. The most common chromosomal abnormalities leading to secondary autism have been found on the X-chromosome, chromosome 7 and chromosome 15. A minority of autistic disorders are also associated with Fragile X syndrome. Recently, some rare instances of subtelomere deletions have been reported in autistic patients.

Mental retardation (MR), defined as an individual having an intelligence quotient (IQ) below 70, is present in approximately 1-3% of the general population. Both genetic and environmental factors can cause MR. More than half of MR cases are categorized as idiopathic mental retardation (IMR), of which cryptic subtelomeric rearrangement is a significant cause. Conventional cytogenetic analysis, based on G-banded chromosomes at the 450-550 band levels, can not detect cryptic subtelomeric rearrangement, because the resolution at this level is not sensitive enough to detect deletion sizes smaller than 3-5 Mb. As the subtelomeric regions of human chromosomes are gene-rich, so chromosomal rearrangements in this region result in physical abnormalities and mental retardation. One of the commonly used techniques for investigating subtle chromosomal abnormalities is fluorescence *in situ* hybridization (FISH), which uses a full set of 41 subtelomeric-specific probes located within 0-3 Mb from a telomere.

Autism and mental retardation are closely related. Although the nature of the relationship between the two conditions is not well understand, cryptic rearrangements involving the subtelomeric regions of chromosomes have emerged as an important cause of IMR, although rarely reported in autism. Subtelomeric rearrangements have never been investigated in Thai patients, so this study was designed to help fill in this gap by examining the frequency of

subtelomeric rearrangements in Thai patients with IMR, and including some autistic patients. Subtelomeric FISH would be a useful diagnostic test for diagnosis in IMR in the future, and enable appropriate genetic counseling when subtelomeric rearrangements are found.

Review of Literatures

Autism (MIM 209850) is a complex neurodevelopmental disorder, which is described by impairment in three domains: 1) language, communication; 2) social interaction; and 3) repetitive stereotypic behaviors. Autism is one of pervasive developmental disorders (PDDs), which also include Rett syndrome, Asperger syndrome, Childhood disintegrative disorder, and pervasive developmental disorder not otherwise specific (PDD-NOS). The development of this disorder usually appears in the first 3 years of life and occurs in males more than females (4:1). The prevalence of autism is about 4-5 cases per 10,000 children in the general population, but the incidence has been increasing depended on the study group, ranging from 16.8 to 31 cases per 10,000 for children with PDDs (Honda et al., 1996; Jorde et al., 1990). The first noticeable impairment is in poor communication skills with other people, including speech, gestures and facial expression. The impairment of social interaction includes avoiding eye contact, separating themselves from other people, no shared interests and a lack of symbolic-imaginative play. Autistic children usually also have repetitive or stereotypic behaviors, and cannot be flexible in their daily activities. Some autistic children have isolated, prominent skills such as superior musical ability, exceptional artistic talent, or the ability to instantly calculate dates. Approximately 50 to 70% of autistic children have mental retardation, as assessed by nonverbal IQ testing (Skjeldal et al., 1998; Miles and McCathren, 2003).

There are several standard tools that are used for diagnosing autism, such as the criteria from the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV), the diagnostic checklist of the Childhood Autism Rating Scale (CARS), and the International Classification of Diseases, 10th Edition (ICD-10) (Deuel 2002; Miles and McCathren, 2003).

The causes of autism can be divided into 2 groups:

(1) Idiopathic autism (90-95%), which can be further divided into two subgroups:

1.1 Essential autism, found in 70% of cases, which has no physical abnormalities, and

1.2 Complex autism, occurring in 30% of cases, which shows dysmorphic features, microcephaly and/or a structural brain malformation.

(2) Secondary autism (5-10%) can be divided into environmental causes and heritable causes. The heritable causes are chromosomal abnormalities and single gene disorders, such as Fragile X syndrome (FXS), Rett syndrome and Tuberous Sclerosis Complex (TSC). (Miles and McCathren, 2003; Volkmar and Pauls, 2003).

