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Ph.D in Oncology and Genetics

Strategies for identification of new mental retardation genes

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Thesis suitable for the title of “Doctor Europaeus”

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1. INTRODUCTION
1. Introduction

Mental retardation (MR) is a frequent cause of serious handicap and it represents the most common reason for referral to genetic services. It is also one of the most important unsolved problems in healthcare throughout the world. Since 2002 MR is defined as "a disability characterized by significant limitations both in intellectual functioning and in adaptive behavior as expressed in conceptual, social and practical adaptive skills, originating before 18 years of age." It is estimated that 2-3% of the general population is affected by MR with a sex ratio (male to female) of 1.5. Increasingly, the term 'intellectual disability' is being used instead of 'mental retardation'.

Intellectual functioning is commonly determined by performance in a series of standardized tests (O.M.S. 1980) that allow the definition of the "Intelligence Quotient" (IQ) of an individual. Assuming a population mean IQ of 100, MR can be divided into four degrees of severity, based on IQ measurements (World Health Organization, WHO; American Psychological Association APA):

1- MILD with an IQ between 50 and 70
2- MODERATE, with an IQ between 35 and 50
3- SEVERE, with an IQ between 20 and 35
4- PROFOUND with an IQ below 20

It is calculated that moderate to profound MR has a prevalence of 0.3-0.5% in the population, and that this prevalence increases to about 1-1.5% when mild MR is included in the estimate. There are more than 1000 Mendelian disorders listed in the OMIM database (Online Mendelian
Inheritance in Man; [http://www.ncbi.nlm.nih.gov/Omim/searchomim](http://www.ncbi.nlm.nih.gov/Omim/searchomim) in which MR is one or the only clinical feature. When MR is the only clinical feature, the condition is referred to as nonspecific or non-syndromic; if MR is accompanied by other physical or neurological features, the condition is referred to as specific or syndromic. \(^6\,^7\). The underlying causes of MR are extremely heterogeneous \(^8\) (Fig.1). Establishing the cause of MR is essential for prognosis, management, and genetic counseling and frequently represents a challenge.

![Figure 1: Causes of mental retardation (modified from Stevenson et al., 2003)](image)

In the majority of diagnosed cases the cause of MR is unknown; about 25-40% of severe MR and most of mild MR cases remain unexplained \(^5\). The interaction between genetic and environmental factors seems to play an important role in the pathogenesis of mild MR \(^3\). In the portion of cases with known etiology, environmental causes including very premature birth,
head trauma, brain infections, fetal alcohol syndrome or exposure to toxic agents, are estimated to underlie 10-30% of moderate to profound MR and about 15% of mild MR. An additional 30-40% of moderate to profound MR and 15% of mild MR are due to chromosomal or single-gene alterations.

Patients with chromosomal rearrangements belong to syndromic MR. These include well known and relatively common disorders such as Down syndrome (trisomy 21), Prader-Willi and Angelman syndromes (15q11.3 deletion), William syndrome (7q11.2 deletion) or Wolf-Hirschhorn syndrome (deletion of distal 4p). In addition, subtle subtelomeric rearrangements missed by standard cytogenetic analysis were estimated to account for about 7% of children with moderate to profound MR and 0.5% of children with mild MR. Diagnosis in patients with small rearrangements is now being greatly facilitated by recent technological developments that allow the investigation of the human genome at a resolution that is 50-100 times higher than that of routine chromosome analysis by karyotyping. In particular Array CGH, which in analogy with karyotyping has been termed "molecular karyotyping, is able to detect rearrangement as small as 10 kb, and it can provide a diagnosis in an estimated 4-17% of previously undiagnosed MR patients.
1.1 X-linked mental retardation (XLMR)

In 1938 Penrose reported the observation that mental retardation is significantly more common in males than in females, with the ratio of affected males to females being 1.3:1. Subsequent studies confirmed this observation and the description of large families with X-linked inheritance pattern laid the concept that X-linked gene defects play an important role in the etiology of MR. The concentration of genes causing MR may be twice as high on the X chromosome compared to autosomes.

The identification of X-linked conditions is easier due to the hemizygosity of males, who inevitably show the phenotype when harbouring a mutant allele. In females the normal allele on the second X chromosome can compensate for a mutation in an X-linked gene, thus resulting in the absence of a phenotype or in a milder phenotype, possibly because of skewed X-inactivation. The low percentage of affected females in familiar cases of X-linked mental retardation (XLMR), suggests that the majority of XLMR cases have a recessive inheritance. The prevalence of recessive inheritance of XLMR in familial cases is not unexpected, since MR patients usually do not reproduce and consequently, it is more likely that dominant conditions lacking healthy carriers remain limited to the single individual.

XLMR is estimated to cause about 10% of all inherited cases of MR in males with a frequency of 1:600 males. However, the male excess in XLMR cannot be caused by X-linked gene defects alone. Different explanations have been given for this phenomenon over the years, including social biases and hormonal contribution. Another explanation could be different gene-dosage in the two sexes. Many genes on X chromosome escape from X-inactivation totally or at least partly. For most of these,
there is no functionally equivalent homologue on Y chromosome, which could explain gender specific differences in the predisposition for certain diseases. Furthermore Y chromosome genes could be directly responsible for these sex differences, as SRY which has a male-specific effect on brain function \(^{23,24}\) hypothesized the existence of X-linked risk factors for MR, as X-linked polymorphisms. Such polymorphisms affect cognitive abilities, but without causing evident MR in most, and thus causing little or no decrease in reproductive fitness. Such alleles would result in MR only when associated with predisposing genetic combinations (of X-linked or autosomal alleles) or environmental conditions. X-linked modifiers would be expected to have a stronger effect on the frequency of mild MR and this is compatible with the observation that the male excess is significantly higher in mild than in severe forms of MR.

Clinical and genetic observations have shown that XLMR includes a very heterogeneous set of conditions responsible for a large number of inherited MR cases. The classification of MR can be based on the underlying causes (genetic, environmental or their combination) and/or on the timing of onset (pre-, peri-, postnatal) \(^{25}\).

In 1991, Kerr et al. proposed the classification of XLMR into syndromic (MRXS) and non-syndromic (MRX) \(^6\). Patients with MRX only have reduced mental capacities, whereas patients with MRXS have additional clinically recognizable features, such as neuromuscular abnormalities, dysmorphisms and congenital malformations. It is estimated that 2/3 of XLMR is non-syndromic \(^1\). Syndromic XLMR conditions were subdivided into four classes \(^{26-31}\): (a) syndromes, with concurrent physical anomalies in various combinations, (b) neuromuscular conditions, with associated neurologic and/or muscular symptoms, (c) metabolic conditions and (d) dominant conditions. However, classes (c) and (d) are not based on a clinical
presentation, but on the biochemical mechanism (c) or on the mode of inheritance (d). Hence more recently XLMR conditions have been categorized into only three classes based on clinical presentation: (a) syndromes, characterized by multiple congenital anomalies and defects in organs/tissues other than (but also including) the brain; (b) neuromuscular disorders, characterized by neurological or muscular symptoms (epilepsy, dystonia, spasticity, muscle weakness, and so on) but no malformations and (c) nonspecific conditions (MRX), where MR is the only consistent clinical manifestation among the affected individuals. The importance of this classification is represented by its practical value. The list of genes that have been implicated in non-syndromic and syndromic forms of XLMR continues to grow. In the last update of the on-line XLMR database (http://xmr.interfree.it/home.htm) 215 XLMR conditions are reported subdivided by clinical presentation and according to their mapping status (97 mapped and 82 cloned genes). (Tab. 1)

<table>
<thead>
<tr>
<th></th>
<th>Total count</th>
<th>Mapped</th>
<th>Cloned</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syndromes</td>
<td>98</td>
<td>31</td>
<td>38</td>
</tr>
<tr>
<td>Neuromuscular</td>
<td>51</td>
<td>16</td>
<td>28</td>
</tr>
<tr>
<td>Nonspecific/MRX</td>
<td>66</td>
<td>50</td>
<td>16</td>
</tr>
<tr>
<td>Total conditions</td>
<td>215</td>
<td>97</td>
<td>82</td>
</tr>
</tbody>
</table>

Table 1: Count of XLMR conditions by clinical presentation: syndromes, neuromuscular conditions and nonspecific/MRX conditions (Chiurazzi et al., 2008)
In MRX patients have no biochemical abnormalities or evident phenotypic manifestations other than MR to distinguish them from unaffected males in the family \(^7\). The absence of distinctive features arranges the linkage analysis an objective method for genetic classification but only in familiar cases with multiple occurrences of MRX. Hence, MRX loci localized to non-overlapping regions were designed by the “MRX” acronym and a progressive number as MRX1, MRX2, MRX3 and so on \(^6\). In 1988 Suthers et al. mapped the first MRX family (MRX1) \(^33\). During the years other families have been identified and mapped to defined regions of X chromosome. For the first families, MRX symbols were assigned on the basis of mapping to overlapping intervals, that is different families mapping to the same region were assigned the same MRX number. However, it was soon evident that this method was not working well for different reasons. The naming was not coordinated and different groups accidentally assigned the same MRX symbol to different MRX loci. It was possible that a new family could have mapped to a region overlapping with more than one established MRX locus. Furthermore, new markers became available and the localizations for existing MRX loci were being refined. In 1992, in order to overcome these problems, new nomenclature guidelines were introduced. It was established that each family for which a linkage interval could be determined (LOD score value \(> 2\)) should be considered as a distinct entity irrespective of the possible overlapping with other MRX loci \(^7\). At present there are 92 distinct MRX loci mapped on the X chromosome (http://www.ggc.org/xlmr.htm) (Fig. 2)
Figure 2: MRX genes. (Modified from "Greenwood Genetic Center XLMR Update").
For long time, the identification of new MRX genes seemed to be an almost impossible task, given the extensive genetic heterogeneity of this condition. However, in spite of this difficulty, the progress in genome analysis and the establishment of large collaborations between clinical and molecular research teams have led to great progress since the identification of FMR2 in 1996. Until now 34 MRX genes have been identified (http://www.ggc.org/xlmr.htm). (Fig. 2).

