CYCLIN DEPENDENT KINASE LIKE 5 (*CDKL5*) MUTATION SCREENING IN RETT SYNDROME AND RELATED CLINICAL DISORDERS

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DECLARATION

This thesis contains no material, which has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge this thesis contains no material previously published or written by another person, except when due reference is made in the text.

Rose White

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"I have fought a good fight,
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I have kept the faith."

2 Timothy 4:7

ABSTRACT

Rett Syndrome (RTT) is a severe neurodevelopmental disorder affecting almost exclusively females, with an incidence of 1 in 8,000 by 15 yrs of age. The disorder encompasses a wide spectrum of clinical phenotypes. Up to 95% of classical RTT and a lesser proportion (20%-60%) of those with atypical forms of RTT patients carry mutations in the X-linked *MECP2* gene (Methyl-CpG binding protein 2), the first gene discovered to be associated with this disorder. Mutations in the X-linked *CDKL5* gene (cyclin-dependent kinase-like 5) have also been found to cause atypical RTT (in particular, the early onset seizure variant), autism, X-linked infantile spasm syndrome (ISSX), intellectual disability and other severe neurological disorders.

This study investigates the speculation that *CDKL5* deficiency may underlie a significant proportion of RTT cases that were not linked to *MECP2* mutations. It is also investigates the possibility that *CDKL5* mutations may lead to other non-RTT syndrome neurological disorders.

The clinical cohort consisted of *MECP2* mutation-negative RTT patients (91), males with X-linked mental retardation (8), patients with ISSX (52), patients with autism (59) and other patients with intellectual disability with or without seizures (39). The 21 coding exons of *CDKL5* were screened by Denaturing High Performance Liquid Chromatography and direct sequencing.

In all, six polymorphic variations and one probably pathogenic mutation were identified, accounting for 46 of the 249 patients in this cohort (17%). Each individual identified with a variation had the clinical feature of seizures.

Based on other studies, it appears that the key feature that points to a *CDKL5* mutation will be the presence of severe early onset seizures, particularly infantile spasms. The results of mutation screening in this cohort

lead us to conclude that pathogenic *CDKL5* mutations are unlikely to be identified in neurological disorders unless a RTT-like phenotype with severe early-onset seizures are present.

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LIST OF ABBREVIATIONS

µg	microgram
µl	microlitre
µM	micromolar
3'	3 prime
5'	5 prime
A_{260}	absorbance at 260 nanometre wavelength
A_{280}	absorbance at 280 nanometre wavelength
ARX	*Aristaless* related homeobox gene
bp	base pair
CDKL5	Cyclin-dependent Kinase-like 5 gene
DHPLC	Denaturing high performance liquid chromatography
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethyl diamine tetraacetic acid
ELB	Erythrocyte Lysis buffer
g	gram
kb	kilobases
IS	infantile spasm
ISSX	X Linked Infantile Spasms/X-linked West syndrome
L	litre
MAP	Microtubule-associated protein 2 gene
MBD	methyl binding-domain
MECP2	methyl-CpG binding protein 2 gene
MeCP2	methyl-CpG binding protein 2 protein
MeCP2_e1	MeCp2 isoform incorporating exons 1, 3 and 4
MeCP2_e2	MeCp2 isoform incorporating exons 2, 3 and 4
mg	milligram
ml	millilitre
MLPA	Multiplex Ligation-dependent Probe Amplification

mm	millimetre
mM	millimolar
MQ	Milli-Q
MR	mental retardation
mRNA	messenger ribonucleic acid
NLS	nuclear localization signal
NTNG1	Nertin G1 gene
OMIM	Online Mendelian Inheritance in Man
PCR	polymearse chain reaction
PPM-X	X linked mental retardation with psychosis, pyramidal signs and macro-orchidism
RNA	ribonucleic acid
Rpm	revolutions per minute
RT	room Temperature
RTT	Rett Syndrome
RS	Juvenile X-linked Retinoschisis
SAP	Shrimp Alkaline Phosphate
SE	Salt and EDTA buffer
SNP	Single nucleotide polymorphism
STK9	Serine-threonine kinase
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
TRD	Transcription repression domain
Tris	tris (hydroxymethyl) aminomethane
UV	ultra-violet
UBE3A	ubiquitin protein ligase E3A
UTR	untranslated region
XLAG	X-linked lissencephaly with abnormal genitalia
XCI	X chromosome inactivation
XLMR	X-Linked mental retardation

1 LITERATURE REVIEW

1.1 Introduction

Rett syndrome (RTT; Online Mendelian Inheritance in Man No. 312750) is a severe neurodevelopmental disorder that predominantly affects females (Hagberg et al. 1983; Armstrong et al. 1995; Glaze 1995), with a prevalence of approximately 1: 8,000 by the age of 15 years (Laurvick et al. 2006). In the classic form, following 6-18 months of apparently normal development there is a progressive loss of intellectual functioning and fine and gross motor skills, seizures, ataxia, development of stereotypic hand movements and acquired microcephaly (Hagberg 1995b).

The clinical features of RTT overlap with other neurodevelopmental disorders, therefore the clinical diagnosis is made according to a set of diagnostic criteria and a specific developmental profile (Trevathan et al. 1988). More recently these criteria have been revised to clarify previous ambiguities (Hagberg et al. 2002). The clinical presentation can include a wide spectrum of recognised deviations from the "classic" or "typical" RTT, as initially defined by Hagberg (Hagberg et al. 1985). In 1999, the leading cause of RTT was identified as being linked to mutations in the methyl-CpG binding protein 2 *(MECP2)* gene, which is located on the X-chromosome (Amir et al. 1999). Since that time *MECP2* mutations have been found in approximately 90-95% of patients with classic RTT (Williamson and Christodoulou 2006), *MECP2* mutations are less common in patients with atypical forms of RTT (Hoffbuhr et al. 2002).

Recently, mutations in cyclin-dependent kinase-like 5 (*CDKL5*), also known as serine-threonine kinase 9, (*STK9*), have been found to cause a RTT-like phenotype with early onset seizures. *CDKL5* is also located on the X-chromosome (Xp22). A mutation in this gene also caused a severe neurological impairment in a male from early infancy. A similarly affected

sister developed a combined RTT phenotype that included autism, intellectual disability and seizures, and she had an identical twin sister with mild developmental delay and autism (Tao et al. 2004; Weaving et al. 2004).

It is unclear whether *MECP2* and *CDKL5* alone are responsible for RTT syndrome, or whether other gene mutations can cause RTT syndrome or act as a modifier for *MECP2* and *CDKL5*. Netrin G1 (*NTNG1*) was recently revealed as a candidate gene for RTT, however further studies will be needed to elucidate the function of this gene. (Borg et al. 2005a). In addition *MAP2* is also considered as a gene causing RTT. This is based on identification of a chromosome 2 deletion in a patient with autism and RTT-like features (Pescucci et al. 2003).

1.2 Historical background

In 1966, Dr. Andreas Rett, an Austrian paediatrician, was the first to identify patients with the RTT disorder, when he observed two girls sitting in his waiting room, both wringing their hands in the same manner. Upon further review, both girls had the same profile of developmental histories; early normal development followed by regression, loss of purposeful hand use and compulsive constant hand movements (Rett 1966; Hagberg et al. 1983; Hagberg et al. 1993).

However, it was not until 1983 that RTT received worldwide recognition when Dr. Bengt Hagberg's report was published in the English literature (Hagberg et al. 1983). This seminal publication described RTT as a specific condition, as opposed to its common misdiagnosis as a "type of autism", cerebral palsy, non-specific developmental delay, organic brain syndrome without any known aetiology, or mental retardation (Tsai 1992; Mazzocco et al. 1998). Hagberg described 35 patients, all females from three countries, France, Portugal and Sweden, with a uniform and striking progressive encephalopathy. Since that time, there have been numerous case reports in

all ethnic groups and from all the parts of the world. The frequency of RTT was estimated to be 1 in 10,000 to 15,000 live female births (Hagberg and Hagberg 1997). Based on comprehensive epidemiologic studies in Australia, the estimated prevalence has been more recently revised to 1 in 8,000 by 15 years of age (Laurvick et al. 2006), making RTT a major cause of severe mental impairment in females (Ellaway and Christodoulou 1999).

The genetic basis of RTT proved difficult to establish, because over 99% of cases are sporadic. Moreover, there have been several reports of cases of males with RTT syndrome with more severe clinical features (Philippart 1990; Schanen and Francke 1998; Kankirawatana et al. 2006). Most males with a *MECP2* mutation suffer severe fatal encephalopathy or non-specific X-linked mental retardation, although occasional males with a more typical RTT phenotype are observed. These latter cases are either mosaics for a *MECP2* mutation or have a *MECP2* mutation and a 47XXY karyotype (Leonard et al. 2001; Schwartzman et al. 2001; Hoffbuhr et al. 2002).

1.3 Diagnostic Criteria of Rett Syndrome

Previously, the diagnosis of RTT was based on a consistent constellation of clinical features and the use of established diagnostic criteria (Trevathan et al. 1988). More recently refined diagnostic criteria have been established clarifying some earlier inconsistencies, and with additional information allowing the classification of atypical variants of RTT (Hagberg et al. 2002) (Table 1). The discovery of mutations in the *MECP2* gene led to genetic diagnostic testing, which has strengthened confidence in the specificity of the clinical criteria and allowed refinement of diagnostic criteria for the diagnosis of RTT.

Table 1:1 Revised Diagnostic criteria for classical and variant RTT

Classical RTT

Necessary criteria
Apparently normal prenatal and perinatal history
Psychomotor development normal during the first 6 months (may be delayed from birth)
Normal head circumference at birth
Postnatal deceleration of head growth (most individuals)
Loss of purposeful hand skills between 0.5–2.5 years
Stereotypic hand movements
Evolving social withdrawal, communication dysfunction, loss of acquired speech, cognitive impairment
Impaired or deteriorating locomotion

Supportive criteria
Breathing disturbances while awake
Bruxism (grinding of the teeth)
Impaired sleeping pattern from early infancy
Abnormal muscle tone accompanied by muscle wasting and dystonia
Peripheral vasomotor disturbances.
Progressive scoliosis (abnormal lateral curvature of the spine) or kyphosis (an abnormal, convex curvature of the spine)
Growth retardation
Hypotrophic (progressive degeneration of an organ or tissue caused by loss of cells) small and cold feet and/or hands

Exclusion criteria
Organomegaly (abnormal enlargement of the viscera called also *splanchnomegaly, visceromegaly*) or other evidence of a storage disorder
Retinopathy, cataract, or optic atrophy.
History of perinatal or postnatal brain damage
Identifiable inborn error of metabolism or neurodegenerative disorder
Acquired neurological disorder due to severe infection or head trauma

Variant RTT
At least 3 of the 6 main criteria
At least 5 of the 11 supportive criteria

Main criteria
Absence or reduction of hand skills
Reduction or loss of speech (including babble)
Hand stereotypies
Reduction or loss of communication skills
Deceleration of head growth from early childhood
Regression followed by recovery of interaction

Supportive criteria
Breathing irregularities
Air swallowing or abdominal bloating
Bruxism
Abnormal locomotion
Scoliosis or kyphosis
Lower limb amyotrophy
Cold, discoloured feet, usually hypotrophic
Sleep disturbances, including night time screaming
Inexplicable episodes of laughing or screaming
Apparently diminished pain sensitivity
Intense eye contact and/or eye pointing

*Table derived from Hagberg et al 2002

1.3.1 Classical Rett Syndrome

RTT is a clinically defined condition (Hagberg et al. 2002). The diagnostic criteria for classical RTT include essential clinical observations, supportive clinical observations and exclusion criteria. If all of the criteria apply, the diagnosis of "classical RTT" can be established. However, symptoms vary in severity and may only manifest with age (Weaving et al. 2005; Williamson and Christodoulou 2006) or may occur co-incidentally with other disorders thus delaying the diagnosis (Ellaway and Christodoulou 2001).

Based on the Trevathan system, (Trevathan et al. 1988) necessary diagnostic criteria include a normal prenatal and perinatal period, normal developmental progress and normal head circumference at birth. Subsequently, there is a slowing of the rate of head growth usually leading to microcephaly. In addition, there is a loss of purposeful hand skills, stereotypic hand movements, social withdrawal and a jerky truncal ataxia. There is epidemiological evidence to suggest that some children with classical RTT can show subtle manifestations at birth, such as reduced mean head circumference, reduced birth weight, placidity and hypotonia, (Opitz and Lewin 1987; Leonard and Bower 1998; Jellinger 2003). More recent studies of family videos investigating RTT infants in the first six months of life strongly suggest subtle behavioural abnormalities (Burford et al. 2003; Einspieler et al. 2005). Also, developmental irregularities have been conjectured to be present in the foetus, although this has not been generally accepted (Nomura and Segawa 1990).

Supportive criteria are not required for a diagnosis of classical RTT, but most patients have respiratory dysfunction, teeth grinding, abnormal sleep patterns, loss of muscle tone, peripheral vasomotor disorder, scoliosis, growth retardation, poor circulation of the lower extremities with cold small feet and small thin hands (Trevathan et al. 1988).

Exclusion criteria allow the clinical diagnosis of RTT to be ruled out. Such features include enlargement of liver or spleen or other signs of a storage disorder, loss of vision due to a retinal disorder or optic atrophy, evidence of perinatal or postnatal brain damage, an identifiable metabolic disorder or other inherited degenerative disorder, and an acquired neurological disorder resulting from infections or head trauma (Hagberg et al. 2002). However, as stated above, the existence of RTT with other genetic disorders may delay the diagnosis (Watson et al. 2001; Hammer et al. 2002).

1.3.2 Atypical Rett Syndrome

There are clear indications that females with RTT may present with a much broader phenotype, either more or less severe. Patients fulfilling some but not all necessary criteria for RTT are recognised as "atypical RTT" or "RTT variants". A model has been developed to delineate cases of atypical RTT, which may be subdivided into a number of different subgroups:

Early onset - cases where seizures dominate and regression becomes apparent before the age of 6 months but with an otherwise classical phenotype (Hagberg et al. 1993).

Congenital RTT - a form in which regression is not clearly identified but in which the clinical picture is otherwise classical (Lin et al. 1991; Hagberg et al. 1993).

Late onset - regression occurs at a much later stage, eventually progressing to the classical form (Hagberg and Witt-Engerstrom 1987).