A genetic component for autism has been strongly suggested by twin studies that show a higher concordance rate in monozygotic than dizygotic twins. Autistic disorder can be associated with neurogenetic disorder such as FXS, untreated PKU, TSC and Prader-Willi (PWS)/Angleman (AS) syndrome (Rapin and Dunn, 2003). Fragile X syndrome (FXS) is significantly associated with autism, in which 5-60% of individuals with FXS are on the autistic spectrum. However, the frequency of FXS among individuals with autism ranges from 0-20% (Muhle et al., 2004; Xu et al., 2004). Recently, Reddy (2005) found that the incidence of fragile X mutation was 2.2% in autistic patients. Similar to autosomal dominant disorder, neurofibromatosis may also be associated with autism, but the frequency is less than TSC or FXS (Muhle et al., 2004).

Approximately 3-9% of autistic patients have chromosomal anomalies (Reddy, 2005; Wassink et al., 2001; Xu et al., 2004). The most common chromosomal abnormalities in autism are chromosomes 7, 15, and X. Abnormalities of chromosome 15 at the 15q11-13 locus, PWS/AS region, have been reported in approximately 1-4% of autistic cases. According to Reddy (2005), the most common chromosomal abnormality in this region was duplication and a supernumerary isodicentric 15q chromosome which was leading to trisomy or tetrasomy of genes at the 15q11-q13 region. Association studies have implicated several candidate genes in this region, including *GABRB3*, *GABRA5* and the *GABA* genes, which are strongly involved in the pathogenesis of autism. Another gene at the 15q11-q13 region is the maternal-derived AS gene, *UBE3A* (E6-AP ubiquitin-protein ligase gene), which is expressed mainly in the human brain and is regulated by a complex mechanism involving imprinting and silencing by antisense RNA transcribed from the paternal chromosome. The phenotypes of an individual with chromosome 15

duplication include language delay, epilepsy, mental retardation and facial dysmorphology. This phenotype overlaps with the autistic phenotype (Muhle et al., 2004; Xu et al., 2004; Reddy, 2005). The duplication of 15q11-q13 in autistic children is derived exclusively from maternal origin. Moreover, chromosomal translocation involving the 7q22-q33 region of chromosome 7 is a site which contains many candidate genes for autism, for example *AUTS1* on 7q32, *WNT2* gene on 7q31.31 and *RELN* gene on 7q22.1 (Hutcheson et al., 2003; Xu et al., 2004). In addition, Roubertie et al. (2001) reported on a 22q11 deletion in autistic children using the FISH technique. Also, some reports have suggested that the 22q13 deletion may be associated with hypotonia, developmental delay, absent or severely delayed speech and autistic behaviors (Manning et al., 2004).

Several cytogenetic analyses have identified chromosomal abnormalities in autism. Lists of chromosomal abnormalities in autistic patients are summarized in table1.

Table 1 Summary of chromosomal abnormalities in autistic patients.

Reference	Chromosomal abnormalities	Technique
Weidmer-Mikhail et al., 1998	del(8q), XYY, tetrasomy 15	Chromosome
Roubertie et al., 2001	del(22)(q11)	Chromosome+FISH
Steele et al., 2001	del(13)(q14q22)	Chromosome
Smith et al., 2001	del(2)(q37.3)	FISH
Wolff et al., 2002	del(2)(q37)	FISH
Borg et al., 2002	t(2;8)(q35;q21.2)	Chromosome+FISH
Ana et al., 2002	+der(15)	Chromosome+FISH
van Karnebeek et al., 2002b	mos 45,X/46,XY,+mar	Chromosome
Oliveira et al., 2003	mos 46,XY,add(12)(p13.3)/ 46,XY	Chromosome+FISH
Tonk et al., 2003	t(3;18;8)	Chromosome+FISH
Keller et al., 2003	dup(5)(?p14?p15.1), dup(15)(q11q13)	Chromosome+FISH