Syndromic X-linked mental retardation (MRXS) is characterized by MR and abnormalities like growth alteration, distinctive craniofacial appearance, neuromuscular findings, behavioural abnormalities, or metabolic disturbances. Up to now 70 MRXS genes have been identified (http://www.ggc.org/xlmr.htm) (Fig. 3).
Figure 3: MRXS Genes. (Modified from "Greenwood Genetic Center XLMR Update").
Among MRXS, Fragile X syndrome (FRAXA) (MIM 309550) is probably the most common XLMR condition and the best studied. This condition has an estimated prevalence of 1 in 4000-6000 in men and 1 in 8000-10000 in women. The overall prevalence is 1.5 per 10000 individual. It accounts for 15-25% of all patients with XLMR. Patients show mental retardation and specific physical (long face, large, prominent ears, a high arched palate, flat feet, and macroorchidism) and behavioral anomalies (hyperactivity, avoidance of eye contact, and repetitive speech as well as autistic features). At molecular level the disorder is due to a dynamic mutation caused by expansion of a CGG repeat located in the promoter region at 5′ end of FMR1 gene. The expansion of the repeat above 200 CGG results hypermethylation of the promoter region, aberrant heterochromatinization and silencing of FMR1 gene resulting in the absence of the gene product (FMRP) and this in FRAXA.

However, it should be noted that several genes, such as MECP2, ATRX, SLC6A8, RSK2, OPHN1, ARX, PQBP1, MTC8, AP1S2, FGDY and JARIDIC (Tab. 2), which were initially identified as causative genes for MRXS, have been found mutated in patients with isolated mental retardation. These data suggest that mutation type or other factors, such as genetic background, might modulate the phenotype resulting from an alteration in one of these genes. We could assert that the original distinction between MRX and MRXS is not so straightforward as initially believed. However, the terms MRX and MRXS could be still useful in a clinical practice, providing a first phenotypic classification of the family, before performing molecular analysis.
<table>
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<tr>
<th>OMIM</th>
<th>Gene</th>
<th>Locus</th>
<th>Condition</th>
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<tr>
<td>300005</td>
<td>MECP2</td>
<td>Xq28</td>
<td>Rett</td>
</tr>
<tr>
<td>300032</td>
<td>ATRX (XNP)</td>
<td>Xq13.2</td>
<td>ATR-X</td>
</tr>
<tr>
<td>300036</td>
<td>SLC6A8</td>
<td>Xq28</td>
<td>Creatine transporter deficiency</td>
</tr>
<tr>
<td>300076</td>
<td>RSK2</td>
<td>Xp22.1</td>
<td>Coffin-Lowry</td>
</tr>
<tr>
<td></td>
<td>(RPS6KA3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>300127</td>
<td>OPHN1</td>
<td>Xq12</td>
<td>Cerebellar ataxia</td>
</tr>
<tr>
<td>300382</td>
<td>ARX</td>
<td>Xp22.1</td>
<td>West, infantile spasm</td>
</tr>
<tr>
<td>300463</td>
<td>PQBP1</td>
<td>Xp11.23</td>
<td>Sutherland-Haan/MRXS3</td>
</tr>
<tr>
<td>300823</td>
<td>SLC16A2/</td>
<td>Xq13.2</td>
<td>Allan-Herndon-Dudley</td>
</tr>
<tr>
<td></td>
<td>MCTB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>300630</td>
<td>AP1S2</td>
<td>Xp22.2</td>
<td>Turner</td>
</tr>
<tr>
<td>306400</td>
<td>PGBD</td>
<td>Xp11.22</td>
<td>Aarskog-Scott</td>
</tr>
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<td>314690</td>
<td>JARID1C/</td>
<td>Xp11.22</td>
<td>JARID1C-related XLMR</td>
</tr>
<tr>
<td></td>
<td>SMCX</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.2 Known XLMR genes

The XLMR gene products can be found in all cell compartments: 30% in the nucleus, 28% in the cytoplasm, 16% in the organelles and 22% are either membrane bound or secreted (Fig. 4a) \(^{32}\). Gene Ontology analysis revealed that these proteins have different functions inside the cell. Some of them are related to signal transduction (19%) and regulation of transcription (22%). Furthermore, a large number of XLMR proteins is involved in other biological pathways: metabolism (15%), DNA and RNA processing (6%), protein synthesis (3%), regulation of cell cycle and ubiquitin pathways (7%) (Fig. 4b) \(^{32}\). These fundamental processes may disproportionately affect cognition because of a brain-specific expression pattern of the corresponding genes. It is important to note that XLMR genes may be expressed in the brain but not in neurons; for example, PLP1 encodes for the proteolipid protein 1, a major component of myelin that is expressed exclusively in oligodendrocytes, that is, in the supporting cells belonging to the glia \(^{42}\).
Figure 4: Pie charts illustrating (a) subcellular localization and (b) the molecular function of proteins encoded by the 82 known XLMR genes, according to the available Gene Ontology annotations (www.geneontology.org). (Modified from Chiurazzi et al., 2007)

For all XLMR genes identified, to date knowledge of the pathogenesis is very limited. However, common pathways have been identified for some of them. In particular, three pathways appear to be targeted from mutations in multiple MRX genes: regulation of actin
cytoskeleton through Rho-GTPases signaling; synaptic vesicle transport; chromatin remodeling and gene expression regulation.

1-Regulation of actin cytoskeleton through Rho-GTPases signaling

The neuron contains a specialized structural network named cytoskeleton, composed of three types of filamentous structures: actin microfilaments, microtubules and neurofilaments. The actin cytoskeleton of neurons is essential for morphologic differentiation, including development of specialized dendritic morphology, neurite outgrowth, establishment of cell polarity, synapse formation, synaptic plasticity and protein transport. In addition, for the correct functioning of fully differentiated neurons, it is essential that the actin cytoskeleton remains modifiable to allow dendritic plasticity, a process which is considered essential for learning, memory and cognition. The members of Rho family of GTPases are small GTP-binding proteins which have a key role in signalling pathways controlling the organization of actin cytoskeleton; in the neuronal cells they are known to regulate growth cone morphology as well as growth cone guidance. Rho proteins cycle between an active form (when complexed to GTP) and an inactive form (when complexed to GDP). Their activity is directly regulated by two main classes of proteins: Guanine-nucleotide exchange factors (GEF), which stimulate GDP release and GTP uptake, thereby switching GTPases 'on'; and GTPase activating proteins (GAP) which stimulate GTPase activity thus favoring the conversion from the active GTP-bound to the inactive GDP-bound state, switching GTPases 'off'. Once activated, Rho GTPases activate downstream effectors, such as PAK proteins, which mediate their effect on actin cytoskeleton reorganization. The discovery of XLMR genes that encode for proteins involved in Rho GTPases signalling
suggests that MR result from altered development or plasticity of neuronal networks \(^{43}\) (Tab. 3).

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Protein name</th>
<th>Potential protein function</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>OPHN1</td>
<td>OPHN1</td>
<td>Rho-(G)TPase-activating protein for RhoA, Rac1 and cdc42</td>
<td>Billuart P., 1998</td>
</tr>
<tr>
<td>PAK3</td>
<td>PAK3</td>
<td>serine-threonine protein kinase downstream of Rac1 and cdc42</td>
<td>Allen K., 1998</td>
</tr>
<tr>
<td>ARHGEF6</td>
<td>(\alpha)-Pix</td>
<td>Guanine-nucleotide exchange factor protein for Rac and cdc42</td>
<td>Kurtsche H., 2000</td>
</tr>
<tr>
<td>TM4SF2</td>
<td>TM4SF2</td>
<td>Membrane protein interacting with integrins</td>
<td>Zemni R., 2000</td>
</tr>
<tr>
<td>FMR1</td>
<td>FMRP</td>
<td>RNA-binding protein; regulator of gene expression at the post-transcriptional level</td>
<td>Rousseau F., 1994</td>
</tr>
<tr>
<td>FGD1</td>
<td>FGD1</td>
<td>RhoGEF: possible role in stimulation of neurite outgrowth</td>
<td>Label R.R., 2002</td>
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</table>

In agreement with this hypothesis, alterations in dendritic spines have been observed in mouse and cellular models of various genes (PAK3, ARHGEF6, FMR1) \(^{48,49}\). An additional member of this group is ACSL4 gene. ACSL4, also known as FACL4 (long-chain fatty acyl-CoA ligase 4), has been found mutated by our group in three MRX families \(^{50,51}\). ACSL4 protein adds Coenzyme A to long chain fatty acids, with high preference for arachidonic acid \(^{52,53}\). The exact function of ACSL4 in brain is presently unknown. However we have recently shown that neurons lacking Acsl4 present considerably less spines and an increased percentage of filopodia which are generally considered as precursors of dendritic spines \(^{54}\). Since has been reported that arachinoid acid, ACSL4 substrate, is involved in the
regulation of actin cytoskeleton, it is not unlikely that also the absence of Acsl4 might result in alterations of actin dynamics, as consequence of increased levels of free arachidonic acid 55.