Forme fruste - the most common group of atypical variants occurring between 1 and 3 years of age (Hagberg et al. 2002). Symptoms are not as severe, with developmental stagnation rather than regression and reduced stereotypical hand movements (Lin et al. 1991; Hagberg et al. 1993). This has been seen in familial cases in which (for example) a girl with classical RTT has a sister or maternal aunt with the "forme fruste" variant (Anvret et al. 1990; Journel et al. 1990).

Preserved speech - following the regression period, the child is still able to use phrases or sentences and has limited motor functions (Zappella 1997).

Some males have been diagnosed with RTT, however these cases remain very rare. There have only ever been 17 published reports of males with pathogenic *MECP2* mutations to date, and nine of these boys represent familial cases which were only detected because of the presence of other affected female family members. The phenotype in males with *MECP2* mutations may also be very broad. Several reports have documented cases of males with classical Rett syndrome who were found to have co-existing Klinefelter's syndrome (47XXY) (Schwartzman et al. 2001) and males with somatic mosaicism (Clayton-Smith et al. 2000; Topcu et al. 2002).

1.3.3 Stages of Disorder

The natural history of the disorder can be defined by a staging system (Fig 1). Clinical characteristics and the differential diagnosis of RTT vary according to the stage of disease (Hagberg and Witt-Engerstrom 1986; Naidu et al. 1986).

Stage I, or ***early onset***, generally begins between 6 and 18 months of age and may last for months or even more than a year. Long before

developmental regression is noted, the child's development stagnates and deceleration of head and brain growth begins at two to four months (Armstrong et al. 1999). Deceleration of head growth is one of the main diagnostic criteria for RTT, even deceleration within the normal range particularly in girls with the forme fruste variant (Hagberg 1995a). During this stage, the infant may begin to show less eye contact, have reduced interest in toys, and may develop sporadic hand waving. There may be delays in gross motor skills such as sitting or crawling (Kerr 1995).

Stage II, or the ***rapid destructive*** stage, usually begins between one to three years of age. This stage is characterised by cognitive and motor regression and may possibly last weeks or months. The characteristic hand movements of RTT begin to emerge during this stage and often include wringing, clapping, or tapping as well as repeatedly moving the hands to the mouth. Self-abusive behaviour (in 25% of cases) such as chewing fingers has also been noted (Oliver et al. 1993). The child displays loss of acquired skills and communication, and is often distressed and withdrawn, displaying autistic-like symptoms. Up to 85% of individuals may have some form of seizures, which commence during this stage. The seizures are associated with smaller head circumference and typically show slowing of brain waves on EEG (Hagberg et al. 1993; Glaze 1995; Steffenburg et al. 2001). Gait patterns are unsteady and initiating motor movement can be difficult (Jian et al. 2006). Respiratory abnormalities, present in over 75% of RTT girls, first manifest during this stage. Irregularities such as episodes of apnoea and hyperventilation may be observed, however these abnormalities only occur while awake (Hagberg et al. 1993).

Stage III, is known as the ***Pseudostationary*** stage. Its onset occurs at three to ten years of age and can last for years. Seizures, loss of motor skills, gait ataxia and apraxia are prominent during this stage. However, there may be improvement in social behaviour, attention span, alertness and communication skills often with disappearance of the autistic-like features

(Naidu 1997). The child acquires a generally happy disposition with less irritability and crying.

Other common features are teeth grinding (53%), shakiness/ imbalance of the torso, increased muscle spasticity, rigidity, and hyper-reflexia (30%) (Kerr 1995). Breathing irregularities are also common (Hagberg et al. 1993; Morton et al. 2000). At this stage progressive physical problems such as scoliosis and spasticity often become major management issues.

Stage IV: or the ***late motor deterioration*** stage can begin as early as five years of age and can last for decades. Areas of improvement are seen, such as eye contact, emotional and communicative abilities, a decrease in repetitive hand movements, and reduced seizure frequency. However, this stage is characterised by reduced mobility, with 25-85% of RTT girls becoming wheelchair bound and developing muscle wasting (Kerr 1992; Hagberg et al. 1993). Rigidity, spasticity, dystonia, scoliosis (in up to 80% of patients; (Nomura and Segawa 1990), and trophic disturbances of the feet all become significant issues (Naidu et al. 1986).

The age of death of RTT girls varies and ranges from two to adulthood. The mortality rate in RTT syndrome is 1.2% per annum, and death may be sudden and unexpected in up to 26% of cases. Further studies suggest that sudden death in RTT girls may be linked to an electrical instability of the cardiac conduction system and not to abnormalities of cardiac structure (Guideri et al. 2004; Ager et al. 2006).

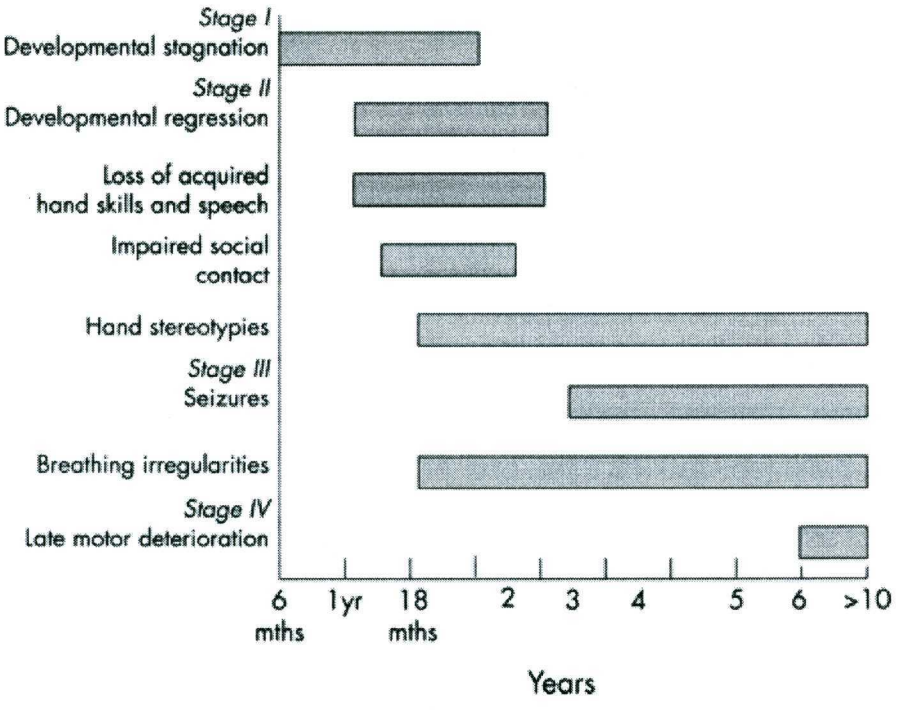

Figure 1:1 The staging system for classical Rett syndrome

*Taken from Weaving et al 2005 with the permission of the authors.

1.4 Genetic Aetiologies of RTT Syndrome.

In 1999 the leading cause for RTT was identified when mutations were found in the *MECP2* gene, which is located on the X-chromosome (*Amir et al 1999*). It has recently been discovered that a small proportion of RTT cases are caused by mutations in the X-linked *CDKL5* gene (Tao et al. 2004; Weaving et al. 2004; Scala et al. 2005). In 2005, a third gene *NTNG1* located on chromosome 1 was identified as a possible candidate for RTT (Borg et al. 2005a; Archer et al. 2006b). At this stage, the genetic basis of many cases remains unaccounted for, this amount to 5%-10% of classical and up to 60% of atypical RTT.

1.4.1 *MECP2*

The occurrence of rare familial cases of RTT initially indicated a genetic cause of the disease. The fact that mainly females were affected and male RTT cases were extremely rare suggested an X-linked dominant inheritance with lethality in males. The proposed theory of inheritance as a dominant mutation on the X-chromosome gained strength when a balanced translocation involving Xp22.11 and 3q13.31 was identified in a RTT patient (Zoghbi et al. 1990). Cytogenetic studies suggested that RTT was probably caused by the abnormal inactivation of maternally inherited late replicating chromosomes (Martinho et al. 1990).

However, linkage studies were complicated due to two factors. Firstly, affected females almost never bore children and secondly 99.5% of cases were sporadic (Wan et al. 1999).

An exclusion-mapping strategy was employed in the 1990's using DNA from a small number of familial RTT syndrome cases, narrowing the candidate region distal to Xq27.3-Xqter (Archidiacono et al. 1991; Ellison et al. 1992; Curtis et al. 1993; Schanen et al. 1998). Identifying the concordance of RTT in monozygotic twins and discordance amongst dizygotic twins in rare familial cases, not only confirmed RTT to be a genetic disease, but also suggesting some cases may be due to germ-line mosaicism (Archidiacona *et al* 1991).This work facilitated the identification of the region likely to harbour the mutated gene, and in 1998 Xiang reported linkage to the Xq28 region in a group of familial RTT cases (Xiang et al. 1998), which was later confirmed by others (Schanen and Francke 1998; Sirianni et al. 1998; Webb et al. 1998).

The histopathology observed in the neurons of the cortex (Armstrong et al. 1995) suggested that the causative gene was involved in neuronal

development. As RTT is a developmental disorder of the nervous system, the candidate genes of interest initially were GABARA3 (Gamma Aminobutyric Acid Receptor 3) and L1CAM (L1 Cell adhesion Molecule) (Webb et al. 1998). Systematic exclusion by mutation screening of several candidate genes in the 10Mb region encompassing band Xq28 was undertaken (Wan and Francke 1998; Amir and Zoghbi 2000; Xiang et al. 2000). A breakthrough was made when 5 out of 21 girls diagnosed with RTT were found to have either missense or nonsense mutations in the *MECP2* gene (Amir et al. 1999). The *MECP2* gene encodes the transcriptional repressor, methyl-CpG- binding protein 2. RTT became the first disease identified that was caused by mutations in a transcriptional regulator (Dragich et al. 2000).

Extensive mutation screening projects in classical and atypical RTT have been undertaken world wide in populations with varied backgrounds and ethnicities. It has been estimated that up to 95% of classical RTT and 20-40% of atypical RTT cases carry mutations in *MECP2* (Cheadle et al. 2000; Giunti et al. 2001; Huppke et al. 2002; Weaving et al. 2003). This supports the concept that RTT is a pan-ethnic disorder with the most common underlying genetic deficiency being in *MECP2* (Amir et al. 1999; Amir and Zoghbi 2000; Bienvenu et al. 2000; Hoffbuhr et al. 2001).

1.4.2 Males with RTT syndrome

A small number of males have been diagnosed with RTT syndrome. To date there are published reports of 17 males with severe neonatal encephalopathy and pathogenic *MECP2* mutations (Kankirawatana et al. 2006). Nine of these boys represent familial cases which were only detected because of the presence of other affected female family members; so many more cases probably remain undiagnosed.

Gomot *et al.* suggested that there are three categories of males with *MECP2* mutations (Gomot et al. 2003). The first category are males with a severe lethal form of RTT born to mildly symptomatic female carriers with classical RTT (Villard et al. 2000).The second category is a severe form of RTT characterised by severe encephalopathy. The third category is males with clinically diagnosed X-linked mental retardation (XLMR) (Couvert et al. 2001). Also, *MECP2* mutations have been identified in males associated with manic-depressive psychosis, pyramidal signs, Parkinsonian features, and macro-orchidism (PPM-X syndrome) (Dotti et al. 2002; Klauck et al. 2002). In addition, there are reports of males with *MECP2* mutations who have either Klinefelter syndrome (Hoffbuhr et al. 2001) or somatic mosaicism (Clayton-Smith et al. 2000; Topcu et al. 2002).

The rarity of males with RTT has led many people to propose that it is an embryonic lethal disease in males (Villard et al. 2000). This hypothesis was consistent with studies of a knockout mouse model for *MECP2*, in which no male animals were born (Tate et al. 1996). However, this was subsequently shown to be an artefact of the molecular system used to generate the knockouts. Jaenisch *et al.* studies reported that *Mecp2*-null male mice were normal until 5 weeks of age, when they began to develop the disorder, leading to death between 6 and 12 weeks. MeCP2 deficient neurons show features similar to RTT (Jaenisch and Bird 2003). The viability of *Mecp2*-null animals proves that the absence of MeCP2 does not cause embryonic lethality in mice, contrary to a previous conclusion (Guy et al. 2001).

In 1996 Thomas proposed that there maybe a very high male: female ratio of germline mutations for RTT and other X-linked dominant genetic diseases, which could explain transmission to females (Thomas 1996). Trappe *et al.* tested this hypothesis and reported in 26 of 27 females affected with RTT the mutation arose from the father's germ cells (Trappe et al. 2001). However the reason for increased rate of mutations in the male germ-line is still not clear. Recently some researchers have reported cases

of males with pathogenic *MECP2* mutations, who do not have a somatic mosaicism or an X-chromosome aneuploidy but have a much more severe clinical phenotype (Kankirawatana et al. 2006).

1.4.3 MeCP2 / *MECP2:* Structure and Function

The gene Methyl-CpG Binding Protein 2 (*MECP2*) was first identified in 1992 (Lewis et al. 1992), characterised in 1996 (D'Esposito et al. 1996), and its function identified in 1998 (Jones et al. 1998). The MeCP2 protein can act as an epigenetic modifier, resulting in transcriptional repression of target genes (Nan et al. 1997). MeCP2 can bind to methylated CpG islands, a function facilitated by its methyl-CpG binding domain (MBD). Upon binding, MeCP2 protein has been shown to repress transcription from a methylated promoter (D'Esposito et al. 1996; Nan et al. 1997; Jones et al. 1998).

The *MECP2* gene is approximately 75kb in size, located on the chromosomal band Xq28, and is subject to X-chromosome inactivation in mice and humans. The gene has 4 exons, and two isoforms, and has an extremely large 3'UTR of 8.5 kb sections of which are highly conserved across species. There are two splice variants of the *MECP2* gene that code for MeCP2 protein isoforms known as MeCP2_e2 (previously MeCP2-β or MeCP2A) and MeCP2_e1 (previously known as MeCP2-α or MeCP2B). The two isoforms differ only in their N-terminal region, with the rest of the protein sharing a common open reading frame, and 4 functional domains (Meehan et al. 1992; Nan et al. 1993; Nan et al. 1996):

(1) The two nuclear localisation signals (NLS), located between aa.255-271 in the MeCP_e1 isoform directs the synthesised protein to the nucleus.

(2) MBD, spans exon 3 and exon 4. The MBD, consisting of 85 amino acids, selectively binds to at least one CpG dinucleotide that is methylated at position five of cytosine (Lewis et al. 1992).