Sauter et al., 2003	mos 46,XY,del(20p)(p12.2)/ 46,XY	Chromosome+FISH
Manning et al., 2004	del(22)(q13)	Chromosome+FISH
Lukusa et al., 2004	del(2)(q37.3)	FISH
Verri et al., 2004	del(10)(p14p15.3)	Chromosome+FISH

Table 1 (continued)

Reference	Chromosomal abnormalities	Technique
Nair-Miranda et al., 2004	r(22)	FISH
Reddy, 2005	mos 47,XY,+mar/46,XY, mos 47,XX,r/46,XX, +mar(2cases), dup(15)(q11.2q13), del(2)(q37.3), del(3)(p25), del(12)(q21.2q23.3), del(13)(q13.2q14.1), inv(10) (p11.2q21.2), mos 6,XY,inv(14)(q11.2q33) /46,XY, add(17)(q23), t(1;14)(q25;q31.2), der(14;18)(q10;q10)	Chromosome+FISH
(Koochek et al., 2006)	der(15)t(14;15)(q11.2;q13.3)	Array-CGH

However, several previously studies reported that subtelomeric rearrangement was rarely found in autistic children (<1%). Wolff et al. (2002) reported a terminal 2q deletion in one out of ten autistic children. Medne et al. (2003) identified one child of 108 autistic children he examined had an unbalanced subtelomeric translocation; der(18)t(13;18)(q34;q23), which was not detected by conventional cytogenetics. In contrast, van Karnebeek et al. (2002b) examined 25 mentally retarded adults with autism, and no subtelomeric rearrangements were found. Also, Battaglia and Bonaglia (2006) found no subtelomeric rearrangements in 71 patients with PDD, using the Cytocell Multiprobe-T system (Cytocell).

Mental retardation (MR) is commonly defined as a person who has an intelligence quotient (IQ) below 70, which is further sub-classified into mild MR (IQ 50-70) and moderate to severe MR (IQ < 50). The prevalence of MR is about 1-3% worldwide (Baralle, 2001). One of the main causes of MR is chromosomal rearrangement. Approximately 4-28% of patients with MR have chromosomal abnormalities (Joyce et al., 2001; Flint and Knight, 2003) . More than half of MR cases are categorized as idiopathic mental retardation (IMR), in which 4.8-10% of the patients have shown cryptic subtelomeric rearrangements in various studies (i.e. Knight et al., 1999; de Vries et al., 2003). Conventional cytogenetic analysis can not detect subtle chromosomal rearrangements involving the telomere region. This is because the terminal bands have a negative G-band and similar morphological characteristics, and cryptic chromosomal rearrangements may not be easily detected as the resolution of conventional cytogenetic analysis is not very sensitive in detecting deletion sizes smaller than 3-5 Mb. One of the commonly used techniques in investigating subtle chromosomal abnormalities is fluorescence *in situ* hybridization (FISH) (Slavotinek et al., 1999; Knight and Flint, 2000a).

The end of the human chromosome is divided into 3 regions: 1) telomere or simple tandem repeat, 2) telomere-associated repeat region (TAR), and 3) a unique sequence (Fig.1). The telomere or simple tandem repeat is a TG-rich region, which is (TTAGGG)n, ranging from 2 to 20 kb in length and all chromosomes have the same sequence. The function of this region serves as a protective cap for linear eukaryotic chromosomes. Loss of telomere function is associated with genetic instability and loss of cell viability. The telomere participates in the process of chromosome repair as evidenced by the capture of de novo synthesis of telomere repeats. Next to this region is a telomere-associated repeat region (TAR), which consists of 100-300 kb of a few copies of a repetitive sequence (TTAGGG)n and has been shown to be highly polymorphic. This unique sequence is susceptible to a change in the number of copies because these regions are gene-rich. Subtelomeric rearrangements can cause physical abnormalities, mental retardation, recurrent miscarriage and hematological malignancies (Knight et al., 1999; Knight and Flint, 2000a; Clarkson et al., 2002; Rodriguez-Revenga et al., 2004).