The observed spine anomalies might thus be a secondary effect of an abnormal actin organization due to ACsL4 absence.

2- Synaptic vesicle transport

A second pathway potentially altered in MRX patients is that of synaptic vesicle transport and recycling regulating neurotransmitter release. The transfer of information in the nervous system, which is essential for cognitive functions, depends on intercellular communication through synapses. The synaptic vesicles are located at the pre-synaptic terminal. After membrane depolarization, synaptic vesicles fuse with the pre-synaptic membrane and release neurotransmitters in the synaptic cleft. The vesicles are then recycled and filled again with neurotransmitter molecules. The correct regulation of this process is essential for correct synapse functionality and thus normal brain functioning. This process is regulated by another family of GTPases, Rab proteins 56. These proteins cycle between an active GTP-bound form inserted in vesicle membrane, and an inactive GDP-bound cytoplasmic form. Their activity is tightly regulated by GTPases-activating proteins (GAPs) and GDP dissociation inhibitors (GDI) which are required to retrieve the GDP-bound form of Rab proteins from the membrane and to make them available as a soluble cytoplasmic reservoir for subsequent re-use 57.

Among MRX genes, six participate in this process, suggesting that MR in these patients could be a consequence of an alteration of synaptic vesicle cycling process resulting in altered synaptic function (Tab. 4)
Gene expression is an important process that can be altered in MRX patients. Selective expression of certain genes and inhibition of others is necessary for the correct differentiation and functioning of all cell types, including neurons. Gene expression can be regulated either by the modulation of chromatin structure or by the regulation of activity of the molecules involved in the different steps of the process which lead from the gene to the mature functional protein. Inactive genomic regions are usually characterized by a condensed chromatin structure enriched in hypoacetylated forms of H3 and H4 histones. In addition, the promoters of inactive genes are usually hypermethylated, while expressed genes have an

<table>
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<th>Gene symbol</th>
<th>Protein name</th>
<th>Potential protein function</th>
<th>Reference</th>
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<tr>
<td>GDI1</td>
<td>α-GDI</td>
<td>GDP-dissociation inhibitor for Rab proteins</td>
<td>D'Adamo P, 1998</td>
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<td>IL1RAPL1</td>
<td>ILRAPL</td>
<td>Unknown; involved in stimulated neurotransmitter exocytosis?</td>
<td>Carrie A, 1999</td>
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<td>NLGN4</td>
<td>HNLX</td>
<td>Postsynaptic membrane protein; involved in induction of presynaptic structures; linked to NMDA-type-glutamatergic receptors</td>
<td>Laumonnier P, 2004</td>
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<tr>
<td>DLG3</td>
<td>SAP102</td>
<td>Postsynaptic scaffolding protein linked to NMDA-type-glutamatergic receptors</td>
<td>Tarpey P, 2004</td>
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<td>AP1S2</td>
<td>AP1S2</td>
<td>Adaptin protein in the assembly of endocytic vesicles</td>
<td>Tarpey P, 2006</td>
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<tr>
<td>SLC6A8</td>
<td>SC6A8</td>
<td>Neurotransmitter transporter</td>
<td>Salomons G, 2001</td>
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</table>

3-Chromatin remodelling and gene expression regulation

Gene expression is an important process that can be altered in MRX patients. Selective expression of certain genes and inhibition of others is necessary for the correct differentiation and functioning of all cell types, including neurons. Gene expression can be regulated either by the modulation of chromatin structure or by the regulation of activity of the molecules involved in the different steps of the process which lead from the gene to the mature functional protein. Inactive genomic regions are usually characterized by a condensed chromatin structure enriched in hypoacetylated forms of H3 and H4 histones. In addition, the promoters of inactive genes are usually hypermethylated, while expressed genes have an
open chromatin structure with unmethylated promoters. Hypoacetylation and hypermethylation can be stably maintained through mitosis and can thus be passed on to daughter cells.

Mutations in genes belonging to this pathway genes suggest that MR could arise from an alteration of epigenetic mechanisms regulating gene expression and silencing (Tab. 5). A member of this group is MECP2 (Methyl-CpG-binding Protein 2) gene which is responsible of Rett syndrome (see below for the description).

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Protein name</th>
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<td>RP56K13</td>
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<td>ATP-dependent DNA-helicase</td>
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<td>MECP2</td>
<td>MeCP2</td>
<td>Methyl CpG binding protein</td>
<td>Couvert P, 2001</td>
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<td>Role in chromatin remodelling</td>
<td>Jensen LR, 2006</td>
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<td>Zinc-finger protein; transcriptional repressor?</td>
<td>Sheichet S, 2003</td>
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<td>Kleefstra T, 2004</td>
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<td>ARX</td>
<td>ARX</td>
<td>Transcription factor?</td>
<td>Stromme P, 2002</td>
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<td>FMR2</td>
<td>FMR2P</td>
<td>Transcription factor</td>
<td>Gecz J, 1996</td>
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<td>PQQBP1</td>
<td>PQQBP1</td>
<td>Poly-glutamine Binding Protein 1; interacts with components of the spliceosome; transcriptional repressor</td>
<td>Kalscheuer V, 2003</td>
</tr>
<tr>
<td>FTSJ1</td>
<td>FTSJ1</td>
<td>RNA methyltransferase</td>
<td>Freude K, 2004</td>
</tr>
<tr>
<td>CDKL5</td>
<td>CDKL5</td>
<td>Serine-Threonine kinase</td>
<td>Kalscheuer, 2003</td>
</tr>
</tbody>
</table>
**Orphan mechanisms**

Little is known about the function of the other MRX genes, for which no common pathways have been identified so far. *SLC16A2* encodes for a creatine transporter belonging to a protein superfamily of transporters responsible for a neurotransmitter uptake \(^5\). Thus mutations in *SLC16A2* might result in an alteration in neurotransmitter release, as for *SLC6A8*. *AGTR2* encodes for AT2, a seven transmembrane domain G-protein-coupled type 2 receptor for angiotensin II \(^5\). Its function in brain is unknown. It seems to be involved in several functions in neurons, including ionic fluxes, cell differentiation, and axonal regeneration suggesting that an alteration in one of these pathways following *AGTR2* absence might lead to mental retardation \(^60\text{–}^63\) .

| **Table 6.** MRX genes involved in orphan mechanisms |
|-------------------------------|----------------------------------|---------------------------------------------|
| **Gene symbol**               | **Protein name** | **Potential protein function** | **Reference** |
| *SLC16A2*                     | MCT8              | Creatine transporter             | Friesema EC, 2004 |
| *AGTR2*                       | AT2               | Receptor for angiotensin II      | Vervoort V, 2002  |
1.3 Rett syndrome

Rett syndrome (RTT, OMIM#312750) is a progressive neurodevelopmental disorder affecting almost exclusively girls with an incidence of 1:10000 female births. It represents the second genetic cause of severe mental retardation in females. The early observation that the syndrome occurred exclusively in females suggested an X-linked dominant inheritance with possible male lethality. Since RTT familial cases are extremely rare, it took years of research to find data supporting this genetic model. Typically, RTT is characterized by a period of nearly normal development followed by regression with loss of social motor, and communication skills, combined with the occurrence of specific features, including hand stereotypies, microcephaly, autonomic disturbances or epilepsy (Fig. 5).
Figure 5: Onset and Progression of RTT Clinical Phenotypes After a period of normal development, a healthy-looking baby girl falls into developmental stagnation, followed by rapid deterioration, loss of acquired speech, and the replacement of purposeful use of the hands with incessant stereotypies, a characteristic of the syndrome. Patients also develop social behaviour abnormalities and are often misdiagnosed as having autism. The condition worsens with loss of motor skills and profound cognitive impairment. In addition, patients suffer from anxiety, seizures, and a host of autonomic abnormalities (Chahrour & Zoghbi, 2007).

According to the criteria of Hagberg, classical and atypical RTT cases could be distinguished. Atypical or variant RTT can be divided into several subgroups. These variants have some, but not all diagnostic features of RTT and can be milder or more severe. They include: i) the infantile seizure onset variant, with seizure onset before regression; ii) "forme fruste" with a milder and incomplete clinical course; iii) the congenital variant, lacking the normal perinatal period; iv) the late regression variant, which is rare and still controversial; v) the Zappella variant (previously known as the preserved speech variant, PSV), in which girls recover the ability to speak.
in single words or third person phrases and display an improvement of purposeful hand movements. Furthermore, in our Italian cohort of patients, we have described a “highly functioning PSV” associated with acquisition of more complex language function including use of first person phrases \(^68\). In this variant, girls acquire a better control of their hands and they are able to draw figures and write simple words. The degree of mental retardation in these girls is also milder than in Z-RTT and their IQ can be as high as 50.

Since more than 99% of RTT cases are sporadic, it was very hard to map the disease locus by traditional linkage analysis. Using information from rare familial cases, exclusion mapping identified the Xq28 candidate region, and subsequent screening of candidate genes in RTT patients revealed mutations in MECP2 (Methyl-CpG-binding Protein 2) gene \(^69\). MECP2 mutations account for approximately 95% of cases with classic RTT and for a lower percentage (20-40%) of variant patients \(^64\).

A large degree of phenotypic variability has been observed in individuals with MECP2 mutations. In 2000, we described a family in which a MECP2 mutation segregated in male patients with recessive X-linked mental retardation (XLMR) and spasticity \(^70\). Other studies identified MECP2 mutations in males with nonspecific mental retardation, males with severe neonatal encephalopathy, males with language disorder and schizophrenia, males with X-linked syndrome of psychosis, pyramidal signs, and macro-orchidism (PPM-X), Angelman syndrome and infantile autism \(^71-81\).