(3) The transcription-repressor-domain (TRD) is located wholly within exon 4. The TRD is 103 amino acids long, between aa.207-310 in the MeCP_e1 isoform and overlaps with one of the two nuclear localization signals. It recruits other transcription factors such as Sin3A (Nan et al. 1998) or ski/NCoR to the transcription repression machinery (Nomura and Segawa 1990).

(4) The fourth domain located between aa.310-487 is the WW domain. The binding region, from residue 337 to the C terminus, specifically binds to group II WW domains of splicing factors, including formin binding protein II and Huntington yeast protein C (HYPC) (Buschdorf and Stratling 2004), suggesting that MeCP2 has a direct role in splicing regulation (Young et al. 2005). In addition, the C-terminal domain exhibits homology to neuron-specific transcription factors and contains evolutionarily conserved poly-histidine and poly-proline regions (Lewis et al. 1992; Chandler et al. 1999).

MeCP2 exerts its effect by binding to symmetrically methylated CpG dinucleotides via its MBD, and to the transcriptional co-repressor proteins via the TRD, to the silencing complex and ultimately histone deacetylase, HDAC I or II (Jones et al. 1998). Interaction between this transcription repressor complex and chromatin bound MeCP2 leads to deacetylation of the ends of core histones H3 and H4 causing chromatin compression resulting in transcriptional repression (Grunstein 1997). Disruption of either the TRD or the MBD abolishes the ability of MeCP2 to act as transcriptional repressor *in vitro.* These regions are highly conserved across species and so are likely to be sensitive to mutations *in vivo* (Nan et al. 1993; Nan et al. 1997).

As previously noted, the *MECP2* gene encodes two isoforms of the MeCP2 protein, MeCP2_e2 and MeCP2_e1. The MeCP2_e2 isoform is translated from a start codon in exon 2, and exon 1 forms part of the 5' UTR. It incorporates exon 2, 3 and 4 and produces a protein of 486 amino acids. MeCP2_e2 was initially the only isoform identified and so had been the main focus of investigation. In 2004 the MeCP2_e1 isoform was discovered. It is translated from a start codon in exon 1, has exon 2 spliced out, and utilises exons 3 and 4 to produce a protein of 498 amino acids. MeCP2_e1 is more closely related to ancestral MeCP2 isoforms (frog, mouse zebrafish), and the mRNA is 10-fold more abundant in the brain tissue than that of MeCP2_e2 (figure 1.2) (Kriaucionis and Bird 2004; Mnatzakanian et al. 2004).

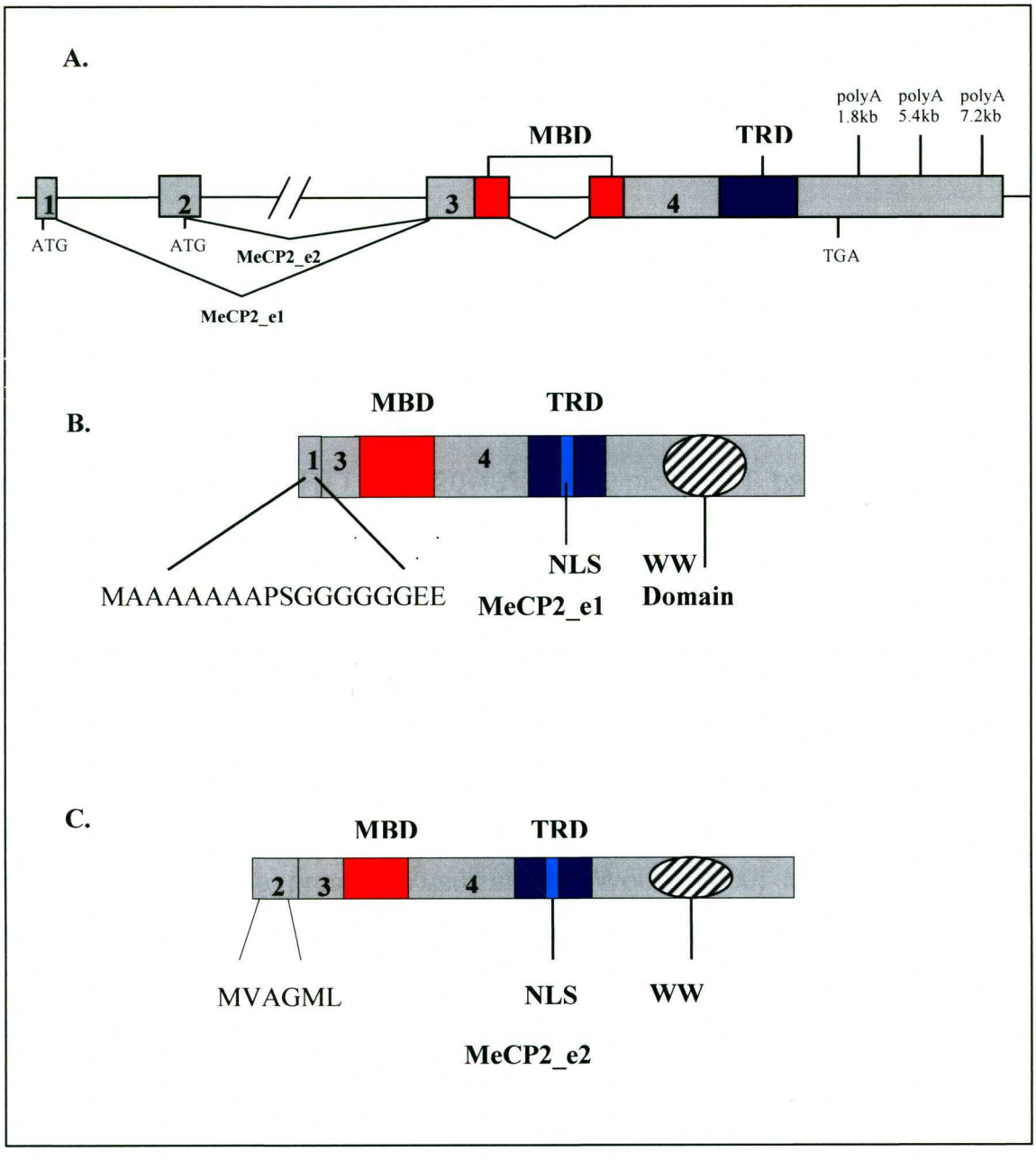

Figure 1:2 The molecular structure of MECP2/MeCP2

(**A**) The gene structure and splicing of *MECP2*. (**B C**) Schematics of MeCP2_e1 and MeCP2_e2 protein isoforms. These proteins consist of methyl-binding domain (MBD), a transcription repression domain (TRD), an embedded nuclear localisation signal (NLS) and a C-terminal WW domain.

1.4.4 *MECP2* Mutation Analysis

To date, over 300 different *MECP2* mutations have been identified in 2,100 patients. This is detailed in the RettBASE database (http://www.chw.edu.au) (Christodoulou et al. 2003).

Up to 95% of classical RTT and 20-40% of atypical RTT patients have *MECP2* mutations (Evans et al. 2005). Mutations are spread across the whole gene; the majority of mutations lie within exon 3 and exon 4 and include missense, nonsense, truncation and frameshift mutations. Missense mutations are predominately centred in the MBD and truncation mutations in the TRD. Nonsense mutations are scattered between the MBD and the TRD or in the TRD itself, and deletions are generally restricted to a 100bp region containing repetitive elements (c1096-c1197), leading to a loss of the C-terminal region of the protein (Yusufzai and Wolffe 2000; Miltenberger-Miltenyi and Laccone 2003). There are 10 common mutations which make up 70% of all pathogenic *MECP2* mutations identified in RTT (Table 1.2). Wan *et al* found that the majority of mutations were cytosine to thymine (C >T) transitions at so called CpG mutational "hotspots" (Wan et al. 1999), and suggested a mutational mechanism, namely spontaneous deamination of methylated cytosine residues which causes the C to T transition. This could account for the predominance of affected females by means of *de novo* mutations derived from the heavily methylated male germ cells (Trappe et al. 2001).

Table 1:2 The ten most common *MECP2* mutations identified in RTT patients

Amino acid change	Frequency	Percentage of total
p.T158M	191	9.05
p.R168X	186	8.82
p.R255X	166	7.87
p.R270X	146	6.92
p.R294X	118	5.59
p.R306C	96	4.55
p.R133C	92	4.36
p.R106W	71	3.36
intronic variation	56	2.65
p.L386fs	48	2.27

*Data compiled from RettBASE as at May 2007 http://www.chw.edu.au/ (Christodoulou et al 2003).

Mnatzakanian *et al.* recently reported that exon 1 mutations were found in two out of 19 patients with classical RTT. In this study one patient had an 11 bp deletion which led to a truncated 36 amino acid protein, and another patient had an exon 1 deletion detected by Multiplex Ligation-dependent Probe Amplification (MLPA) (Mnatzakanian et al. 2004). Prior to this discovery, exon 1 was not typically screened for MECP2 mutations. However, exon 1 mutation analysis has since been carried out on cohorts of patients previously identified as *MECP2* mutations negative.

For instance, Amir *et al.* screened 63 RTT patients (38 classical and 25 atypical) who previously tested negative for *MECP2*. Two mutations were reported, the first was a *de novo* mutation at the intron 1 splice donor site (c.62+1delGT) in a classic RTT patient. The second patient with atypical RTT had an 11bp deletion (Amir et al. 2005) identical to one recently

reported by Mnatzakanian *et al.* As this mutation is in a region with an AGG repeat, this locus could be particularly prone to recurrent *de novo* deletions.

Ravn *et al.* identified a 5 bp duplication in exon 1 of the *MECP2* gene in a Danish patient with classical RTT, resulting in a frameshift and premature truncation of the protein (Ravn et al. 2005a). Bartholdi *et al.* identified one girl with a novel exon 1 mutation (c.30delCinsGA) by sequencing and identified three with genomic rearrangements by MLPA (Bartholdi et al. 2006). Saxena *et al.* screened RNA samples from 20 females with classical or atypical RTT. A previously reported 11 base pair deletion in exon 1 was detected in one subject with a milder phenotype (Saxena et al. 2006).

While mutations in *MECP2* exon 1 appear to be rare, in view of aforementioned studies, it is likely that exon 1 screening will become increasingly incorporated into genetic testing for RTT.

1.4.5 Large Deletions and Gross Rearrangements

The majority of genetic screening for RTT is restricted to the coding parts of the *MECP2* gene. This is performed by conventional direct sequencing or denaturing high performance liquid chromatography (DHPLC). However, mutations may be missed because of limited sensitivity of these screening methods or the presence of intronic mutations. These approaches will also miss gross rearrangements in females. A number of techniques have evolved to address this issue.

Gross rearrangements, deletions and duplications of the *MECP2* gene have been successfully identified using Southern blot analysis (Bourdon et al. 2001c; Schollen et al. 2003; Laccone et al. 2004), and more recently the dosage assays including quantitative fluorescent PCR (QF PCR) or Real time PCR (qPCR) (Laccone et al. 2004), and MLPA (Erlandson et al. 2003). In one report Southern screening identified 6 rearrangements out of 41

previously mutation-negative classical RTT cases (Bourdon et al. 2001b; Bourdon et al. 2001c; Yaron et al. 2002; Schollen et al. 2003).

Using quantitative real-time PCR, Ariani *et al.* reported a deletion in 1 out of 8 classical RTT patients and a duplication in 1 out of 6 preserved speech variant (PSV) patients previously diagnosed as mutation-negative (Ariani et al. 2004). Erlandson *et al.* made use of the MLPA technique to screen a cohort of 22 RTT patients, previously classified as mutation negative, and reported three patients with large deletions. The first and second of these deletions included exons 3 and 4 and the third deletion included exons 1 and 2 (Erlandson et al. 2003).

As a result of these studies, quantitative methods have shown deletions in approximately 29% of females with classical RTT who had no detectable *MECP2* mutations by sequencing or DHPLC. Currently, *MECP2* mutation screening can include at least one of these methods, thereby increasing the mutational detection rate of RTT (Bourdon et al. 2001b; Bourdon et al. 2001c; Erlandson et al. 2001; Erlandson et al. 2003; Schollen et al. 2003; Ariani et al. 2004; Laccone et al. 2004; Ravn et al. 2005b; Archer et al. 2006c).

1.4.6 Genotype / Phenotype Correlations

Initially, it was considered that genotype-phenotype correlations could not be drawn due to conflicting results. This was related in part to the use of different clinical severity scales, different diagnostic criteria, confounding variables such as age and the influence of X-chromosome inactivation. Bienvenu *et al.* suggested that favourable (skewed) X-inactivation can spare a patient from the effects of mutant *MECP2,* and that they would display only the mildest learning disability or no phenotype at all (Bienvenu et al. 2000; Huppke et al. 2000). In support of this Weaving *et al.* concluded that it

is likely that X-inactivation modulates the phenotype in RTT (Weaving et al. 2003).

Despite the relatively few cases of males with a classical RTT phenotype, the mutations identified are different to those in females. It is speculated that the same mutation appears as a milder phenotype in females, which may not be diagnosed as RTT. It has also been noted that a number of female patients with the same mutations display different phenotypes (Cheadle et al. 2000; Huppke et al. 2000).

The correlation study based on a large cohort of 123 RTT patients noted the following results: missense mutations or deletions located within the 'hotspot' for deletions resulted in a milder phenotype. Truncation mutations either completely or partially truncating the region coding for the nuclear localisation signal (NLS) are associated with a more severe phenotype than other truncating mutations. Missense mutations within the MBD and mutations truncating the entire TRD resulted in a similar outcome, and finally, missense mutations in the NLS were more severe (Huppke et al. 2002). Schanen *et al.* also found that patients with missense mutations in TRD had a less severe clinical presentation including better preservation of head growth and language skills. It was also concluded that patients with the p.R306C mutation displayed later regression, better language and less motor impairment (Schanen et al. 2004).

Bartholdi *et al.* reported that *MECP2* mutations involving exon 1 resulted in a severe phenotype (Bartholdi et al. 2006). They also found that some RTT patients with large *MECP2* deletions had additional congenital anomalies, such as cleft palate, atretic right ear and absent auditory meatus (Archer et al. 2006c).

The behavioural patterns of patients carrying *MECP2* mutations were compared recently by Robertson *et al.* and it was concluded that some behaviours differ according to genetic mutations. Behaviours such as fear/anxiety were more commonly reported in those patients with R133C and R306C mutations. Patients with the R294X mutation were more likely to have mood difficulties and body rocking, whereas hand behaviours were more commonly reported with R270X or R255X mutations (Robertson et al. 2006).