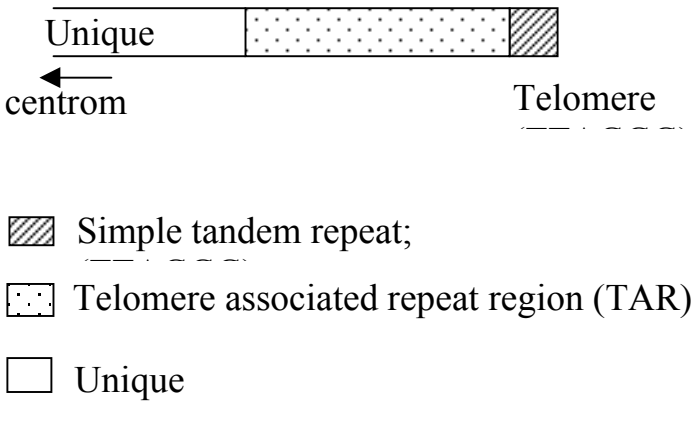

Figure 1 Diagram of human

A complete set of subtelomeric-specific probes, consisting of 41 different regions for all chromosome ends with the exception of the short arm of 5 acrocentric chromosomes and located 0-3 Mb from the telomere, was developed (Knight et al., 2000b). These probes made it possible to detect all chromosome ends and assess them for possible subtelomeric rearrangements. Knight et al. (1999) reported that the prevalence of subtelomeric rearrangements in patients with moderate to severe mental retardation was 7.4%, while in children with mild retardation rearrangements were found in only 0.5%. Half of the cases were de novo because chromosomal rearrangements were not found in their parents. For this reason, several methods have been developed for detecting subtelomeric rearrangement (Flint and Knight, 2003). The molecular tools which used to identify chromosomal rearrangements are FISH, which uses a gene-specific or subtelomeric probe, and Comparative Genomic Hybridization (CGH). Many studies have found 0-11.9% subtelomeric rearrangement in patients with idiopathic mental retardation (Anderlid et al., 2002; Barker et al., 2002; Bocian et al., 2004; Clarkson et al., 2002; Harada et al., 2004; Helias-Rodzewicz et al., 2002; Joyce et al., 2001; Knight et al., 1999; Popp et al., 2002; Rio et al., 2002; Rodriguez-Revenga et al., 2004; Slavotinek et al., 1999; van Karnebeek et al., 2002a; Sogaard et al., 2005; Velagaleti et al., 2005). Subtelomeric FISH results of screening subtelomeric rearrangements in IMR patients are summarized in Table 2.

Table 2 Summary of cryptic subtelomeric rearrangement in IMR.

References	No. of cases	No. of Subtelomeric rearrangements	Method used
Slavotinek et al., 1999	27	2 (7.4%)	Microsatellite marker
Knight et al., 1999	466	22 (4.7%)	Subtelomeric FISH
Joyce et al., 2001	200[a] 150[b]	0 (0.0%) 2 (1.33%)	Subtelomeric FISH
Anderlid et al., 2002	111	10 (9%)	Subtelomeric FISH + SKY
Clarkson et al., 2002	50	3 (6%)	Subtelomeric FISH + SKY
Helias-Rodzewicz et al., 2002	33	3 (9%)	Subtelomeric FISH
Popp et al., 2002	30	4 (13.3%)	M-TEL[c]
Barker et al., 2002	250	9 (3.6%)	Subtelomeric FISH
van Karnebeek et al., 2002	184	1 (0.5%)	Subtelomeric FISH
Bocian et al., 2004	84	10 (11.9%)	Subtelomeric FISH
Harada et al., 2004	69	4 (5.8%)	Subtelomeric FISH + Microarray CGH
Rodriguez-Revenga et al., 2004	30	2 (6.7%)	Multiprobe FISH+ DNA polymorphism analysis
Velagaleti et al., 2005	18	2 (11%)	Subtelomeric FISH
Sogaard et al., 2005	132	9 (6.8%)	Subtelomeric FISH
Total	1834	83 (4.5%)	

[a]=IMR study group,

[b]=control group consisted of relatives of probands with numerical or structural chromosome abnormality,