The spectrum of mutation types includes missense, nonsense, and frameshift mutations, with over 300 unique pathogenic nucleotide changes described (http://mecp2.chw.edu.au/; http://www.biobank.unisi.it) \(^82,83\), as well as deletions encompassing whole exons \(^84-86\). Eight missense and nonsense mutations account for 70% of all mutations, while small C-terminal
deletions account for another 10%, and complex rearrangements constitute 6%. Furthermore, duplications spanning the MECP2 locus have been found in several patients with severe mental retardation and progressive spasticity and in members of three families with non specific mental retardation \(^{87,88}\).

**MECP2** gene (OMIM #300005) encodes for a broadly expressed nuclear protein that was originally characterized as a transcriptional repressor \(^{89}\). MeCP2 is a member of the methyl-CpG binding protein family has two conserved functional domains, the methyl-CpG binding domain (MBD) and the transcription repression domain (TRD) \(^{90}\). MeCP2 binds to methylated DNA through the MBD and effects gene silencing by imparting changes in chromatin structure via the interaction with the corepressor Sin3A and the histone deacetylase complex \(^{91}\). MeCP2 also associates with the corepressors c-Ski and N-CoR via the TRD and forms complexes with these repressors independently from its interaction with Sin3A \(^{92}\). In addition, MeCP2 inhibits transcription directly at the level of the preinitiation complex through the interaction of the TRD domain with the transcription factor IIB \(^{93}\). MeCP2 also contains a nuclear localization signal (NLS) within the TRD domain, necessary for transport of the protein into the nucleus \(^{94}\), and a C-terminal domain, which may affect the stability and function of the entire protein \(^{95}\). MeCP2 also interacts with the RNA-binding protein Y box-binding protein 1 (YB1) to regulate splicing of reporter constructs \(^{96}\). In order to identify new neuronal target of epigenetic regulation of MeCP2 “ChIP on chip” analysis was performed \(^{97}\). These studies reveal several important new findings of high significance to understanding MeCP2 function and its relation to Rett syndrome. Unexpectedly, they showed that the majority of MeCP2 bound promoters are on active genes. Therefore, MeCP2 promoter occupancy is not consistent with only gene silencing. Furthermore, these analysis show that
the majority of promoters with the highest methylation levels are not bound by MeCP2 and a large number of MeCP2 binding sites are found outside of transcription units and CpG islands. These data suggest to consider MeCP2 as a “transcriptional regulator” instead on a “transcriptional repressor”.

The apparent lack of MECP2 mutations in a proportion of clinically well defined RTT cases suggests the existence of additional RTT loci supporting the idea of genetic heterogeneity. In 2005, mutations in CDKL5 were identified in patients with the onset early seizure variant of RTT syndrome 98-104. CDKL5 (OMIM #300203; also known as serine threonine kinase 9 (STK9)) is located in Xp22 encodes for a product belongs to the serine-threonine kinase family, which shares homology with members of the mitogen-activated protein and cyclin dependent kinase (CDK) families 105. CDKL5 is a large protein of 1030 amino acids with an estimated molecular weight of 116 kDa containing a conserved serine-threonine kinase domain within its N-terminus and a large C-terminal region 105. It is a ubiquitous protein mainly expressed in the brain, testes and thymus 106, whose expression varies in different brain areas and during development 107. Cdkl5 shuttles between nucleus and cytoplasm 107. Its subcellular distribution seems to be modulated by its C terminal tail, which is responsible for an active nuclear export mechanism 107. Moreover this big tail seems to act as a negative regulator of the catalytic activity of CDKL5 106 and probably this function is enclosed in the last 240 residues 108.

The observation that mutations in MECP2 and CDKL5 cause similar phenotypes suggested that these genes may be involved in the same molecular pathway. To verify this hypothesis, previous study compared the expression patterns of Mecp2 and Cdkl5 in embryonic and postnatal mouse brains dimostrating that the two genes have an overlapping temporal and
spatial expression profile during neuronal maturation and synaptogenesis. Furthermore, the two proteins physically interact and that CDKL5 is indeed a kinase able to phosphorylate itself and to mediate MeCP2 phosphorylation (Fig. 6).

Figure 6: Interaction between CDKL5-MeCP2. A region of MeCP2 including the last residues of TRD and residues belonging to the C-terminal domain represents the main interacting surface (red circle). CDKL5 is a kinase able to phosphorylate itself and mediates the phosphorylation of MeCP2. The green circles represent the phosphate groups.
1.4 Autosomal MR

With the assumption that 8-10% of moderate-to-severe forms of MR are X linked, the majority of the gene defects underlying MR must be autosomal. Severe dominant forms of MR are rarely familial because affected individuals do not reproduce. Genes causing autosomal dominant familial non-syndromic MR have not been reported, although several genes assumed to be involved in autosomal dominant non-syndromic MR have been identified by mapping of the chromosomal breakpoints in patients with balanced chromosomal aberrations 109. Functional considerations and epidemiological data suggest that the majority of the gene defects that give rise to the disease will be inherited as recessive traits. The autosomal recessive forms may be considered common and they may be due to mutations in many hundred if not thousand different genes 110. By analogy with XLMR, non-syndromic forms of autosomal MR are supposed to be more common than syndromic forms 111.

In contrast to the significant progress in XLMR research, there is still a gap in the knowledge about the genetic basis of autosomal non-syndromic MR. Only five genes causing autosomal recessive nonsyndromic MR have been identified: PRSS12 on chromosome 4q26, CRBN on 3p26, CC2D1A on 19p13, GRIK2 on 6q16 and TUSC3 on 8p22 112-116. Mutations in PRSS12, CRBN and CC2D1A genes cause a similar degree of severity of MR in all the affected members of the same family, on the contrary mutations in TUSC3 can cause MR varying from mild to severe among different members of the same family. Interestingly, all mutations in these genes identified to date are protein-truncating mutations. It could be hypothesized that milder missense mutations or sequence variants in these and other genes might cause an additive effect in the pathogenesis of mild
cognitive impairment. Although all these molecules are expressed in the brain, their neuronal functions are not yet completely clear.

The classic observation that various forms of mental retardation are associated with abnormalities in the morphology of dendritic spines suggests that disruption of pathways involved in synaptic plasticity may be a common mechanism of this disorder 117. Nearly all synapses on dendritic spines use the excitatory neurotransmitter glutamate to activate N-methyl-D-aspartate (NMDA) receptors which are associated with a large complex of transmembrane receptors, scaffold proteins, and signaling molecules with numerous attachments to actin filaments 118,119. SYNGAP1, a GTPase-activating protein that is selectively expressed in the brain and is a downstream component of the NMDA-receptor complex 120. Mice heterozygous for SYNGAP1 have impaired synaptic plasticity and learning, whereas mice that lack SYNGAP1 die shortly after birth. Hamdan et al. found three de novo protein protein-truncating mutations in 3 of 94 patients with autosomal dominant nonsyndromic mental retardation 121. The disruption of SYNGAP1 appears to be associated with a homogeneous clinical phenotype that is characterized by moderate-to-severe mental retardation accompanied by severe language impairment.

In the only systemic effort to map and identify new genes for non-syndromic autosomal recessive MR, Najmabadi et al 122,123 used DNA array-based SNP typing to perform homozygosity mapping in more than 100 consanguineous Iranian families with at least two intellectual disability children. These studies revealed various novel loci for non-syndromic autosomal recessive MR, indicating extreme heterogeneity of the condition. These results confirmed the idea that mutations in the currently known genes are responsible for only a small percentage of the cases of autosomal recessive non-syndromic MR 124.
2. RATIONALE, AIM and OUTLINE of the study
2. Rationale, aim and outline of the study

MR is characterized by significant limitation in intellectual function and adaptive behaviour arising during childhood. It represents a serious medical and social problem. The high incidence of MR (2-3% in most populations) makes this condition one of the major unsolved problems in modern medicine. Until now more than 300 MR genes have been identified but the field of MR is still huge. The etiologies of MR are diverse and include teratogenic and environmental factors (5-13%), perinatal asphyxia (2-10%) and genetic causes (two-thirds of cases).

During the first 3 years of the Ph.D. course, my research activity has been focused on one relatively common syndromic form of MR: Rett syndrome. Despite the discovery of MECP2 and CDKL5 mutations in classic and atypical RTT cases, a fraction of patients are still without a molecular diagnosis, suggesting the existence of additional loci. During the last ten years, the Medical Genetic Unit of Siena has collected a large cohort of RTT patients, including classic, variant and RTT-like cases. In 2007, this collection has been connected to an on-line database (http://www.biobank.unisi.it) to share data/samples with the scientific communities and accelerate progresses in this research field (Result3.1).

For long time MECP2 has been the only one gene associated with RTT syndrome. In 2004, different groups firstly reported the identifications of CDKL5 mutations in patients with RTT features. One year later, the Medical Genetics Unit of Siena contributed to the clarification of the phenotype associated with CDKL5 mutations, reporting four new cases classified as early seizure variant of RTT. Successively, performing CDKL5 molecular analysis in all RTT patients included in the Italian database, we identified additional five mutated patients that allowed the
detailed characterization of the phenotype and the delineation of specific diagnostic criteria for this RTT variant (Result 3.2).