1.5 CDKL5

Despite advances in genetic screening detecting an increasing number of *MECP2* mutations, there remained no genetic basis for a proportion of clinically well defined RTT cases. This led to the search for a possible second gene causing RTT.

Linkage analysis of a family comprising a girl with RTT syndrome, her identical twin sister with autism and a deceased brother with severe intellectual disability and seizures led to the discovery of mutations in the *CDKL5* as a cause for RTT (Weaving et al. 2004). Weaving *et al.* identified a deletion of nucleotide 183 of the *CDKL5* coding sequence (c.183delT) in the affected family members, and in a screen of 44 RTT cases identified a single splice mutation, IVS13-1G>A, in a girl with severe phenotype overlapping that of RTT. *CDKL5* mutations have also been identified in a female patients with early-onset infantile spasms with clinical features that not only overlaps that of RTT and X-linked infantile spasm (ISSX; also known as X-linked West syndrome) but also Angelman syndrome (Tao et al. 2004).The mutations p.C152F and p.R175S were located within the protein kinase domain and affected highly conserved amino acids. Mutations in CDKL5 reported to date are summarised in Table 1.3.

However, the first *CDKL5* defects identified were found in two girls with ISSX. Each patient carried a *de novo* balanced X-autosome translocation which disrupted the *CDKL5* gene at Xp22.3 (Kalscheuer et al. 2003a). Additionally, one male patient with X-linked juvenile retinoschisis (RS) and a large deletion involving a C-terminal deletion of the *CDKL5* gene had seizures, which are otherwise not associated with RS (Huopaniemi et al. 2000).

Most cases reported to date of missense, nonsense or frameshift *CDKL5* mutations have been associated with early onset seizure variant of RTT, the so-called *Hanefield variant* (Tao et al. 2004; Weaving et al. 2004; Evans et al. 2005; Scala et al. 2005). Thus, the phenotype observed as a result of *CDKL5* mutations not only overlaps that of RTT and ISSX, but also severe neonatal onset encephalopathy, and autism. The overlapping phenotypic spectrum raises the question whether other clinical phenotypes might have *CDKL5* mutations, such as ISSX and autism with or without seizures.

Table 1:2 *CDKL5* variations

Type of variant	Nucleotide change*	Amino acid change	Comments	Phenotype **	Reference
Splice site	c.404-1G>T (IVS6)		Pathogenic, predicted to skip exon 7	MR, hypotonia, epileptic seizures and ISSX	(Archer *et al.* 2006a)
	c.978-2A>G (IVS11)		Pathogenic, predicted to skip exon 12	MR, hypotonia, and epileptic seizures	(Archer *et al.* 2006a)
	c.464-2A>G (IVS7)		Pathogenic, results in exon 8 skipping	Severe encephalopathy and seizures	(Evans *et al.* 2005)
	c.2047-1G>A (IVS13)	p.G683fs	Pathogenic, activates an alternative splice acceptor site	Atypical RTT- early onset seizures	(Weaving *et al.* 2004)
	c.2376+1G>C (IVS16)		Pathogenic, results in exon 16 skipping	Atypical RTT- early onset seizures	(Evans *et al.* 2005)
	c.2376+1G>A (IVS16)		Pathogenic, predicted to skip exon 16	MR, hypotonia, and epileptic seizures	(Archer *et al.* 2006a)
Deletion/ insertion	c.163_166del4	p.E55fs	Pathogenic	Atypical RTT- early onset seizures	(Scala *et al.* 2005)
	c.183delT	p.M63fs	Pathogenic	Atypical RTT, autism, neonatal encephalopathy	(Weaving *et al.* 2004)
	c.838_847del10	p.D281fs	Pathogenic	RTT, seizures	(Mari *et al.* 2005)
	c.2343delG	p.R781fs	Pathogenic	RTT, seizures	(Mari *et al.* 2005)
	c.2362_2366del5	p.K788fs	Pathogenic	MR, hypotonia, epileptic seizures and ISSX	(Archer *et al.* 2006a)
	c.2635_2636del2 (CT)	p.L879fs	Pathogenic	RTT- early onset seizures	(Scala *et al.* 2005)
	del678_691 ins683_673		Pathogenic	MR, hypotonia, epileptic seizures and ISSX	(Archer *et al.* 2006a)

Type of variant	Nucleotide change*	Amino acid change	Comments	Phenotype**	Reference
Missense	c.215T>A	p.I72N	Pathogenic, de novo, conserved amino acid	Atypical RTT- early onset seizures	(Evans *et al.* 2005)
	c.455G>T	p.C152F	Pathogenic	Atypical RTT, Angelman	(Tao *et al.* 2004)
	c.525A>T	p.R175S	Pathogenic	RTT, infantile spasm (IS)	(Tao *et al.* 2004)
	c.539C>T	p.P180L	Pathogenic, de novo, conserved amino acid		(Archer *et al.* 2006a)
	c.680T>C	p.L227P	Likely to be pathogenic, conserved amino acid		(Archer *et al.* 2006a)
	c.1330C>T	p.R444C	Not pathogenic: present in unaffected father		(Archer *et al.* 2006a)
	c.1399A>G	p.H467R	Not pathogenic: present in unaffected mother		(Evans *et al.* 2005)
	c.2372A>C	p.Q791P	Known SNP		(Kalscheuer *et al.* 2003)
	c.2378T>C	p.V793A	Unknown pathogenicity, seen in 1 patient		(Archer *et al.* 2006a)
	c.2995G>A	p.V999M	Unknown pathogenicity, seen in 1 male patient		(Archer *et al.* 2006a)
Nonsense	c.175C>T	p.R59X	Pathogenic	MR, hypotonia, and epileptic seizures	(Archer *et al.* 2006a)
Exon 1 & promoter*	c.-440G>T (promoter)		Present in 1 male patient, not seen in 69 controls		(Evans *et al.* 2005)
	c.-391G>T (promoter)		SNP: present in 2/69 male controls		(Evans *et al.* 2005)
	c.-265C>G (exon1)		SNP: present in 2/69 male controls		(Evans *et al.* 2005)
	c.-189C>T (exon1)		Present in 1 female patient, not seen in 69 controls		(Evans *et al.* 2005)

Type of variant	Nucleotide change*	Amino acid change	Comments	Phenotype**	Reference
Intronic	c.145+4_5dupAT/delAT (IVS4)		Polymorphic [AT]n repeat		(Evans *et al.* 2005)
	c.283-43G>A (IVS5)		Seen once, unknown pathogenicity		(Evans *et al.* 2005)
	c.464-37_40delCTTT (IVS7)		Seen once, unknown pathogenicity		(Evans *et al.* 2005)
	c.463+22T>C (IVS7)		Seen once, unknown pathogenicity		(Evans *et al.* 2005)
	c.555-19C>G (IVS8)		Seen in 2 patients, unknown pathogenicity		(Archer *et al.* 2006a)
	c.978-42_50del9bp (IVS11)		Seen once, unknown pathogenicity		(Archer *et al.* 2006a)
	c.2152+48C>T (IVS14)		Seen once, unknown pathogenicity		(Evans *et al.* 2005)
	c.2376+118T>A (IVS16)		Common SNP (minor allele freq 33%)		(Evans *et al.* 2005)
	c.2496+17A>G (IVS17)		Rare haplotype with c.3003C>T and c.3084G>A		(Tao *et al.* 2004)

* Mutations are named on based on the CDKL5 cDNA sequence where the A of the ATG is +1.

** ISSX, X-linked infantile spasm; IS; infantile spasm; RTT; Rett syndrome; MR, mental retardation

1.5.1 *CDKL5* Structure / Function

During transcriptional mapping in the Xp22 region, Montini *et al.* identified the *CDKL5* gene located on the X-chromosome (Montini et al. 1998). Kalscheuer *et al.* identified two alternative 5' UTRs, describing the resulting transcripts as isoform I (with exon 1 as a part of the UTR) and isoform II with 2 exons (exon 1a and 1b) as a part of the UTR (Kalscheuer et al. 2003a). The coding region of the *CDKL5* transcript was also redefined with the previously named exon 1 becoming exon 2 and several of the exon-intron boundaries were changed, with exon 7 decreasing in size from 169 bp to 60 bp, exon 17 decreasing from 121 bp to 120 bp, exon 18 increasing from 214 bp to 217 bp and exon 20 increasing in size from 76 bp to 183 bp (Kalscheuer et al. 2003b). These transcripts produce an identical protein product utilising a start codon in exon 2 and a stop codon in exon 21. The gene spans the region marker DXS8000; near the *XLRS1* gene which is orientated in the opposite direction of *CDKL5* (Montini et al. 1998; Kalscheuer et al. 2003a)(Figure 1.3).

CDKL5 is a 118 kDa protein, and was observed to have a Thr-Xaa-Tyr motif. This motif is characteristic of serine-threonine kinases and based on this shared homology it was predicted to function as a kinase (Kalscheuer et al. 2003a). Phosphorylation of Thr and Tyr residues in a Thr-Xaa-Tyr motif are essential for activation of the MAPK (Mitogen-activated protein kinase) family of proteins. This dual-phosphorylation motif is present in serine-threonine kinases as well as p56 KKIAMRE (CDKL2) and p42 KKIALRE (CDKL1), which are the proteins showing the highest homology with CDKL5 (Montini et al. 1998). An antibody specific to the phosphorylated Thr-Xaa-Tyr motif confirmed that all CDKL5 constructs that were capable of phosphorylating MeCP2 were also phosphorylated at the Thr-Xaa-Tyr motif. In addition, they also demonstrated by using a kinase dead mutant that CDKL5 is capable of trans-autophosphorylation (Bertani et al. 2006) (Figure1.3).

CDKL5 is localised primarily in the nucleus of the cell. Lin *et al.* noted that removal of the C-terminus results in an increase both expression and kinase activity and suggested that the C-terminus regulates CDKL5 function (Lin et al. 2005b). Bertani *et al.* added that a portion for the C-terminal domain is responsible for a permanence in this cellular compartment probably through protein-protein interactions (Bertani et al. 2006). CDKL5 is widely distributed in all tissues, with highest levels in brain, thymus and in the testes. Weaving *et al.* found that in the mouse brain, CDKL5 expression overlaps, but is not identical to, that of MeCP2, and that its expression is unaffected by the loss of MeCP2 (Weaving et al. 2004).

Considering the overlapping phenotypic spectrum caused by mutations in *MECP2* and *CDKL5,* it has been suggested that dysfunction of either gene acts in a common pathogenic process (Tao et al. 2004). Subsequent work by Mari *et al.* shows that CDKL5 is a nuclear protein whose expression in the nervous system overlaps with that of MeCP2, during neural maturation and synaptogenesis. In addition, it has been reported that CDKL5 is capable of mediating MeCP2 modification *in vitro* and *in vivo*. One publication (Mari et al. 2005) indicated that CDKL5 is capable of phosphorylating MeCP2. This however, was disputed by a second group (Lin et al. 2005a), who suggested that MeCP2 is not a direct substrate of CDKL5. However, the most recent study by the first group confirmed the phosphorylation of MeCP2 by CDKL5 (Bertani et al. 2006).

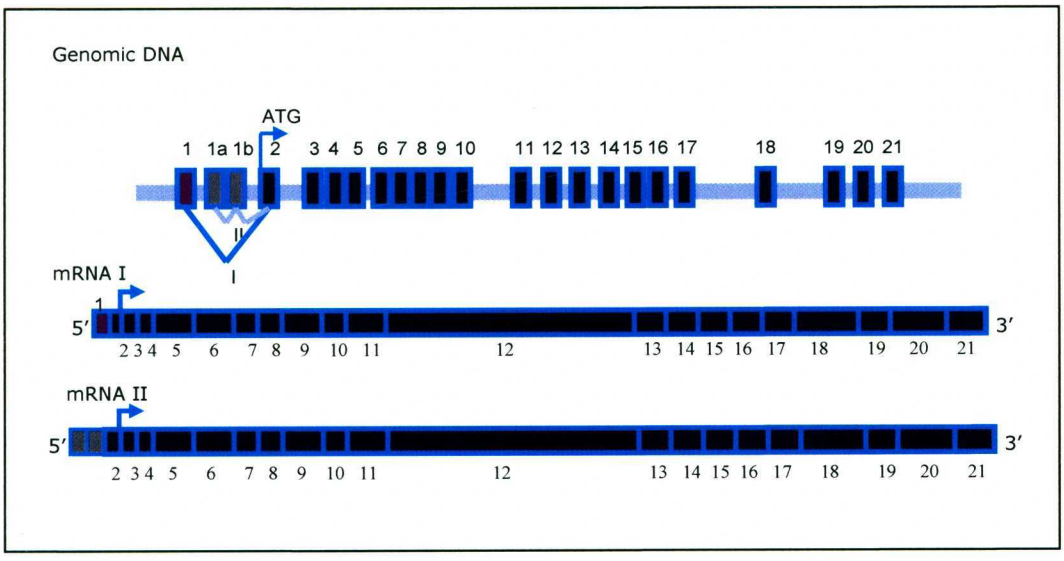

Figure 1:3 CDKL5 genomic and protein structure

1.5.2 Phenotype / Genotype Correlations

Some speculations about the CDKL5 mutations and genotype/phenotype correlations of CDKL5 mutations have been made. Phenotypes associated with CDKL5 mutations can range from ISSX and infantile epileptic encephalopathy to atypical RTT and possibly even autism (Evans et al. 2005). However, no CDKL5 mutations have been identified in classic RTT patients and lethality in males is still to be determined (Evans et al. 2005). Mutations in *CDKL5* are more likely to be seen in patients whose seizures begin before the age of 6 months. All but one of the 18 RTT patients with mutations reported had early-onset of seizures with a subsequent severe mixed seizure disorder (Tao et al. 2004; Weaving et al. 2004; Evans et al. 2005; Mari et al. 2005; Scala et al. 2005; Archer et al. 2006a). Since CDKL5 is subject to X-chromosome inactivation (XCI), the severe phenotype, with early-onset encephalopathy, was present in the females who carried the X-autosome translocations, with the normal X-chromosome presumably being inactivated (Kalscheuer et al. 2003a). Moreover, a hemizygous male with a frameshift mutation (c.183delT) was very severely affected. Interestingly,

this male had identical twin sisters. The first twin, the most mildly affected patient, was diagnosed with autism and has never had seizures. The other twin was moderately affected and had a diagnosis of atypical RTT (Weaving et al. 2004). The difference in phenotype between the twins did not appear to be due to X-inactivation status, raising the possibility that other epigenetic factors could be at play. Patients with missense mutations were relatively mildly affected, and able to walk (Tao et al. 2004), whilst truncating mutations were associated with variable phenotypes and did not clearly correlate with the position of the mutation. Kalscheuer et al. studies concluded that protein truncating mutations at the N-terminus cause severe, early onset phenotype of infantile spasms (Kalscheuer et al. 2003a). Truncations more towards the C-terminus end cause atypical RTT in girls, a severe neurological disorder in boys, or autistic disorder with intellectual disability (Tao et al. 2004; Weaving et al. 2004)

In the future, genotype-phenotype studies in additional patients with CDKL5 mutations may lead to a better correlation between these characteristics.