[c]=Multiplex FISH telomere integrity assay

High-Resolution Peripheral Blood Cytogenetic Analysis (Lawce and Brown, 1997; Clousto, 2001)

Chromosome analysis is done on the peripheral blood of patients clinically diagnosed for chromosome abnormality or family members at risk for an abnormality previously confirmed in a relative. Lymphocytes in peripheral blood rarely undergo spontaneous cell division and must be stimulated to divide by exposure to mitogens, such as phytohemagglutinin. Prior to harvest, the culture is treated with colchicine (colcemid®) to arrest the cell cycle in the metaphase of mitosis when the chromosomes are condensed. Then the cultures are treated with hypotonic solution to swell the lymphocytes which are subsequently fixed. Slide preparations are made, stained and karyotyped.

High-resolution analysis is a special technique of the routine stimulated blood procedure. This technique provides a number of mitotic cells in late prophase or prometaphase and the chromosomes are longer and less condensed. Consequently, the level of band resolution of these chromosomes is at 850 or greater and subbands not seen in routine analysis can be detected (Figs 2, 3). The advantage of this method is that it can rule out microdeletion or rearrangement of specific chromosomes, such as Prader-Willi syndrome (15q-), Retinoblastoma (13q-) and Wilms' tumor (11p-). It is also useful for fine breakpoint determination.

Figure 2 G-banded karyotype at 400 band levels. (Conventional routine cytogenetic)