In order to identify the genetic cause in mutation-negative RTT patients, we employed array-CGH technique that allows the detection of submicroscopic deletions/duplications throughout the entire genome in a single experiment. Actually the size of the alterations that can be identified with this technique varies from few Kb to several Mb. This means a huge resolution in comparison with that of the standard karyotype or conventional CGH (5-10 Mb)\(^{125,126}\). Previous studies has demonstrated that array-CGH represents a powerful disease gene identification strategy\(^ {127-132}\). In order to identify genomic regions containing candidate genes for RTT, eight mutation-negative RTT-like patients were selected from the Italian database and analysed by 105A oligo array-CGH (Agilent Human Genome CGH Microarray Kit 105A). The analysis revealed a “de novo” 14q12 deletion of 3 Mb in a girl with dysmorphic features and a RTT-like clinical course\(^ {133}\). Gene content analysis of the deleted region, revealed the presence of only five genes. Among them, we selected FOXG1 since it encodes for a brain-specific transcriptional repressor. Subsequent mutational analysis in RTT mutation negative patients identified FOXG1 mutations in two patients affected by the congenital variant of RTT (Result 3.3). FOXG1 analysis, extended to an international cohort of patients, allowed the characterization of 4 new FOXG1 mutated cases that significantly contributed the clarification of the clinical phenotype (Result 3.4).

During these years, I have been also involved in the search of new genes responsible for other X-linked MR forms. The Medical Genetic Unit of Siena has collected a large number of MR patients with possible X-linked inheritance, both syndromic and non-syndromic. Given the genetic
heterogeneity of XLMR, in order to facilitate the molecular diagnosis, the Unit of Siena together with 12 laboratories and 27 clinical centers has created an Italian network. Participating members collect patients with detailed clinical information and analyse known XLMR genes (Result 3.5). This network is connected to a bank of biological samples (lymphoblastoid cell lines and DNA) that are stored in the different centers and that are available for research projects. The biobank has a dedicated on-line database available at the address http://www.biobank.unisi.it. This database allows researchers to rapidly search for samples of interests on the basis of specific clinical and/or molecular data (Result 3.5).

In the last year of the Ph.D. course, I spent a six-months research period in the laboratory of Human Genetics, Radboud University in Nijmegen (The Netherlands). This collaboration arises with the aim of identifying new XLMR genes thought the following strategies: i) linkage analysis, using a collection of 16 simple-tandem repeat (STR) markers evenly spaced across the X chromosome; ii) analysis of copy number variations by whole-genome SNP microarray (Result 3.6). By these two approaches, we analysed five patients with at least another affected family member, selected from the XLMR Italian biobank. In all cases, the analysis revealed interested candidate genes (Result 3.6).
3. RESULTS
Result 3.1

Italian Rett database and biobank.


DATABASES

Italian Rett Database and Biobank

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Rett syndrome is the second most common cause of severe mental retardation in females, with an incidence of approximately 1 out of 10,000 live female births. In addition to the classic form, a number of Rett variants have been described. MECP2 gene mutations are responsible for about 90% of classic cases and for a lower percentage of variant cases. Recently, CDKL5 mutations have been identified in the early onset seizures variant and other atypical Rett patients. While the high percentage of MECP2 mutations in classic patients supports the hypothesis of a single disease gene, the low frequency of mutated variant cases suggests genetic heterogeneity. Since 1996, we have performed clinical evaluation and molecular analysis of a large number of Italian Rett patients. The Italian Rett Syndrome (RTT) database has been developed to share data and samples of our RTT collection with the scientific community (www.biobank.unisi.it). This is the first RTT database that has been connected with a biobank. It allows the user to immediately visualize the list of available RTT samples and, using the “Search by” tool, to rapidly select those with specific clinical and molecular features. By contacting bank curators, users can request the samples of interest for their studies. This database encourages collaboration projects with clinicians and researchers from around the world and provides important resources that will help to better define the pathogenic mechanisms underlying Rett syndrome. Hum Mutat 0, 1–7, 2007. © 2006 Wiley-Liss, Inc.

KEY WORDS: RTT; Rett syndrome; MECP2; CDKL5; molecular database; clinical database; biobank; biological samples

INTRODUCTION

Rett Syndrome (RTT; MIM# 312750) is a neurodevelopmental disorder that predominantly affects girls and is the second most common cause, after Down syndrome, of severe mental retardation in females [Hagberg, 1999]. It has an incidence of approximately 1 out of 10,000 live female births [Leonard et al., 1997]. RTT patients show a well-defined clinical course and peculiar characteristics. Clinical criteria for the diagnosis of RTT were defined in the 1980s [Hagberg et al., 1985; Tevisian and Moser, 1988] and recently revised in 2001 to clarify previous ambiguities in interpretation of clinical features [Hagberg, 2002]. In classical RTT girls, birth and early development appear to be normal, although several investigators consider RTT to be a developmental disorder manifesting very soon after birth [Einspieler et al., 2005; Kerr, 1995]. After this apparently normal period, the clinical course is characterized by a stagnation of development followed by regression lasting for several months and usually occurring between 1 and 3 years of age. The fully developed clinical picture is dominated by mental retardation with autistic features, reduction of communication skills, loss of purposeful hand movements combined with hand stereotypes, progressive postural microcephaly, abnormal locomotion, and other various signs such as seizures, breathing abnormalities, and other autonomic dysfunctions. In classic RTT, it is possible to appreciate a variability in disease severity. Furthermore, several RTT variants have been described, including the “preserved speech variant” (PSV), characterized by preservation of some degree of speech [Fukuda et al., 2005; Yamashita et al., 2001; Zappella, 1992; Zappella et al., 1998]; the “congenital variant,” recognized from birth; the “early onset seizures variant” with seizures onset before regression; the “forme fruste,” with a milder, incomplete clinical course (regression between 1 and 3 years); and the “late regression variant.” Patients showing a striking preservation of their abilities in comparison with PSV girls, especially concerning language and hand use levels, have been reported and classified as highly functioning PSV [Zappella et al., 2003]. Up to 50 to 60% of classic RTT and 60 to 80% of RTT variants are caused by mutations in the X-linked MECP2 gene encoding for MeCP2, a transcriptional repressor that plays a critical role in the regulation of gene expression. The Supplementary Material referred to in this article can be accessed at http://www.interscience.wiley.com/pages/1059-7794/suppmat.

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methyl CpG binding protein 2 (MIM* 300059) [Weaving et al., 2005]. Recently some groups [Evan et al., 2005; Marti et al., 2005; Nectoux et al., 2006; Scala et al., 2005; Weaving et al., 2005] identified mutations in the CDKL5 gene encoding for cyclin dependent kinase-like 5 (MIM* 302029) in patients with the diagnosis of the early onset seizures variant and in other phenotypes overlapping with RTT. While the high percentage of MECP2-positive classic RTT patients supports the hypothesis of a single gene causing this phenotype, the low frequency of “solved” RTT variant patients supports genetic heterogeneity in RTT variants, as already demonstrated by CDKL5-mutated cases.

To date, three RTT databases have been developed: one established at the University of Edinburgh and the other two funded by the International Rett Syndrome Association (IRSA). Originally developed as a MECP2 mutation collection, the first database has been successively improved with clinical data to allow genotype-phenotype correlations (www.mecp2.org.uk). In 2001, RettBASE was established to collect both published and unpublished data on MECP2 pathogenic mutations, benign polymorphisms, and sequence variations of uncertain significance from around the world (www.chr.uwa.edu.au/rett/retta) (Fyfe et al., 2001). All these databases are excellent electronic tools that are very useful for genotype-phenotype correlations. However, none of them is connected to a biobank of RTT patients.

Since 1998, the Medical Genetics Unit of the University Hospital of Siena has collected DNA and lymphoblastoid cell lines from a large number of RTT patients. During the last years, the collection of samples has significantly expanded. The rapid enlargement of the samples collection required the establishment of an online database for data management and sharing of resources with the scientific community (www.biobank.unisi.it). At present (September 2006), the site contains 221 entries corresponding to RTT patients included in the bank. For each entry, the site contains clinical and molecular information about each patient and indicates the biological samples available for patients and relatives included in the bank. The database is a useful tool for researchers working on RTT since they can rapidly search for RTT patients with specific clinical and/or molecular features and, by contacting the bank curators, they can request the biological samples for their studies.

RTT Bank

At present (September 2006), the bank contains 631 DNA samples (219 RTT patients and 412 relatives), 49 lymphoblastoid cell lines, and 52 leukocytes in dimethyl sulfoxide (DMSO) medium for a total of 63 probands and 13 lymphoblastoid cell lines and 61 leukocytes of relatives, also in DMSO medium.

Concerning the phenotype, among the total of 233 probands, 126 are classified as classic RTT and 61 as RTT variants, according to the international diagnostic criteria [Hagberg et al., 2002]. Among RTT variant cases, 41 are PSV, three are “highly functioning PSV,” 10 are early onset seizures variants, five are “forme fruste,” and two are congenital variants. Among the remaining 36 patients, 18 have been classified as RTT-like, i.e., cases who do not completely fulfill the international clinical criteria for RTT, and 18 is not determinable (ND), when the very young age of the patient does not allow a definitive clinical classification.

MECP2 mutations have been identified in 113 out of 126 classic cases (mutation detection rate: 90%), in 27 out of 61 variant cases (mutation detection rate: 44%), in 17 out of 18 ND cases, and in five RTT-like patients. CDKL5 mutations have been found in 4 out of 10 early onset seizures variant RTT patients. In the other 57 cases (13 with a classic phenotype, 30 with variants, one ND, and 13 RTT-like patients), MECP2 and/or CDKL5 mutation screening failed to identify any pathogenic change.