1.5.3 Related Disorders with Phenotypes Overlapping with RTT

There are features of various intellectual disability disorders that overlap with RTT, suggesting that these could be candidates for *CDKL5* mutation screening. The phenotypic spectrum of *MECP2* mutations is diverse, including autism (Lam et al. 2000; Carney et al. 2003), Angelman syndrome-like features in females (Imessaoudene et al. 2001; Watson et al. 2001), neonatal-onset encephalopathy (Schanen and Francke 1998; Villard et al. 2000), and nonsyndromic XLMR (Couvert et al. 2001; Yntema et al. 2002), and syndromic XLMR (Klauck et al. 2002).

Mutations in the *CDKL5* gene particularly the *Hanefeld variant* of RTT, with characteristic early-onset seizures, usually of the infantile spasm type, potentially provides a phenotypic overlap with X-linked infantile spasm (ISSX) (also known as X-linked West syndrome), and X-linked mental retardation XLMR (Kalscheuer et al. 2003a; Weaving et al. 2004; Scala et al. 2005; Archer et al. 2006a). It was noted a male RTT patient had a rare phenotype of seizures, which are otherwise not associated with RTT, and this patient was found to have a large deletion that disrupted the *CDKL5* gene (Huopaniemi et al. 2000). In addition, Tao *et al.* reported that *CDKL5* may be associated with clinical features that overlap with those of other neurodevelopmental disorders such as autism, and Angelman syndrome (Tao et al. 2004). Another related disorder of interest is Aicardi Syndrome. It is an X-linked dominant disorder mainly affecting girls. The onset of this syndrome generally begins between the ages of 3 and 5 months with infantile spasms. Features include seizures, mental retardation and lesions of the retina that are specific to the disorder (Aicardi 2005). Aicardi syndrome thus has some overlap with RTT due to the fact that both can have infantile spasms.

It is therefore compelling to examine disorders with phenotypes overlapping with RTT related disorders of interest, particularly those with the diagnosis of early onset seizures (Fig 1.4).

1.5.4 X-linked Infantile Spasm

ISSX, (MIM308350) is a serious form of epilepsy with its onset in infancy. It is characterised by the triad of early-onset seizures, hypsarrhythmia, and severe to profound mental retardation (Bruyere et al. 1999). The prevalence of ISSX is 1 per 2,000-5,000 live births and 90% of cases manifest within the first year of life (Wong and Trevathan 2001). Moderate to severe mental

retardation occurs in 60-70% of children with infantile spasms (Bruyere et al. 1999). Some of these patients have been shown to carry mutations in the *ARX (*aristaless related homeobox) gene, which maps to Xp21.3-p22.1. Mutations in *ARX* were found to cause mental retardation (syndromic and non-syndromic), epilepsy and X-linked lissencephaly with abnormal genitalia (XLAG) (Bienvenu et al. 2002; Stromme et al. 2002). Interestingly *ARX* and *CDKL5* are both located in the human chromosome Xp22 region, and mutations in both of these genes have been found in patients with ISSX. Kalscheuer *et al.* reported two females with an identical ISSX phenotype with early-onset severe infantile spasms, profound global developmental arrest, hypsarrhythmia, and severe mental retardation, and who both had *de novo* X:autosome translocations where the X-chromosome breakpoint disrupted the *CDKL5* gene. It was concluded that *CDKL5* can be second X-chromosomal locus for this disorder (Kalscheuer et al. 2003a).

1.5.5 Autism

Autism is a common neurodevelopmental disorder with an onset in the first three years of life that is characterised by severe impairments in reciprocal social interactions and communication. Autistic children also show patterns of interests and behaviours that are restricted and stereotypical (Bailey et al. 1996; Risch et al. 1999). Mental retardation is present in 75% of individuals with autism, and seizures in 15% to 30% (Smalley 1997). Recently, there has been controversy about the incidence of autism and whether it is increasing. Two reasons are commonly suggested for the perceived increase; a lack of consistency of diagnosis and changes in the diagnostic classification system

The population prevalence of core autism was for many years reported to be in the range of 1 per 2,000- 2,5000 births (Smalley et al. 1988), although recent surveys indicate a prevalence of 1 in 600 (Chakrabarti and Fombonne 2001). In Australia, a recently reported study from the Barwon

region of Victoria reported an incidence of autism spectrum disorders of 1 per 2,350 person-years in children aged 2-17 years (Icasiano et al. 2004). Autism is 3-4 times more common in males than females; one explanation for the predominance of male patients could be the involvement of X-chromosome genes (Skuse 2000). Moreover, genome screens have identified several, but non-overlapping, regions with small linkage scores on the X-chromosome, mostly in Xq (Folstein and Rosen-Sheidley 2001).

Autism and RTT share overlapping phenotypes to a certain extent, and are both listed as pervasive developmental disorders (Beyer et al. 2001). Both may be characterised by a period of relatively normal development in infancy following by a period of regression with loss of social, cognitive and language skills during the first 2-4 years of life, with repetitive stereotypic behaviours in both disorders (Lopez-Rangel and Lewis 2006). Interestingly, as mentioned above, Weaving *et al.* reported identical twin sisters with the same *CDKL5* mutations. One girl had a diagnosis of autism, the other twin had a diagnosis of atypical RTT (Weaving et al. 2004). This observation raises the possibility that *CDKL5* mutations may be found in a proportion of patients with autism particularly those with seizures. In view of the overlapping phenotypic spectrum seen with *CDKL5, ARX* and *MECP2* mutations, it is tempting to speculate that mutations of these three genes play a role in a common pathogenic process.

1.6 Netrin G1 (*NTNG1*)

An apparent lack of both *MECP2* and *CDKL5* mutations in a small cohort of clinically well-defined RTT may imply the existence of a third gene causing RTT. *NTNG1* is a predominantly brain-expressed gene that has an important role in the developing central nervous system, particularly in axonal guidance, signalling and N-methyl-D-aspartate (NMDA) receptor function and is a good candidate gene for RTT.

This understanding led Borg *et al.* to further investigate a girl with characteristic features of RTT who had no mutations in *MECP2* or *CDKL5* but carried a *de novo* balanced translocation involving chromosomes 1 and 7. Sequence analysis indicated that on chromosome 1, *NTNG1* was disrupted. The chromosome 1 breakpoint was located within intron 6 of the *NTNG1* and affected alternatively spliced transcripts. However, no known gene was disrupted by the chromosome 7 breakpoint (Borg et al. 2005a). In another study, Archer *et al.* screened 115 patients with RTT (females: 25 classical and 84 atypical; 6 males) with no mutations in the *MECP2* or *CDKL5* genes. No pathogenic mutations were identified in the coding sequence of *NTNG1* gene, suggesting that mutations in the *NTNG1* gene are a rare cause of RTT, and the role of this gene in RTT remains uncertain (Archer et al. 2006c).

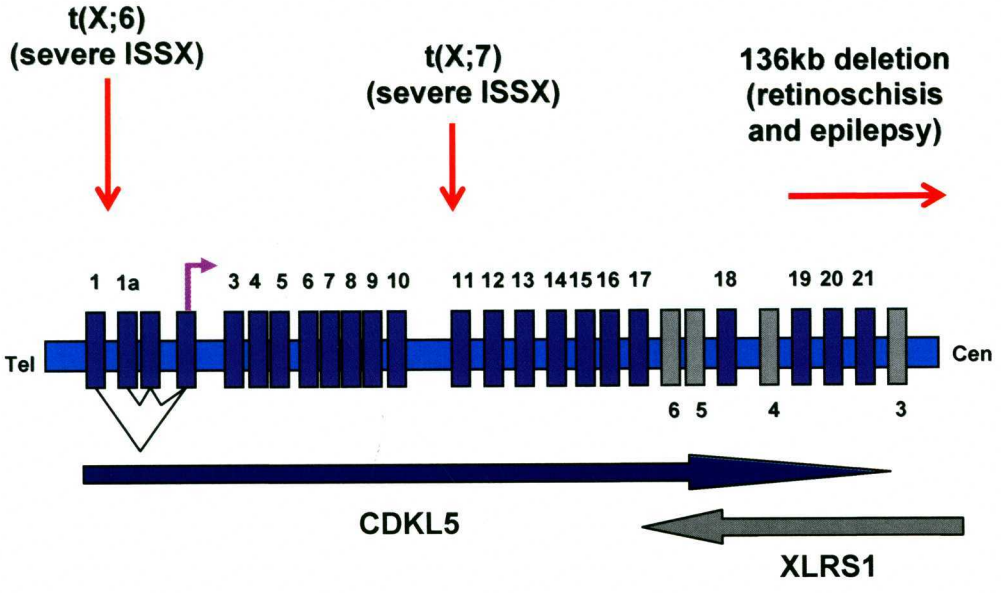

Figure 1:4 Genomic structure of the region containing CDKL5 and XLRS1

The 21 *CDKL5* exons are indicated by the dark blue boxes, while the 3' prime exons of *XLRS1* is indicated by grey boxes. The purple arrow in exon 2 indicates the transcription start site, while the black lines indicate alternative isoforms of *CDKL5*. The arrows below indicate the direction of transcription of the two genes. The red arrows indicate the initial defects that were described in this region.

1.7 Hypothesis and Aims

The project commenced in 2005, shortly after a case report indicated two patients diagnosed with RTT syndrome exhibited *CDKL5* mutations (Weaving et al. 2004). It was speculated by us that *CDKL5* mutations may underlie a significant proportion of RTT cases that were not due to *MECP2* mutations in an existing patient cohort at the Children's Hospital at Westmead (NSW, Australia). We also considered the possibility that other *CDKL5* mutations may lead to non-RTT syndrome conditions affecting the nervous system.

Hypotheses:

1. *CDKL5* mutations will be identified in *MECP2* mutation-negative RTT patients, particularly those with atypical RTT.

2. *CDKL5* mutations will be identified in other cohorts of patients, including families with XLMR and seizures, individuals with ISSX, and individuals with autism, intellectual disability and seizures.

Aims:

1. To screen patients with atypical RTT for mutations in the *CDKL5* gene.

2. To screen patients with ISSX, XLMR and seizures, and autism, intellectual disability and seizures for mutations in the *CDKL5* gene.

2 MATERIALS AND METHODS

2.1 Patient Cohort

This patient cohort consisted of *MECP2* mutation negative RTT patients, mainly atypical, and other clinical conditions which share some overlap with RTT (Table 2.1). In total 249 patients were recruited for this study.

Table 2:1 Summary of patients forming the study cohort

Patient Category	No. of Patients	No. of males	No. of females
RTT	91	5	86
ISSX	52	47	5
Autism	59	55	4
X-linked mental retardation (non-syndromic)	8	7	1
Other	39	8	31

DNA or blood samples were collected from all of these patients, with appropriate consent for genetic analysis. Samples were kindly provided by the following sources. The clinical features of the cohort are discussed in detail in chapter 3.

2.1.1 RTT

There were two main sources of RTT samples for this study. The first source of samples was from a previously recruited study of Weaving *et al.* (Weaving et al. 2003). The second source of samples was from the Australian Rett Syndrome Study, Dr H. Leonard; Centre for Child Health Research, The University of Western, Australia.

The rest of the RTT cohort was from the following collaborators:

- Dr. E. Gak and Dr. B. Ben Ziev; Sheba Medical Center, Genetic Institute, Israel.
- Dr. H. Zoghbi; Baylor College of Medicine, Houston, USA.
- Dr. M. Freilinger; University Hospital of Vienna, Department of Paediatrics, Austria.
- Dr. M. Bailey; Division of Molecular Genetics, University of Glasgow, Scotland.
- Dr. L. Brown; Children's Hospital of Philadelphia, Philadelphia, USA.
- Dr. D. Chitayat; Division of Clinical and Metabolic Genetics, Hospital for Sick Children, Toronto, Canada
- Dr. O. Rahman; Division of Medical Genetics, Stanford, USA.
- Dr. C. Williams; University of Florida Pediatric Genetics, Gainesville, USA.

2.1.2 ISSX

ISSX samples were obtained from Associate Prof. J. Gecz; Neurogenetics Laboratory, Department of Genetic Medicine, Women's and Children's Hospital, Adelaide, Australia.

2.1.3 Autism

Samples of patients with autism were previously recruited from the Cure Autism Now (CAN) Foundation and forwarded by Associate Prof. J. Gecz; Neurogenetics Laboratory, Department of Genetic medicine, Women's and Children's Hospital, Adelaide, Australia.

2.1.4 Other

A mixed group of patient samples were provided by:

- Dr. J. Allanson; Children's Hospital of Eastern Ontario, Ottawa, Canada.
- Dr. K. Anyane-Yeoba; Division of Clinical Genetics, Columbia, USA.
- Dr .W. Chung; New York Presbyterian Hospital, New York, USA.
- Dr. Tim Cox; Department of Anatomy and Cell Biology, Nursing and Health, University of Melbourne, Australia.
- Dr. M. Davis; Department of Anatomical Pathology, Royal Perth Hospital, Perth, Australia.
- Dr. W. Dobyns; Department of Human Genetics, University of Chicago, Chicago, USA.
- Dr. M. Edwards and Dr C Meldrum; Hunter Genetics, Newcastle, Australia.
- Dr. K. Friend; Department of Genetic Medicine, North Adelaide, Australia.
- Dr. V. Kalra; Department of Paediatrics, All India Institute of Medical Science, New Delhi, India.
- Dr. M. McEntagart; S. W Thames Regional Genetics Service, London, United Kingdom.
- Dr. W. Sanger; Human Genetics Laboratories, University of Nebraska, Nebraska, USA.
- Dr. I. Sinnerbrink and Dr. P Grattan-Smith; South Eastern Sydney Area Health Service, Sydney, Australia.
- Dr. E. Thompson, Women's and Children's Hospital, North Adelaide.
- Dr. R. Zannolli, Department of Paediatrics; University of Siena, Italy.