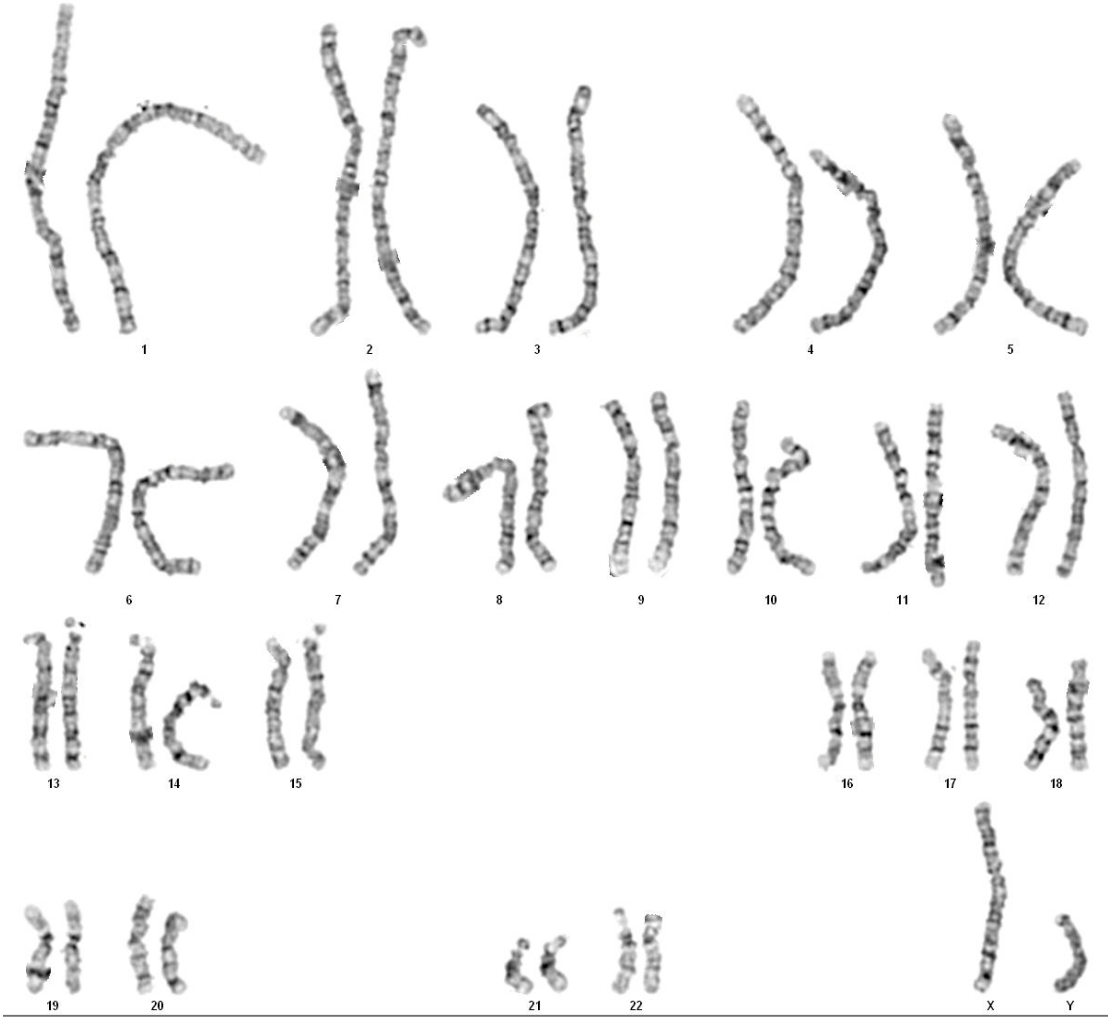

Figure 3 G-banded karyotype at 850 band levels. (High-resolution cytogenetic)

The high-resolution technique involves cell synchronization of peripheral blood culture with methotrexate (MTX). The effect of MTX is to block the cell cycle at the S stage and inhibit thymidine synthesis by blocking the conversion of dihydrofolate to tetrahydrofolate, which is needed to convert uridine to thymidine. Then, as a result of depleted thymidine in the cultures, the cells are unable to complete replication. After overnight MTX exposure, the blocking is released with thymidine and cells blocked in the S stage proceed to the G2 stage and then mitosis (Fig. 4). To further increase the number of high-resolution cells, ethidium bromide is added to the culture at the end of the release time. Ethidium bromide will intercalate in the double-stranded

DNA of each chromosome and will lessen chromosome condensation yielding high resolution chromosomes.

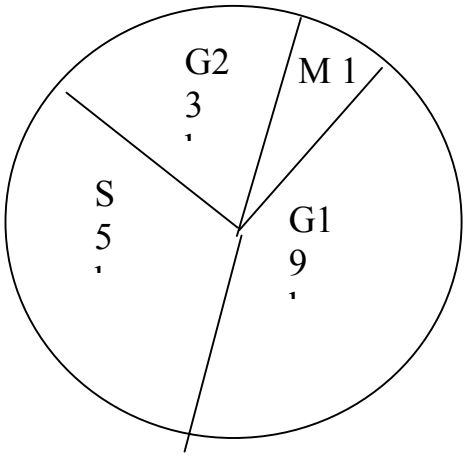

Figure 4 The cell cycle: G1=gap1, S=synthesis, G2=gap2, M=mitosis (prophase, metaphase, anaphase, telophase)

Fluorescence *in situ* Hybridization (FISH) (Montgomery et al., 1997, Kearney and Buckle, 2001**)**

Fluorescence *in situ* hybridization (FISH) uses fluorochrome labeled to specific genes or chromosomal regions. This technique is particularly useful for gene mapping and for identifying chromosomal abnormalities that are generally beyond the resolution of routine cytogenetics.

FISH involves the probe (DNA or RNA sequence) of interest and the locus or target sequence to which the probe hybridizes. The target DNA (metaphase chromosomes or interphase nuclei) is first denatured to separate the DNA double helix structure. The fluorescently labeled probe of interest is then added to the denatured sample mixture and hybridizes with the sample DNA at the target site as it reanneals (or reforms itself) back into a double helix. The

probe signal can then be seen through a fluorescent microscope and the sample DNA scored for the presence or absence of the signal.

Objectives

1. To determine the frequency of chromosomal abnormalities and subtelomeric rearrangements in children with autism and idiopathic mental retardation by using Fluorescence *in situ* hybridization (FISH).

2. To establish Fluorescence *in situ* hybridization (FISH) as a reliable method for detecting submicroscopic chromosomal aberrations.