EDTA peripheral blood samples are used for DNA extraction using a QIAamp DNA blood kit (Qiagen, Hilden, Germany; www.qiagen.com). After the extraction, the quality of the DNA is tested through a spectrophotometer. For each sample an aliquot containing at least 400µg with an Optical Density (OD) 260/280 ratio of 1.8–2.0 is stored in dedicated boxes at −20°C for long-term conservation. Heparin peripheral blood samples are treated for leukocyte isolation in DMSO medium or establishment of Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines. Four aliquots of transformed cell lines and primary leukocytes are stored at −135°C. Two additional aliquots of cell line of each patient are conserved in a liquid nitrogen dewar to avoid the loss of samples in case of electronic damage. Cells are frozen at passage 2 in 1.5–2 × 10^6 cells/ml aliquots.

All cases contained in the bank come from all over Italy and all have been clinically evaluated by the Medical Genetics Unit (University of Siena). Patients fulfilling the clinical criteria for RTT and its variants and those who have a mutation in either MECP2 or CDKL5 are inserted in the database. MECP2 and CDKL5 mutation analysis is performed by a combination of DHPLC for all coding exons (exons 1–4) and real-time quantitative PCR (qPCR).

To protect the patient’s privacy, when a biological sample arrives it is stripped of personal identifiers and for each sample an internal code is assigned: BETT followed by a sequential number. Anonymity of the samples is assured in each step of the research. A consent form has to be signed by the patient’s guardians in order to insert the sample in the bank, according to international standards.

RTT Database

The RTT database has been available (at www.biobank.unisi.it) since 2004. It is maintained and updated every 3 months on the University of Siena server. The website is written in VBScript and takes advantage of an Internet Information Server (Microsoft IIS; Microsoft, Redmond, WA) with Active Server Pages (ASP) (Microsoft) technology. The website contains an Access (Microsoft) database to manage the data.

In the general homepage of the database, there are links to four independent databases: X-Linked Mental Retardation (XLRM); Rett syndrome; Retinoblastoma; and Other. All these databases are managed by the Medical Genetics Unit (Fig 1). The first two databases are funded through a Telethon grant. By accessing to the RTT section of the bank, users can see the complete list of patients contained in the bank.

The general homepage contains useful links important for site navigation for all the four databases: 1) Guidelines, containing a description of the procedures to follow in order to store and request biological samples (the first option is only possible for the XLRM database); 2) Contact information, for contacting the bank administrator; 3) Services offered from the bank; in particular, isolation of leukocytes from human peripheral blood samples, establishment of EBV-transformed lymphoblastoid cell lines from human peripheral blood leukocytes, DNA extraction, cryopreservation of transformed cell lines and primary leukocytes at −135°C, storage of DNA and plasmids at −20°C, and, finally, distribution of the stored biological samples (the latter is the only service
available for the Rett database); 4) Bank organization, describing
the general organization of the bank and its sections; 5) Forms,
containing the forms users have to complete to take advantage of
bank services and the informed consent.

Database Organization

The database is organized on two levels: a “public” level freely
available to the general public and a “curator” level accessible only
to bank curators through the use of a username and password
and containing personal data of patients and relatives and their
detailed clinical information. All users accessing the main page
of the site (Supplementary Fig. S1; available online at
http://www.interscience.wiley.com/pages/1059-7794/suppmat),
can visualize a table with the following information: 1) Code: a
progressive number that identifies the single families, usually
consisting of the affected girl, the parents, and other relatives
when available. The system assigns a progressive number
automatically every time a new case is inserted, and prevents a
double insertion of the same case. 2) Internal code: the personal
code assigned to each individual (proband, parents, other
relatives) coming to our attention. These codes allow single
individuals to be identified without using the personal data.
Together with the personal code, the relationship within the family
(proband, father, mother, etc.) is visible in this column, so that
users can immediately know the individuals with available
biological samples. 3) Phenotype category: the phenotypic
definition of the proband. Seven phenotypic definitions are
available: classic RTT; FS; which is the most common RTT
variant in our series; high functioning FS; fragile frame;
congenital variant; early onset seizures variant; and Rett-like;
including patients with a suggestive phenotype but who can not be
framed in one of the specific phenotypes reported above. In
addition, for very young patients for whom a definitive clinical
classification is not possible the phenotype is indicated as “ND age
less than 4 years.” 4) Gene name: the name of the mutated gene in
each patient (MECP2 or CDKL5). In those patients for whom the
causative mutation has not been identified, the gene name is
designed as “Unknown.” 5) Mutation type: pathogenic mutations
are classified in four general categories: a) missense mutations, in
which a single amino acid has been substituted with a different
one; b) early truncating mutations, which interrupt the protein,
eliminating part or all of the methyl-CpG-binding domain (MBD)
and/or transcription repression domain (TRD) domain; c) late
truncating mutations, which interrupt the protein in the
C-terminal portion after the TRD domain; and d) gene deletion,
either partial or total. 6) Nucleotide change: the change at
nucleotide level is reported according to the standard nomen-
clature [den Dunnen and Antonarakis, 2001]. 7) Amino acid
change: the change at protein level according to the standard
nomenclature [den Dunnen and Antonarakis, 2001]. 8) Addi-
tional info: an icon in this column links to another page containing
additional information about the family. On this page, bank
curators can visualize all available information about the family,
including personal and clinical data of patients and relatives.
At the moment, external users can visualize only information about
the X-inactivation status of the patient (skewed or partially
skewed, with the cut off being 90% and 70%, respectively) [Sharp
et al., 2002] and the mutation inheritance (de novo mutation,
apparently sporadic, carrier mother, mosaicism in one parent). 9)
Biological sample available: a list of the biological samples available
for each family (lymphoblastoid cell line, leukocytes in DMSO
medium, DNA, fibroblasts). In addition to the type of biological
sample, the internal code of all family members for whom the
sample is available is reported. Clicking on each sample, only bank
curators, through the use of a protected password, can access to
the information regarding the location of the sample.
MECP2 and CDKL5 Mutations and Variants

On the main page, in addition to the list of all inserted patients, there are three links in the left upper part of the page, having to do with mutations and variants of MECP2 and CDKL5 genes (Supplementary Fig. S1). These links are: 1) “List of Mutations”, which provides a table of all identified mutations ordered by their frequency (Supplementary Fig. S2). For each mutation there is the mutation type column, in which the following fields may be present: missense, early truncating, late truncating or gene deletion. The mutations are identified by a systematic name of nucleotide change (GenBank accession number NM_004992 and NM_001037343) and amino acid change (GenBank accession number NP_004983 and NP_0013150). One column reports the number of patients in whom the mutation has been found. Another column describes the phenotype of each patient carrying a specific mutation. The last column is dedicated to references, which allows the user to view the PubMed references of papers on the selected patients. Data in this table is automatically updated every time a new mutated patient is inserted in the database. 2) “Graph of Mutations”, which contains a dynamic graph showing the position of the pathogenic mutations and their relative frequency (Fig 2). On the X-axis of the graph a scaled schematic representation of the protein with the main identified domains is given. On the Y-axis, the frequency expressed as the percentage of mutated patients bearing a specific mutation is given. Mutations are positioned along the protein scheme according to their amino acid numbering and they are represented as vertical columns; the height of the column represents the frequency of the mutation. Moreover, from this page, a link allows access to a pie chart that reports the frequency of the different types of mutations (missense, early truncating, late truncating) expressed as a percentage of mutated patients bearing a specific mutation type. Both graphs are managed by a dedicated program that reads data in the table of mutations and updates the graphs every time a simple is added. At present, MECP2 gross rearrangements are not counted in the graph of mutation and in the pie chart. 3) “Rare variants”, which contains a table listing all nonpathogenic rare variants identified in the patients of the bank (Supplementary Fig. S11). The table includes the following information: a) ID, a progressive number that identifies the single variant; b) nucleotide change; c) amino acid change; d) number of unrelated samples, i.e. the number of unrelated individuals where the variant has been identified; e) samples, i.e. the internal code of all the individuals in which the variant is present; f) reference, which allows the user to view the PubMed references of papers reporting the specific variant.

“Search by” Tool

The website is interactive, with a user-friendly graphical interface. Users that visit the online database can search for a patient by simply looking through the list of registered cases. Alternatively, the database may be explored using the “Search by” tool located in the upper right part of the main page. The search page allows the user to browse the database selecting for specific fields (Fig 3). The patients of interest can be selected by: 1) mutated gene (MECP2 or CDKL5) or absence of mutation (“Unknown” option); 2) mutation type; 3) nucleotide change; 4) amino acid change; 5) phenotype category; and 6) survival, which allows to search alive patients of specific ages or dead patients. Users can choose to search by one single option or to combine two or more options; e.g. they can search all patients with missense mutations or they can search all patients with nonsense mutations in the MECP2 gene and a PSV phenotype. As a result of the search, they will visualize a table containing all the fields present in the general table and listing all patients fulfilling the requested characteristics. This extremely flexible search option allows users to perform an accurate selection of patients so that they can immediately evaluate whether the type of samples they need are present in the bank and can choose the samples to request before contacting the bank curators.

Security and Quality Assurance

The database has been constructed in compliance with the guidelines of the Italian Society of Human Genetics and Telethon Foundation for biobanking [Dugar Britarello et al., 2003]. The database design assures patients’ anonymity, privacy, and confidentiality, according to international criteria [Godard et al., 2003]. Moreover, the informed consent has been written explicitly, covering all aspects of stored samples and personal data management [Godard et al., 2003].

The RTT bank is available to all users who contact bank curators and fill in and sign a specific form asking for biological samples. Biological samples stored in the bank are distributed only to qualified professionals for research purposes only. The specimen cannot be distributed to other investigators without previous written permission of the bank curator. Researchers are asked to include the name of one of our researchers and/or to acknowledge the bank in any paper that includes results obtained using the bank samples/services. Anonymity of the samples is assured in each step of the research. Only the bank curator has access to the whole information content of the database, including personal data.