2.2 Methods

2.2.1 Isolation of DNA

When blood was received, DNA was extracted using an altered "salting out" method (Miller et al. 1988). Blood was collected into EDTA anticoagulant tubes. 5ml of blood was added to 45 ml ELB (Erythrocyte Lysis buffer:1.5mM NH_4Cl, 50mM $KHCO_3$, 0.1mM Na_2EDTA) and mixed well to lyse the red blood cells. The tubes were placed on ice for at least 20 minutes before centrifugation at 1000 rpm for 10 minutes at 4ºC. The supernatant was carefully discarded, taking care not to disturb the cell pellet (white blood cells). The pellet was resuspended in 30 ml of ELB by vortexing gently, and placed on ice for 10 minutes, followed by centrifugation of the mixture for 10 minutes at 1000 rpm at 4ºC. The supernatant was carefully discarded and the pellet was resuspended in 5 ml SE (salt and EDTA buffer: 0.8MNaCl, 25mM EDTA pH8.0), 250 µl of 20% SDS and 25 µl of 20 mg/ml Pronase was added to the tube. This was then mixed well by vortexing and incubated the sample overnight at 37°C, in a shaking incubator. 1.6 mL of 5M NaCl was added and vortexed vigorously, before centrifugation at 3000 rpm for 25 minutes at RT. Supernatant was carefully transferred into a new 50 ml falcon tube. Two volumes of ice cold 100% Ethanol was added and the sample was inverted gently (to avoid shearing chromosomal DNA) several times. The DNA clump was spooled into an Eppendorf tube using a sterile yellow tip or a sterile needle and allowed to air dry (to evaporate the ethanol). TE buffer (10mM Tris-HCL pH 8.0, 1mM EDTA pH8.0) was added to the air dried DNA (200-600 µl, volume depends on amount of DNA isolated). The sample was incubated at 37°C for 30 minutes. The DNA was quantified using a Beckman DU® 650 Spectrophotometer and sample diluted to 25ng/µl.

Where DNA stocks became depleted, whole genomic amplification of genomic DNA was performed as outlined in the protocol using the Genomiphi™ DNA Amplification Kit (Amersham Biosciences).

2.2.2 PCR Amplification

The 20 coding exons of *CDKL5* (exons 2-21) were amplified by PCR amplification from genomic DNA. Desalted primers were purchased from Invitrogen™ Life Technologies, and are listed in Table 2.2. Primers were resuspended in sterile Milli-Q (MQ) water and diluted to 100µM. Investigation of *CDKL5* transcripts identified an alternative isoform (S. Williamson, personal communication), resulting in the need to redesign the exon 18 primers. In addition patients reported by Weaving *et al.* 2003 were re-screened for the alternate exon 18 sequence. A working primer mix of each exon for use in PCRs was produced by adding 5µM each of the forward and reverse primers to sterile MQ water.

Each reaction of 25µl contained 25ng of genomic DNA, 0.5µM of each primer, 0.2mM of each deoxynucleotide, 0.75U HotMaster Taq DNA Polymerase, 1x HotMaster buffer with a final concentration of 2.5mM $MgCl_2$. Deoxy-nucleotide triphosphates were obtained as individual tubes of 25mM dCTP, dGTP, dTTP, and dATP (Invitrogen ™ Life Technologies), which were subsequently mixed to produce a 2mM stock solution. HotMaster Taq DNA polymerase (Applied Biosystems) was used in all reactions. Amplifications were performed in a Hybaid (PCR Express gradient block) or a GeneAmp 9600 thermocycler (Applied Biosystems).

The thermocycling conditions were as follows: 94°C for 2 minutes (1× cycle); 94°C for 20 sec, 65°C for 10 sec, 70°C for 20 sec (2× cycles); 94°C for 20 sec, 63°C to 55°C [minus 2°C for 10s per cycle], 70 °C for 20 sec (35× cycles); 72°C for 4 min.

Table 2:2 Primer sequences used for amplifying coding regions of *CDKL5*

Exon	Forward Sequence (5'→3')	Reverse Sequence(5'→ 3')	Product size
2	TAAGATTGGTTACTAGAGTACTGC	GACACACATGTGAATTGATATAGG	506
3	GAGAAGCAATGTCAGTATAGCAG	CATGCCCACACGCAAAGACCAC	194
4	CAACTGGAATCCCCAGTCGGA	AGTGTCTGACCAGCTAGATCC	221
5	GAAGTACTCAAAGCAGAAGGTGA	TCGGGCAAATGTGCACATTGGC	285
6	GCTCTGTATTGGATGAATTATTCTAG	GACAGTAACATGTGAAATACTCTTAAC	297
7	TGTCAATCAGGAGAACATAGAACA	TCAAAAGAATGTTCCTCTACCAA	250
8	GCCCATGCGAGAACAGTCATTAC	GCAAATGACAATAGAATCAGCAG	280
9	AGTTGCCAAAATAATCTCTTCCTT	GAACAATGACTCAAATACTGCAG	520
10	AACACTCACAAGCACGTGCA	TTATTTGCCATTCCACATCTCCT	273
11	GACTTTGTAATGTTCTTAACGATC	CTAATTGCATCATTTAAGCAGCC	281
12	TTGTGTGTCAGCTATTGAGGG	GGTATGTTGTTGTTGGTGAGATC	284
12a	TGCACACCAAAACCTACCAAGC	GCTTTTGGCCTTGGTCCTGTAGGA	329
12b	GAGTCGGCATAGCTATATTGACAC	GAATGGCTACTGTCCATGTGC	354
12c	AACGCTGGACTCACGTCGAAC	CCACCAGATTCAGTCAAGGTG	318
13	CTGGTTATGGTCCTAGTTCTACC	GTGGGAGACTGGGTATTAATAC	277
14	AGGCTACAGTAAGCCATGA	GTGTAGGTGAGAAGGCCGCTG	345
15	GAAAAGTCCATCAGTGACTTAC	GGACACTAAAAAGCTCATCCAGA	249
16	GTTTGATTCTTCCCGGCTATAG	GGCTCTGTTGAGAGGATAGTTG	221
17	CTCCTCTTGGGTGTGGTTGC	GCTCAGCCTTACTGTAACATTG	307
18	TCTAACTTGAATCCTGTGTGC	TCCTGGTCACAGAGGACACATG	300
19	GTGGGCAGAAGTGGCCAATA	GTCTAGGGTCGTTATGGCAGC	223
20	ACCTTGGCTTCAGCTGGTGTC	GGGCAATTCCGAGGTACAGC	325
21	GCCAGAGTGCACCTGCTAGC	AAGGAAAACTCAACCTCAGCG	286

2.2.3 Gel Electrophoresis

Following PCR, agarose electrophoresis was performed. Three microlitres of PCR product was mixed with two microlitres of loading buffer (Sambrook *et al* 1989). One microgram of a 100bp ladder (New England Biolabs) was also loaded onto each gel. The gel was run at 70-100 Volts for 20-50 minutes depending on the size of the DNA fragments. Gel concentration of 1.5% (w/v) agarose (Agarose 1, biotechnology grade, Amresco) in 1xTBE (89 mM Tris base, 89 mM Boric acid, 2 mM EDTA pH8.0, Amresco) containing 5 pg/ml of ethidium bromide (500µg/ml aqueous solution, Sigma)was prepared. Products were visualised using UV light and the images collected with Alphamager™ camera and software (Alpha Innotech).

2.2.4 Mutation Screening

Female patients were initially screened via denaturing High-Performance Liquid Chromatography (dHPLC), followed by direct sequencing of any identified heteroduplexes. In contrast male patients were initially screened by direct sequencing. In later experiments all samples were screened by direct sequencing to speed up the screening progress.

2.2.5 Denaturing High-Performance Liquid Chromatography

The detection of DNA variants by DHPLC analysis exploits the differential retention of double-stranded homo-and heteroduplex species under conditions of partial thermal denaturation. Primary screening was performed using the dHPLC Prostar Helix System (Varian Inc.). PCR products (Table 2.2), were denatured for a final 5 min at 95°C after completion of the normal

PCR cycling then allowed to gradually reanneal over 30min from 95°C to 65°C (1% ramp rate), allowing formation of heteroduplexes.

The optimal temperature for analysing each PCR fragment (e g. exon) was initially calculated using the web site *http://insertion.stanford.edu/melt.html* and assessed empirically to determine if the melting temperature was correct. In some cases more than one significant melting domain was present in a fragment, and so repeat injections at multiple temperatures were performed. A mutation positive control was run when available through the DHPLC system for quality control purposes. In addition 0.5µg of a commercially available *Hae* III restriction digest of pUC18 was also run at the start and end of each batch for assessing the quality of the column.

Table 2:3 Optimal temperatures for analysis of each PCR fragment

Exon	Temperature (°C)	Product size
2	56	506
3	56	194
4	57,52,47	221
5	57	285
6	55	297
7	52	279
8	57	280
9	59	520
10	58	273
11	57	281
12	55,60	284
12a	60	329
12b	60	354
12c	60	318
13	59	277
14	60	345
15	57	249
16	54	221
17	60	307
18	62	300
19	60	223
20	62	325
21	62,56	286

2.2.6 DNA Sequencing

PCRs were prepared for DNA sequencing by the addition of 1.3 units of shrimp alkaline phosphatase (SAP) (Promega), and 6 units of exonuclease I (Epicentre® Biotechnologies at 20u/µl). This solution was incubated at 37°C for 30 minutes and heat inactivated at 80°C for 15 minutes. Sequencing reactions contained 2-4µl of the purified template and 6 pmoles of primer, either reverse or forward in a final volume of 12µl. The samples were then sequenced in the forward and reverse directions using an ABI Prism 3100 Genetic analyser with the BigDye Terminator (v.3.1) cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions by the DNA Sequencing Facility at the Westmead Millennium Institute. Two software programmes were used for sequence variation detection in DNA sequencing trace data against a corrected reference sequence from GenBank® (http:/www.ncbi.nlm.nih.gov/Genbank/), namely Vector NTI software (Invitrogen™ Life Technologies) and Mutation Surveyor™ (SoftGenetics LLC). When a sequence variation was identified, parents were screened when available, in order to determine inheritance. In addition, the conservation and type of amino acid change, if any, was examined. For example, *de novo* changes of conserved amino acids in conserved domains were considered to be probably pathogenic. Mutations that were inherited from the unaffected father were considered to be polymorphisms. In the case of mutation inheritance in an unaffected mother, bioinformatics analysis was used to assess pathogenicity (Chapter 3). To determine amino acid conservation with the *CDKL5* family from different species, protein alignment was performed using the European Bioinformatics Institute (EBI) database (http://www.ebi.ac.uk/clustalw/).

2.2.7 Determination of Pathogenicity

Two methods were used to verify mutations in the 300 control samples as well as parental samples, where available. One method was restriction analysis in cases that the sequence variation created or destroyed a restriction site. Genomic DNA sequences were amplified by PCR and digested using appropriate restriction enzymes. The second method utilised a Custom TaqMan® SNP Genotyping Assay (Applied Biosysytems) for genotyping single nucleotide polymorphisms (SNPs), as per the manufacturer's protocol.

2.2.8 Restriction Endonuclease Digestion

Control alleles and parental samples were screened for the p.S196L missense mutation to examine whether it could be simply a rare polymorphism. The *Ahd* I restriction site is lost by the variation c.586C>T, the size of the uncut product is 280 bp, with the *Ahd* I site located 60bp from one end. The control samples that is digested by *Ahd* I produces two fragments of 60 bp and 220 bp (Figure 3.2). All digests included a sequence verified normal individual to verify that the digests had gone to completion, and the uncut PCR fragment was analysed adjacent to the digests. Restriction enzymes were purchased from New England BioLabs and MBI Fermentas and were used with the recommended 10x restriction buffers at the recommended temperature. The digested fragments were analysed by gel electrophoresis on a 1.5% (w/v) agarose gel. Products were visualised using UV light and the images collected with Alphamager™ camera and software (Alpha Innotech).

2.2.9 Custom TaqMan® SNP Genotyping Assays

Parental samples and control alleles were screened for the IVS11-23T>C variation to determine whether this might be a rare polymorphism. In order to calculate the allelic frequency of the variation a Custom TaqMan® SNP Genotyping Assay (Applied Biosystems, Foster City, CA, USA) was used, in accordance with manufacturer's instructions, to screen 306 normal control alleles. These products use the 5' nuclease assay for amplifying and detecting specific SNP alleles in control individuals. Sequence-specific forward and reverse primers were designed to amplify the SNP of interest. Two fluorescently labelled probes (TaqMan MGB) annealed specifically to a complementary sequence between the forward and reverse primer sites. The fluorescent signal generated by PCR amplification indicated which alleles were present in the sample.

3 RESULTS

CDKL5 mutations have recently been implicated in atypical RTT, ISSX, severe neurological disorders associated with mental retardation, loss of communication and motor skills, infantile spasms and seizures, predominantly in females (Lin et al. 2005a). The aim of this project was to screen RTT patients for mutations in the *CDKL5* gene. In addition, screening was carried out on patients with ISSX, XLMR and seizures, MR and seizures and autism. Thus, patients with a broad range of neurological clinical phenotypes previously associated with *CDKL5* mutations were selected for this study.

A cohort of patient samples (249) was recruited who had one or more of the aforementioned features and were categorised as shown in Figure 3.1. Out of 249 patients in this cohort, 139 (56%) had seizures.

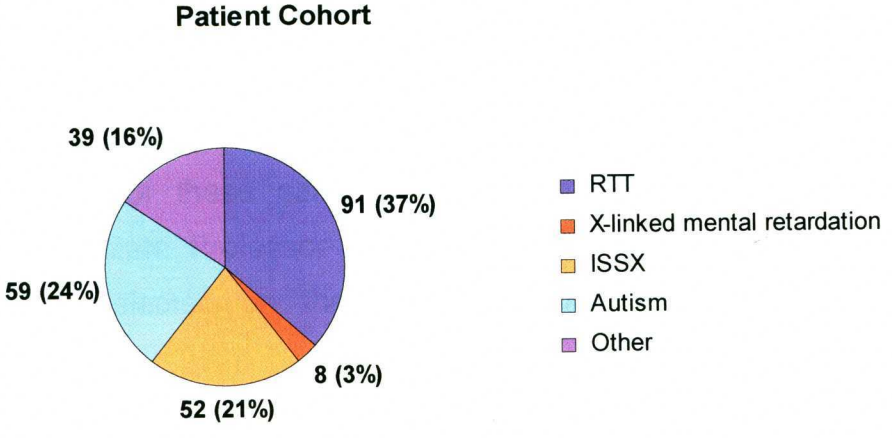

Figure 3:1 Total of 249 patients selected for CDKL5 screening

3.1 Clinical description

3.1.1 RTT

The RTT group formed 37% of patients recruited for this study. All samples were previously proven negative for *MECP2* mutations. The promoter and coding region were screened for 24% and MLPA screening 29% of this group. Out of the 91 RTT patients, 10 had the classical phenotype (11%), 49 with atypical RTT (54%) and 32 were unclassified (35%). Seizures were seen in 40 out of 66 patients for whom this information was available. Of the remaining 26 patients, their seizures status was unknown.

3.1.2 ISSX

The ISSX group formed 21% of subjects in this study. Out of 52 patients with ISSX, 47 were males and 5 were females. *ARX* mutations have been reported to cause IS and XLMR (Bienvenu et al. 2002; Stromme et al. 2002). Thus all of these samples were confirmed negative for *ARX* mutations (Associate Professor J, Gecz personal communication). ISSX patients were selected on the basis of probable X-linked mode of inheritance.

3.1.3 Autism

The autism group formed 24% of patients recruited for this study. Out of the 59 patients with autism, 4 were females and 55 were males. These patients were diagnosed with autism-spectrum disorder. This group includes multiple affected males in one family, suggestive of X-linked mode of inheritance. All affected males had been haplotype matched across the Xp22 region.

3.1.4 Non-syndromic XLMR

The non-syndromic XLMR group formed 3% of this study. This group numbered 8 in total and consisted of 1 female and 7 males. All of these patients participated in an epilepsy genetic study because of their seizures.

3.1.5 Other related disorders

This group accounted for 16% of this study. A sub-group of 7 patients were diagnosed with Aicardi syndrome (details discussed further in chapter 1). The gender of these patients was not supplied. The 32 remaining patients (4 males and 28 females) were incompletely classified but had clinical features of interest according to the selection criteria for this study. Of these, 4 patients were diagnosed with mental retardation, and 7 diagnosed with seizures.

Methods for sequencing and dHPLC screening are described in Chapter 2. Subsets of patients were screened by Ms Gladys Wallis (Rett Syndrome Research, The Children's Hospital at Westmead). Assistance was given by conducting direct sequencing, 22 RTT and 52 Autism samples, on the coding region of *CDKL5* according to previously described methods in Chapter 2.

3.2 *CDKL5* Screening Results

In all, six polymorphic variations and one probable pathogenic mutation were identified (Table 2), accounting for 46 of the 249 patients in this cohort (17%). Largely most individual identified with a variation had the clinical feature of seizures.

Table 3:1 Screening results

MR; Mental retardation, RTT; Rett syndrome, ISSX; X-linked infantile spasm, ND; not determined.

Type of variant	Nucleotide change	Amino acid change	No. of Patients	Diagnosis	Detected in Parent	SNP
Missense	c.586C>T	p.S196L	1 (*de novo*)	RTT	Not carried by both parents	
Missense	c.2372A>C	p.Q791P	18	ISSX, RTT, seizures	ND	Known c.SNP
Intronic	IVS+118T>A	None predicted	21	ISSX, RTT, seizures	ND	Known c.SNP
Intronic	IVS11-23T>C	None predicted	1	Male, seizures, MR	Carried by unaffected mother	Probable polymorphism
Intronic	IVS8+11G	None predicted	1	RTT-like Unknown classification	Carried by unaffected father	Probable polymorphism
Intronic	IVS7+22T>C	None predicted	1	RTT, seizures	Carried by unaffected mother	Probable polymorphism
Silent polymorphism	c.[3003C>T(+) 3084G>A]	p.H1001 p.T1028	3	Atypical RTT	ND	Probable polymorphism

3.2.1 Missense c.586C>T

The missense c.586C>T variation was identified in a three year old female, first born child of non-consanguineous Indian parents. The patient was carried to full term, and the delivery was uneventful. Normal development occurred until 5 months of age when she could sit with support, reach for objects, had social smiling, recognition of her mother, good eye contact and non-specific monosyllabism. Regression was noticed from the age of 5 months, where she no longer reached for objects, lost eye contact and had no useful hand activity. She developed hand washing stereotypies and does not produce any meaningful sounds. Since the age of 5 months she has had 10-15 flexor spasms per day, which increase when she is drowsy. She was treated with ACTH (adrenocorticotropic hormone) for 6 weeks, Valproate up to 60 mg/kg and Clobazam without adequate control of seizures. Currently she has tonic spasms and hypothyroidism and is presently on Lamotrigine. The patient has no history of abnormal respirations, has a head circumference 47 cm (lower 20% percentile band), weighs 14 kg (lower 50% band), and a height of 94 cm (lower 40% band). The patient also has small feet but has normal muscle tone. Her EEG shows hypsarrhythmia.

The patient is heterozygous for the missense mutation c.586C>T in exon 9 which changes the amino acid Serine to Leucine at position 196 of the protein (Figure 3.2). The alignment (figure 3.3) is part of the protein kinase domain, and the change in the amino acid affects a highly conserved residue within this domain. Therefore it is likely to affect the protein kinase activity of CDKL5 causing atypical Rett syndrome. Both of her parents were screened, as were 300 control alleles. The sequence variation was not detected in the either of parents or the control samples. The *Ahd* I restriction site is lost by the variation c.586C>T, the size of the uncut product is 280 bp, with the *Ahd* I site located 60bp from one end. The parents of the patient

both have a PCR product that is digested by *Ahd* I into two fragments of 60 bp and 220 bp (Figure 3.4).

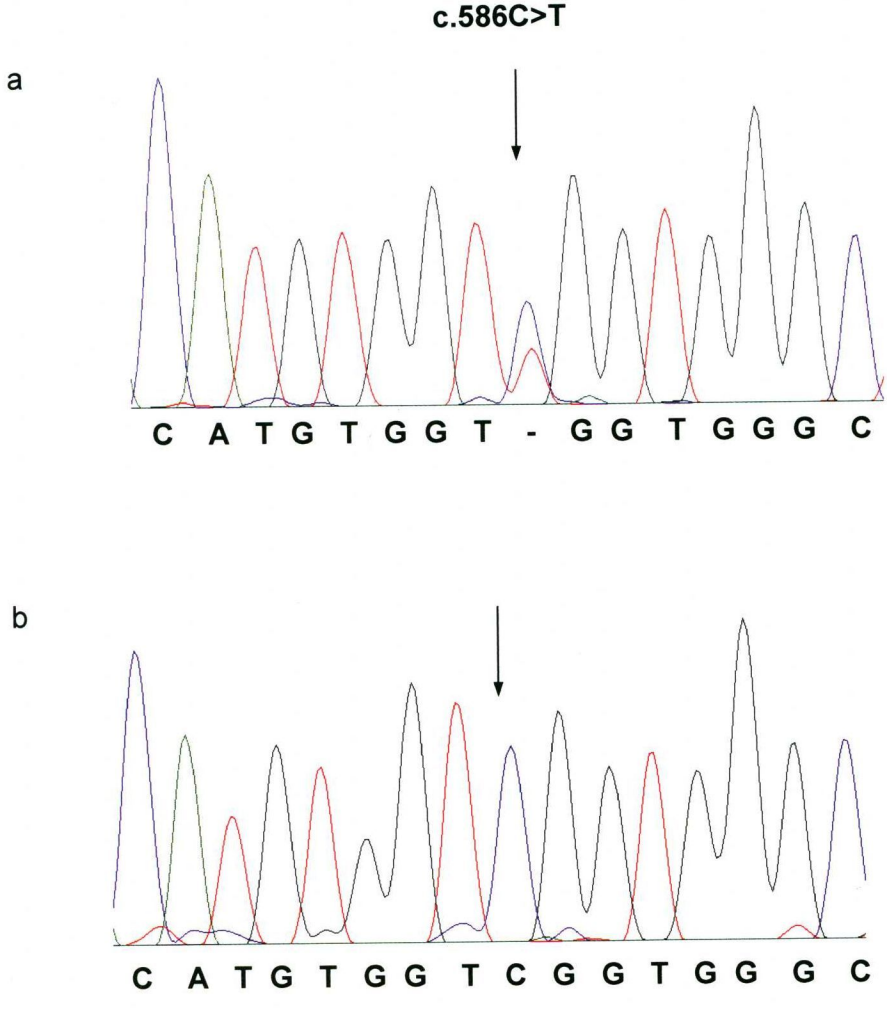

Figure 3:2 Sequence trace of c.586c>T

Sequence trace showing the c.586C>T mutation in an affected patient (a) compared to a normal control (b).

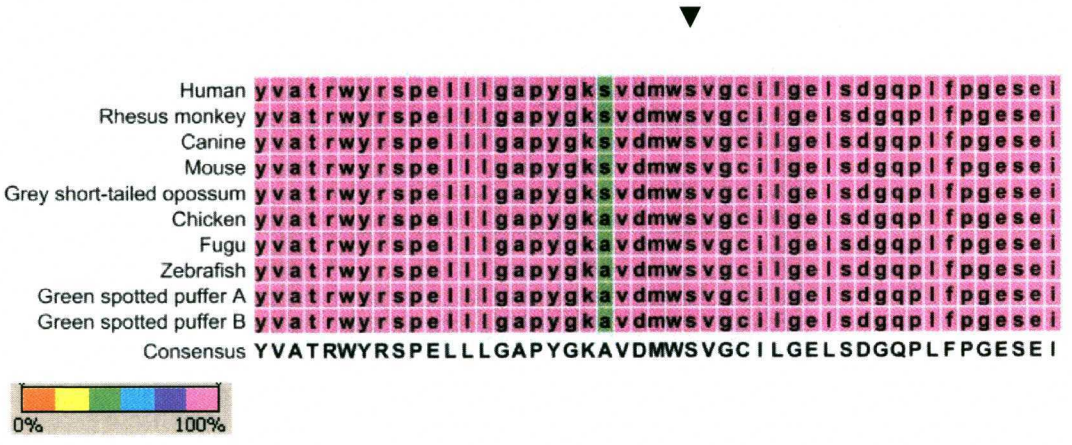

Figure 3:3 Protein sequence alignment of CDKL5 orthologs

Conservation of amino acid p.S196L protein sequence alignment of CDKL5 orthologs. Sequences were aligned using *CLC Free Workbench 3.0.2 (www.clcbio.com)*. Amino acids identical to consensus sequences (below alignment) are shaded according to the legend (<10 %: red, 10-30 %: yellow, 30-50 %: green, 50-70 %: light blue, 70-90 %: dark blue, >90% purple). The black arrow above the alignment indicates 100% conservation of amino acid S at position 196 across all species in a highly conserved region of CDKL5. Protein sequences were identified by Sarah Williamson (Metabolic Research, Sydney Australia) from GenBank (nr, EST and htgs) and Ensembl databases. Human (*Homo sapiens*, NP_003150), rhesus monkey (*Macaca mulatto*, XR_009820), canine (*Canis familiaris*, XP_548881), mouse (*Mus musculus*, NP_001019795), grey short-tailed opossum (*Monodelphis domestica*, XP_001380717), chicken (*Gallus gallus*, XP_425571), fugu (*Takifugu rubripes*, AAD28798), zebrafish (*Danio rerio*, XP_687767), green spotted puffer A (*Tetraodon nigroviridis*, CAF99817) and green spotted puffer B (*Tetraodon nigroviridis*, CAF95446).

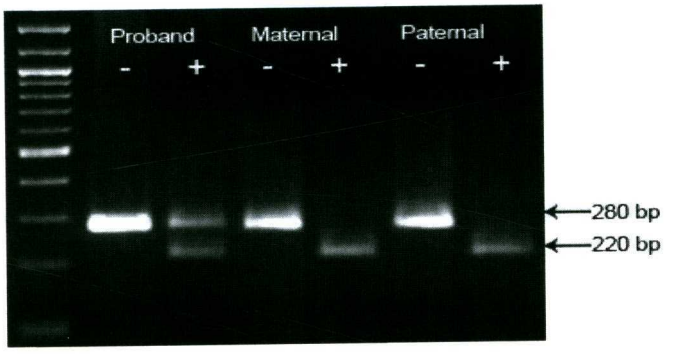

Figure 3:4 Digested and undigested PCR amplicons of c.586C>T

Electrophoresis of digested (+) and undigested (-) PCR amplicons. The *Ahd* I restriction site lost by the variation c.586C>T. The size of the uncut product is 280 bp, with the *Ahd* I site located 60bp from one end. The patient with the variation has a band that remains uncut; whereas the parents of the patient both have a PCR product that is digested by *Ahd* I into two fragments of 60 bp and 220 bp (the 60 bp fragment is not visible on the gel).

3.2.2 Missense polymorphic variation (c.2372A>C; p.Q791P)

A coding Single Nucleotide Polymorphism (c.SNP) was detected at position 2372 (c.2372A>C), exon 16, which results in the amino acid change p.Q791P. This c.SNP was previously detected in 20% of a panel of 50 control chromosomes (Kalscheuer et al. 2003a; Tao et al. 2004). This variation was identified in 18 individuals (7% of the study cohort), 15 RTT and 3 ISSX patients. This group consists of 3 males and 15 females. Of these patients 12 individuals had seizures.

3.2.3 Intronic variation IVS16+118T>A

This variation has been previously reported as a common SNP detected at position 2376 (c.2376+118T>A) in intron 16 with a minor allele frequency of 33% (Evans et al. 2005). The c.2376+118T>A variation was identified in 21 patients (8%) in this cohort, 18 RTT and 3 ISSX patients. This group

consists of 3 males and 18 females. Of these patients 13 individuals had seizure (65%).