DISCUSSION

The Italian RTT database has been developed to share data and samples of our RTT biobank with the scientific community. The database allows to immediately visualize RTT samples contained in the biobank and to rapidly select those with specific clinical and molecular features. Contacting bank curators, users can request the samples of interest, following simple procedures indicated on the homepage of the website. In comparison with the already existing RTT databases, this database is the first one that is connected with a biobank. It is important to underline that all patients inserted in the database are evaluated by the same group of clinicians and the molecular analysis is performed by the same laboratory, allowing a uniform clinical and molecular data collection. The database includes both classic and variant RTT patients and cases who do not completely fulfill the international clinical criteria for RTT, but who have clinical features strongly resembling the RTT phenotype (RTT-like patients). Our cohort of MECP2-mutated patients also comprises those patients with a MECP2 gene deletion. Using the “Search by” tool, users can select...
for patients with a MECP2 mutation or a CDKL5 mutation, or for patients in whom the molecular analysis failed to reveal a pathogenic mutation. The well-characterized collection of mutated patients is very important for researchers working on genetic modifiers of the RTT phenotype or on the functional consequences of a specific mutation, while mutation-negative RTT patients are potentially important for the identification of other genes involved in the syndrome.

During the last years, it has become evident the importance of international data to perform accurate genotype-phenotype studies [Leonard et al., 2005; Robertson et al., 2006]. InterRett, linked to RettBASE, has collected data on a large scale sample of...
cases (2,089) representing 28 countries around the world and it is the most powerful tool for researchers working on genotype-phenotype correlations. For this reason, since our database contains a collection of RTT patients limited to the Italian population, we will make all effort to support this international project. However, it is important to also maintain and to improve this database to preserve its unique features. In addition to the already-mentioned characteristics, our database contains data on the X-inactivation status for a great portion of patients; these data are freely available for the general public. This has been possible thanks to the collection of parents’ DNA samples. Since one of the most important factors in phenotype modulation is the X-inactivation status, this information will allow users to perform more accurate genotype-phenotype correlations once the patients’ clinical section is improved (see Future Prospects). In addition, this information is fundamental for researchers who request biobank samples for expression studies.

In a separated section, the database contains tables indicating MECP2 and CDKL5 polymorphisms/rare variants with the code for the patients in whom they have been identified. This information, together with detailed clinical data (see Future Prospects), is important to study if these variants contribute in modulating the phenotype. In fact, the recent demonstration that MECP2 and CDKL5 interact suggests that the association between mutations in one of the two genes and specific variants in the other can modulate the RTT phenotype [Matt et al., 2003].

Given that for rare disorders such as RTT, the Internet provides an important means of communication, this online database encourages collaborative projects with clinicians and researchers from all around the world. This database, providing the opportunity for researchers to take advantage of this collection of clinically and molecularly well-characterized patients, represents an important resource to accelerate the clarification of the molecular basis of RTT.

**FUTURE PROSPECTS**

A detailed clinical characterization of each patient is a fundamental step for inclusion in the database. At present, all clinical details except for the general classification in classic, variants, or RTT-like cases, are available only for bank curators on the "Additional info" page. We are planning to create, for each case, a table with a set of clinical features and a related clinical score according to data in the literature [Churms et al., 2005; Colvin et al., 2003; Huppler et al., 2002; Kerr et al., 2001; Monros et al., 2001]. The new schedule will be freely available to the general public. Users will access this information by clicking on "Additional info", which already exists on the main page. This anechoization will make possible a better definition of the clinical phenotype of each patient. All these clinical features will be added in the "Search by" tool, making it possible for all users to sort cases by particular fields in the clinical data.

Regarding the X-inactivation data, we plan to ascertain the X-inactivation status of all patients included in the database and to display this information on the "Additional info" page. Furthermore, the bank includes RTT patients with chromosomal rearrangements not involving MECP2 or CDKL5 [Debeloc et al., 1999; Pescucci et al., 2003]. The expanding number of these cases will eventually lead to the identification of patients with overlapping rearrangements. These cases will possibly contribute to the identification of new RTT candidate genes. We plan to add the column "chromosomal rearrangements" to the main page of the database in order to allow users to immediately visualize this information and, eventually, to request the samples.
ACKNOWLEDGMENTS

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REFERENCES


**Result 3.2**

Early-onset seizure variant of Rett syndrome: Definition of the clinical diagnostic criteria.


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Early-onset seizure variant of Rett syndrome: Definition of the clinical diagnostic criteria

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Abstract

Background: Rett syndrome is a severe neurodevelopmental disorder affecting almost exclusively female. Among Rett clinical variants, the early-onset seizure variant describes girls with early onset epilepsy and it is caused by mutations in CDKL5. Methods: Four previously reported girls and five new cases with CDKL5 mutation, ranging from 14 months to 13 years, were evaluated by two clinical geneticists, classified using a severity score system based on the evaluation of 22 different clinical signs and compared with 128 classic Rett and 25 Zappella variant MECPS2 mutated patients, evaluated by the same clinical geneticists. Clinical features were compared with previously described CDKL5 mutated patients. Both the statistical and the descriptive approach have been used to delineate clinical diagnostic criteria. Results: All girls present epilepsy with onset varying from 10 days to 3 months. Patients may present different type of seizures both at onset and during the whole course of the disease; multiple seizure types may also occur in the same individual. After treatment with antiepileptic drugs patients may experience a short seizure-free period but epilepsy progressively relapses. Typical stereotypic hand movements severely affecting the ability to grasp are present. Psychomotor development is severely impaired. In the majority of cases head circumference is within the normal range both at birth and at the time of clinical examination. Conclusion: For the practical clinical approach we propose to use six necessary and eight supportive diagnostic criteria. Epilepsy with onset between the first week and 5 months of life, hand stereotypes, as well as severe hypotonia, are included among the necessary criteria.

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Keywords: Rett syndrome; Early-onset seizure variant; CDKL5; Epilepsy; Diagnostic criteria

1. Introduction

Rett syndrome (RTT; MIM 312750) is a severe neurodevelopmental disorder primarily affecting females. RTT has an incidence of 1:10,000 female births.

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RTT syndrome and firstly described the “early-onset seizure variant of RTT syndrome” [4]. Shortly after this report, Goutières and Aicardi described two additional cases with early and intense seizure activity followed by rapid and profound behavioral deterioration [5]. In one patient the seizures were reminiscent of infantile spasms.

Since no MECP2 mutations have been identified in patients with RTT variant phenotype and early onset of epileptic seizures, the molecular mechanism of this variant remained unknown until 2005 [6,7]. CDKL5 (cyclin-dependent kinase like 5) gene (previously known as STK9), located in Xp22, was initially found in an X-autosome translocation that disrupted the gene and was identified in two female patients with infantile spasms and severe mental retardation [8]. The gene was subsequently analyzed in patients with both classic and atypical variants RTT but mutations were identified only in patients with seizure onset before six months of age [6,7].

Here we report a detailed clinical investigation of nine girls with CDKL5 mutations in order to delineate the specific clinical diagnostic criteria. Five are new cases while four girls were previously reported [6,9–11]. Furthermore, we accurately review the clinical features of the other 34 patients with CDKL5 point mutations so far reported in literature [7,12–16].

2. METHODS

2.1. Patients

The five new described patients were evaluated by two different clinical geneticists (F.M. and A.R.). The same geneticists have re-evaluated four previously reported cases with mutations in CDKL5 [6,9–11].

2.2. Clinical score

For each patient a severity score through the evaluation of 22 different clinical signs was applied [2]. Results were compared with the severity score of 128 classic and 25 Zappella variant (Z-RTT) MECP2-mutated patients, all evaluated by the same clinical geneticists [2]. Statistical analysis was performed using median, chi-square test and Fisher exact test.

2.3. Molecular analysis

Genomic DNA was extracted using QIAamp DNA blood maxi kit, according to the manufacturers’ protocol (Qiagen, Hilden Germany). Mutation analysis was performed by Denaturing High Performance Liquid Chromatography (DHPLC) using the Transgenomic WAVE®TM (Transgenomic, San Jose, CA, USA). PCR products were obtained using the conditions described in Scala et al. and denatured at 95 °C, re-annealed at 65 °C for 10 min, and cooled at 4 °C to generate heteroduplexes [6]. The optimal column temperature for fragments analysis was calculated using the WaveMaker Software (Transgenomic, San Jose, CA, USA). PCR products resulting in abnormal DHPLC profiles were purified and sequenced on both strands by using PE Big dye terminator cycle sequencing kit on an ABI Prism 310 genetic analyser (PE Applied Biosystems, Foster City, CA, USA).