3.2.4 Intronic variation IVS11-23T>C

The IVS11-23T>C variant was identified in a male individual, who had neonatal seizures followed by severe epilepsy in infancy. He has severe mental retardation and phenotypically displays features of both RTT and Angelman Syndrome, for example laughing spells, sterotypies, aphasia, mild microcephaly, broad mouth, and suffers from a sleep disorder. He was also reported to have mild spasticity. Serial MRI scans of the brain have been normal. Investigation for a number of inborn errors of metabolism was negative. In addition, levels of cerebrospinal fluid neurotransmitters, folic acid metabolites, purines and pyrimidines were normal. The patient was negative for mutations of *UBE3A, MECP2* and *ARX*. The BDG Splice Site Predictor program (http://www.fruitfly.org/seq_tools/spilces.html) predicted that this variation would not affect exon or intron splicing. This previously unreported variation was also identified in the unaffected mother but not in 306 normal control alleles. If this variation IVS11-23T>C is indeed pathogenic, the absence of any symptoms in the mother may be explained by a skewed pattern of X-chromosome inactivation, whereby most of the copies of the mutant allele have been silenced. The X-chromosome inactivation assay carried out showed inconsistent results between repetitions and therefore the state of X-chromosome inactivation in the carrier mother remains uncertain. Given the other evidence of splicing prediction and control screening, it is concluded that this variation is most likely non-pathogenic.

3.2.5 Intronic variation IVS7+22T>C

The IVS7+22T>C variant was identified in a female RTT individual, who had well controlled seizures. This previously unreported variation was also identified in the unaffected mother of this patient but not in 306 normal control alleles. The BDG Splice Site Predictor program predicted that this variation does not affect the exon or intron splicing. It is likely that this variation is non-pathogenic.

3.2.6 IVS8+11G

The IVS8+11insG variation was identified in a 3 year old female, RTT-like individual. Clinical data supplied was very limited. This previously unreported IVS8+11G variation was also identified in the unaffected father of this patient. The BDG Splice Site Predictor predicted that this variation does not affect the exon or intron splicing. Therefore it is concluded that this variation is probably non-pathogenic.

3.2.7 Silent polymorphisms: c.3003C>T (+) c.3084G>A

Two silent polymorphisms (c.3003C>T, and c. 3084G>A) occurring together in *cis* 81 base pairs apart were identified in three patients, two males and one female. As two unrelated males carry the mutation in *cis*, this suggests that these two variants occur in *cis* as part of a haplotype and it is reasonable to infer that the female patient is a carrier for this haplotype and that these mutations are in *cis* in her as well. Patient 1; is a female with RTT with severe mental retardation, early onset seizures, progressive microcephaly, stereotypic hand movements, autonomic changes, and scoliosis. Serial MRI scans of the brain were normal. Investigation for a number of inborn errors of metabolism was negative. Patient 2; is a male with Angelman-like features, no methylation abnormality, and is negative for

mutations in the *UBE3A* gene. Patient 3; is a male diagnosed with ISSX and mental retardation, and who had dystonic posturing and dysarthria. These two silent variations have been previously reported (Tao et al. 2004; Huppke et al. 2005). Previously reported nucleotide exchanges, c.3003C>T and c.3084G>A, form a rare conserved haplotype that was found in 2 of 267 controls from Caucasian individuals (Tao et al. 2004). The BDG Splice Site Predictor predicted that this variation does not create the potential for abnormal splicing. Therefore it is concluded that this variation is probably non-pathogenic.

4 DISCUSSION

In recent studies *MECP2* mutations have been found in up to 90%-95% of patients with classical RTT (Williamson and Christodoulou 2006), and in a lesser proportion (20%-60%) of those with atypical forms of RTT (Buyse et al. 2000; Cheadle et al. 2000; Bourdon et al. 2001c; Hoffbuhr et al. 2002). Further investigations of the remaining group of well defined RTT patients with an unknown genetic cause led to the discovery of mutations in a second gene, *CDKL5*, causing RTT (Tao et al. 2004; Weaving et al. 2004; Evans et al. 2005; Scala et al. 2005). Recently, pathogenic mutations in RTT syndrome have also been identified in two other genes *NTNG1* (Borg et al. 2005b; Archer et al. 2006b) and *MAP2* (Pescucci et al. 2003).

The initial report of a *NTNG1* mutation (Borg et al. 2005b) in a RTT patient appears to be an isolated occurrence, as subsequent screening of a large cohort of *MECP2* and *CDKL5* mutation negative RTT patients failed to find any further mutations (Archer et al. 2006b). The screening of *NTNG1* is therefore not recommended as a part of comprehensive genetic screening of RTT at the present time. Pescucci *et al.* proposed that the *MAP2* gene as a good candidate for RTT. This suggestion was based on their identification of a chromosome 2 deletion encompassing the *MAP2* gene in a patient with autism and RTT-like features (Pescucci et al. 2003). However, further studies are required to validate the role of *MAP2* in RTT patients. Future research could focus on the identification of new genes causing a RTT phenotype, with particular focus on the rare families with multiple affected individuals and the rare individuals with chromosomal rearrangements like this patient. Moreover, as the pathogenesis of RTT becomes better understood, new candidate genes may emerge that would warrant mutation screening.

The first *CDKL5* mutations were identified in patients with ISSX and X:autosome translocations (Kalscheuer et al. 2003a). Most cases reported to date with *CDKL5* mutations have been associated with an early onset seizure variant of RTT, the so-called *Hanefeld variant* (Tao et al. 2004; Weaving et al. 2004; Evans et al. 2005; Scala et al. 2005). Additionally, it is now recognised that the phenotype caused by *CDKL5* mutations not only overlaps that of RTT and ISSX, but also severe neonatal onset encephalopathy, seizures, MR and autism (detailed in Chapter 1). This overlapping phenotypic spectrum raises the question as to whether other clinical phenotypes might have *CDKL5* mutations. For instance, ISSX patients, who are negative for *ARX* mutations, could be good candidates for *CDKL5* mutations. Hence, the aim of this study was to screen RTT patients and other related clinical disorders for *CDKL5* mutations.

We screened a cohort of patients for mutations in the *CDKL5* gene. In our study we identified six polymorphic variations and one novel probably pathogenic mutation (refer to Table 3.1). Three of these variants were also seen in unaffected parents and are therefore likely to be rare polymorphisms. The two SNPs identified in this study were previously reported in the literature (Kalscheuer et al. 2003a; Tao et al. 2004; Evans et al. 2005), as was the complex allele with two silent variations in *cis* (Tao et al. 2004; Huppke et al. 2005). None of the intronic variants altered splice sites according to the prediction software used. The most consistent clinical feature of patients with these variations was seizures, which is not surprising given that this was one of the selection criteria for inclusion in this study, and the spectrum phenotype of variants identified was broad including RTT and ISSX.

An Indian patient was identified to be heterozygous for a missense mutation in exon 11 (c.586C>T, p.S196L, Fig 3.1). We screened both of her parents and 300 control alleles and none shared the mutation. Screening control patients of the same ethnicity was instigated but not completed by our

collaborators. The serine at position 196 of the *CDKL5* protein is a highly conserved residue and is located within the kinase domain (Figure 3.2). Therefore, this serine could potentially affect the protein kinase activity of *CDKL5*. Based on this, and the fact that the c.586C>T mutation was a *de novo* mutation, we suggest that this mutation is likely to be pathogenic. Further investigations on functional studies of this missense mutation to determine if it affects the kinase function of *CDKL5* is recommended. The patient exhibits a RTT-like phenotype with hypsarrhythmia. Additional evidence implicating *CDKL5* mutations as a significant cause of infantile spasms comes from the recent work of Archer *et al.* and Buoni *et al.* (Archer et al. 2006a; Buoni et al. 2006). Our findings coupled with the Archer and Buoni studies further strengthens the aetiological link between *CDKL5* mutations and infantile spasms.

Presently, the diagnosis of RTT remains a clinical one and is not made solely on the basis of identification of *MECP2* and *CDKL5* mutations. However, *MECP2* and *CDKL5* mutations can occur in association with non-RTT phenotypes. Genotype-phenotype correlations have been limited because of the small number of *CDKL5* mutation positive patients who have been identified to date. Bahi-Buisson and colleagues recently investigated how genotype might influence the epilepsy outcome in patients with *CDKL5* mutations. They suggested that the course of epilepsy is more severe in patients who carry mutations in the catalytic domain of *CDKL5* as opposed to patients with late truncating mutations (Buisson-Bahi et al. 2007). Further studies of genotype-phenotype correlation will be a useful tool in the understanding of RTT.

Three genes have been associated with phenotypes overlapping with RTT, namely *ARX*, *NTNG1*, and *CDKL5*. Mutations in the *ARX* gene cause IS, X-linked lissencephaly with abnormal genitalia (XLAG) and X-linked mental retardation (Bienvenu et al. 2002; Stromme et al. 2002). *NTNG1* has been reported to be a candidate gene for a RTT-like phenotype (Borg *et al.* 2005),

and *CDKL5* mutations are a significant cause of IS and early epileptic seizures (Archer *et al.* 2006a). An MLPA kit has been devised to screen these three genes to detect duplications and deletions that would be missed by sequencing. *MECP2* deletions occur in approximately 30% of females with classical RTT who had no detectable *MECP2* mutations by sequencing or dHPLC (Bourdon et al. 2001b; Bourdon et al. 2001c; Erlandson et al. 2001; Erlandson et al. 2003; Schollen et al. 2003; Ariani et al. 2004; Laccone et al. 2004; Ravn et al. 2005b; Archer et al. 2006c; Hardwick et al. 2007). MLPA screening for the *CDKL5* gene has not been reported to date. It may be that genetic abnormalities identified by MLPA are more common than point mutations and that MLPA may thus be more widely applicable. It will only be by performing *CDKL5* MLPA analysis on a large cohort that the importance of MLPA screening will be elucidated.

Mutation screening of exon 1 of *MECP2* has recently been included into DNA diagnostic testing even though mutations appear to be rare (Mnatzakanian et al. 2004; Amir et al. 2005; Saxena et al. 2006). A similar argument could apply for the *CDKL5* gene. Evans *et al.* has reported four promoter/exon 1 variants in the *CDKL5* gene. Two out of the four were not seen in controls (Evans et al. 2005), and their functional significance remains uncertain. Although mutations in exon 1 *CDKL5* may be a rare cause of RTT, analysis of this exon would have been interesting to include in this study, as well as screening the *CDKL5* promoter for possible variations of functional significance. Mutations may lie in untested parts of the *CDKL5* gene, apart from whole exon deletions or duplications such as exon 1, the promoter or the 3'UTR. However, it is highly unlikely that these types of mutations would account for a significant portion of the *MECP2* mutation negative patients. It would be expected that if these types of mutations existed in large number they would be outnumbered by missense or nonsense mutations in the *CDKL5* gene, as is the case in other genes such as *MECP2* and *CFTR*.

Based on previous reports it would seem that to date, 15 different *CDKL5* mutations have been identified in atypical RTT patients with an early onset seizure disorder (Li et al. 2007). Screening of *CDKL5* should be restricted to those cases where the phenotype includes early seizures. This is supported by our study, where of the 249 patients screened for *CDKL5* mutations, only one pathogenic mutation was identified. This patient manifested a RTT-like phenotype with early onset seizures. Our data and the findings of other collaborators, strongly suggest that in the interests of cost effectiveness and the good use of resources, *CDKL5* screening in RTT should be restricted to the individuals who have early onset of seizures.

RTT is a neurodevelopmental disorder that mainly affects females (Hagberg et al. 1983; Armstrong et al. 1995; Glaze 1995), while the gender of the autistic and ISSX groups of this study were mainly males, (93% and 90% respectively). We have found no *CDKL5* mutations in these two later cohorts. Based on our findings and those of others, screening females with autism and ISSX for *CDKL5* sequence variations may lead to the identification of additional pathogenic mutations. Taken together, the results presented in this thesis coupled with our previously published results, indicate an overall incidence of *CDKL5* mutations in RTT to be approximately 2% (3/135). Pooling the *CDKL5* screening results of RTT patients reported by others gives an incidence of 3% (4/150). It is concluded that *CDKL5* is not a major cause of RTT and that *CDKL5* screening does not seem indicated in males with ISSX or autism with or without seizures.

4.1 Future directions

The fact that *CDKL5* is not a major cause for *MECP2* negative RTT raises the question as to what genetic aetiology may be in these patients, throwing the spotlight back on the *MECP2* gene.

Variations may lie in regions which have not been sequenced such as the promoter, exon 1, intronic regions and the 3'UTR region. Screening for mutations in these regions is a possibility but this is a large amount of work with probably a low detection rate especially using existing screening strategies. New high throughput sequencing technologies such as the Roche FLX ™ sequencing system may make this more viable and cost effective in the near future. In the case of clinically well-defined RTT patients who are *MECP2* and *CDKL5* mutation-negative, testing of *MECP2* cDNA may reveal mutations which alter splicing such as intronic variants. Currently such testing is performed using the most easily accessible form of mRNA, namely from blood. However, *MECP2* is mainly expressed in the brain, where it plays a key role in its development and function.

It has been hypothesised that some patients with RTT may in fact carry somatic mutations, as recent clinical and molecular findings suggest that multiple forms of mosaicism in RTT patients (skewing of X-inactivation, mosaicism and somatic mosaicism). In this project, the method of choice for identifying mutations relied on direct sequencing of DNA extracted from blood samples. The ability of this method to detect mosaic mutations is poor, which is particularly true when the level of mosaicism is low. Future research could include the use of at least two complementary approaches, such as quantitative methods based on heteroduplex analysis in particular high resolution melt analysis, and pyrosequencing, for an efficient screening of mosaic mutations of the *MECP2* and *CDKL5* genes.

In view of our results, we propose that an effective approach for screening candidate *CDKL5* mutation patients would be to initially screen for *MECP2* mutations. *MECP2* screening should start with exons 3 and 4 as this is where the majority of mutations are located. This should be followed by screening for large exon wide deletions by MLPA and then sequencing of exons 1 and 2. If no mutations are detected in *MECP2*, then *CDKL5* mutation screening in patients with atypical RTT should be conducted.

Additionally, when screening for *CDKL5* mutations in other clinical disorders, selectively screening phenotypes displaying early seizures, should be considered. This should include *CDKL5* screening by DNA sequencing followed by MLPA for detection of large detections, although, evidence of MLPA screening is yet to be instigated.

Using the cohort from this study, RTT patients with diagnosis of seizures have been selected for ongoing research. We are currently screening these patients for large exonic deletions and duplications by the above mentioned *CDKL5* MLPA kit.

This study has shed more light on the *CDKL5* gene. It strongly suggests that in the interests of cost effectiveness and good use of resources, screening is not generally warranted in RTT patients unless they have severe early-onset seizures, or males with ISSX or patients with autism. Additionally, further intense screening of the *MECP2* gene may identify mutations that have been previously missed.

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