3. Results

3.1. Clinical description of the five new cases

Patient 1 (#1567) is the only child of healthy unrelated parents, presently aged 2 years and 7 months. She was born at term, after a normal pregnancy; birth weight was 3800 g (90–97th centile), length 51 cm (50–75th centile), head circumference 35 cm (50th centile) and Apgar index 8 at 1st and 9 at 5th minute. The parents reported feeding difficulties in the neonatal period, associated with poor sucking and regurgitation. At 1 month she developed seizures characterized by eye rolling, head deviation to the right side, tremors of all four limbs and cyanosis, lasting about 30 s to 1 min. EEGs showed spikes and slow waves in the temporal or centro-temporal areas, predominantly on the right side. These episodes occurred several times a day. At the age of 20 months also epileptic spasms appeared. Drug therapy, with valproic acid, phenobarbital and vigabatrin was ineffective. Her psychomotor development was delayed: she sat at 18 months; at present she is not able to walk and has not acquired any language skills. A brain MRI scan, performed at 13 months of age, was normal. Clinical examination at the age of 2 years and 2 months revealed weight 15.5 kg (>97th centile), height 99 cm (>97th centile), head circumference 48 cm (50th centile), strabismus, open mouth with exerted lower lip, generalized hypotonia. She showed hand stereotypes: she clapped her hands and raised them to the mouth. Seizures were characterized by sudden jerk (myoclonic) and episodes of cyanosis, tonic extension of the legs, eyes deviation sideways followed by reaching, two or three per week during sleep and awakening. Moreover episodes of crying and a scared gaze followed by tonic extension of the superior limbs were present two or five times per week, only during sleep. EEG demonstrated slow background activity with diffuse, high voltage, continuous sharp waves. Multifocal spike and sharp wave, predominant in frontal areas and frequent diffuse spike and spike-and-waves discharges more evident during sleep were present. Visual contact and social interaction were poor. She was able to grasp. She presented feeding difficulties with frequent reffusae and constipation and sleep disturbances.

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Patient 2 (#1564) presently aged 2 years and 10 months, is the second child of healthy unrelated parents; she has one healthy older sister. She was born at term, after a normal pregnancy; birth weight was 3200 g (50-75th centile), length 49 cm (50th centile), head circumference 32 cm (10-25th centile) and Apgar index 8 at 1st and 9 at 5th minute. In her perinatal and postnatal period she was drowsy, presented poor sucking and severe reﬂuxes. Her psychomotor development was delayed: she sat at 13 months at the time of our last examination she was able to walk only with support, she had not yet acquired any language. At 3 months of life, during sleep, she developed epilepsy characterized by eyes rolling up and clonic seizures. Seizures occurred daily in sleep and were characterized by bruxism, ﬁxed gaze, stiffening and twitching movements of the lower limbs. EEG demonstrated diffuse theta activity as well as focal and multifocal paroxysmal activity predominant in the left areas during sleep. Brain MRI at 2 years and 3 months was normal. Clinical examination revealed: hypertonic lower limbs, weight 75-90th centile, height 50-75th centile, head circumference 3rd centile, strabismus. She showed bruxism and stereotypic movements of tongue and hands; she raised her hands to the mouth and presented hand washing. Gastroesophageal reﬂux was already severe. Visual contact and manual abilities were poor.

Patient 3 (#1615), 3 years and 4 months old, is the second child of healthy unrelated parents; she has one healthy older brother. She was born at term after a normal pregnancy by cesarean section because of podoic presentation. Birth weight was 3580 g (60th centile), length 50.5 cm (50-75th centile) and head circumference 35.8 (75-90th centile). At 3 months she developed seizures characterized by myoclonic jerks of the right leg. Then she presented infantile spasms and subsequently generalized tonic-clonic seizures. Generalized tonic-clonic seizures occurred once per week while complex partial seizures were more frequent. EEG demonstrated multifocal paroxysmal activity predominant in the posterior areas. Brain MRI at 6 months was normal. Psychomotor development was severely delayed; at the time of our evaluation she was able to sit unsupported but she could not walk. She was able to grasp but she had not acquired any language skills. She presented stereotypic hand movements such as hand-mouth, hand washing and clapping. Visual contact and manual abilities were poor. Gastroesophageal reﬂux, constipation, breathing disorders and cold extremities were not present. Weight was 16.5 kg (75-90th centile), height 102 cm (75-90th centile) and head circumference 51 cm (75-90th centile).

Patient 4 (#132), presently aged 1 years and 2 months, is the second child of healthy unrelated parents; she has one healthy older brother. She was born at 38 weeks of gestation by Cesarean delivery, after a pregnancy complicated by gestational diabetes. Birth weight was 4150 g (75-90th centile), length 53 cm (75-90th centile) and head circumference 38 cm (75-90th centile). There were no perinatal problems. At 40 days of age, at awakening, she presented tonic seizures followed by a series of symmetrical spasms lasting several minutes. In addition, she had brief sporadic tonic-clonic seizures. Seizures rapidly increased up to 20 episodes per day. At the age of 4 months, the EEG background activity was poorly organized, lacking interictal epileptiform activity. She continued to suffer from tonic seizures followed by clusters of spasms. Her psychomotor development was severely delayed and poor; she was not yet able to sit unaided or to handle objects. At clinical examination weight resulted 12 kg (95th centile), height 81 cm (90th centile), head circumference 45.5 cm (25-50th centile). Generalized hypotonia, poor eye contact, inappropriate smile and absence of response to social interactions were also evident. She did not show any purposeful hand use and developed midline stereotypes such as hand wringing and mouthing. Brain MRI, that was normal at the onset of seizures, later revealed progressive cortical and subcortical atrophy.

Patient 5, a 3 year-old girl, is the second child of healthy unrelated parents, born at 37 weeks of gestation, after an uneventful pregnancy and normal delivery. The birth weight was 3200 g (75th centile), the length 52.5 cm (90th centile) and the head circumference 34.3 cm (75-90th centile). Jaundice and breathing distress due to spontaneous pneumothorax complicated the immediate neonatal period. At about 6 weeks of life the parents noted isolated spasm-like movements but the child was brought to medical attention at 8 weeks, after a partial epileptic seizure during sleep. Antiepileptic treatment with Vigabatrin was started with efﬁcacy. At the age of 14 months she developed intractable epilepsy with tonic and myoclonic seizures and absence-like episodes. The interictal EEG showed multifocal, prevalently frontal, epileptic anomalies, such as multifocal spikes and spike-and-wave complexes, with slowing of the background activity and scarce physiological sleep ﬁgures. A type of antiepileptic drug (valproate, ethosuximide, benzodi- azepines, topiramate, hydorcortisone) were given without a satisfactory control of the seizures. Developmental milestones, in the ﬁrst year of age were delayed: social smile was noted at 4 months; complete head control at the age of 6 months; babbling at 8 months; she learnt to roll and to sit unsupported at 12 months. Bruxism and mouthing stereotypes began at about 2 years of age. The developmental progress, after the seizure onset, has been very poor. At the age of 3 years the child still did not stand, even if supported, she did not speak, and she had poor eye contact. Her head circumference was 48.5 cm (25-50th centile). The frequency and semiology of the seizures were unchanged. The EEG was characterized by a markedly slowed activity and by the
persistence of multifocal epileptic anomalies. Several investigations performed in the course of the years have been consistently negative: standard laboratory tests, lactate, ammonia, acylcarnitine, urinary organic acid, plasmatic aminoaides, glucose and lactate in the CSF, visual, auditory and somatosensory evoked potentials, conventional and spectroscopic brain MRI.

3.2. Clinical score

3.2.1. Molecular results

By DHPLC and sequencing analysis we identified 5 de novo CDKL5 mutations. Case 1 bears a truncating mutation, p.Q347X (c.1039C > T) affecting the C-terminal regulatory domain [19,20]. The remaining four cases have missense mutations that lie within the catalytic domain and affect residues highly conserved in different species: p.N71D (c.211A > G), p.V132G (c.396T > G), p.R178W (c.536C > T), p.R203D (c.609C > G) (Fig. 1). The last two aminoacid changes are predicted to affect also the substrate recognition [12].

3.3. Clinical comparison of our 9 CDKL5-mutated cases

This cohort of female patients, ranging from 14 months to 13 years, is a quite homogeneous group with a similar clinical course and comparable degree of severity (Table 1 and Table 2).

3.3.1. Epilepsy

All patients present early epilepsy with an onset between the first week and 5 months of life. The type of seizures at onset appears to be different, while in all the four previously described patients seizures resembling infantile spasms were present at onset of the disease, infantile spasms are reported only in 3 new patients and developed later in life. Seizures episodes were reported at the beginning as short, lasting 10–15 s, but frequency, intensity and duration gradually increased. At onset patients often experienced partial complex seizures and EEGs were normal or minimally abnormal. Background activity was normal, as well. All girls had multiple seizures types and experienced a seizure-free period, usually after the introduction of new antiepileptic drugs, followed by epilepsy relapses after a variable period.

3.3.2. Regression

Our patients do not have a classic regression period, mostly due to the precocious timing of onset of seizures. Although, in all cases the perinatal and postnatal periods were referred as apparently normal, parents notice that the girls were irritable, easy to cry, drowsy and with poorly sucking.

3.3.3. Stereotypes

All patients show stereotypic hand movements; in particular girls continuously raise the hands to the mouth while, less frequently, they manifest hand washing or clapping movements. These stereotypes are present all the time and severely affect the ability to grasp. These movements usually appear in the first year of life and gradually become more evident.

Manual apraxia is more evident when the girls are younger, while the older girls (2,7 years and 3,4 years) began to hold objects such as bottles or glasses. It is worth noting that the psychomotor development is severely impaired in all cases: the older girls can sit unaided but they cannot stand up even if supported, while the youngest girl (1 year and 2 months) has not yet acquired head control.

3.3.4. Head circumference

Interesting the majority of the girls (6/9) have head circumference within the normal range both at birth and at the time of clinical examination. Only one patient shows postnatal microcephaly. The 2 remaining girls present a deceleration of head growth.

3.3.5. Other somatic features

Regarding the other somatic features it is notable that height and weight are above the 50th centile in all
five new cases, while somatic hyperevolution is present in the four previously diagnosed patients. Skeletal or joint alterations such as scoliosis, kyphosis, genu valgus/plana are not appreciable in these girls.

3.4. Clinical and statistical comparison of our 9 CDKL5-mutated cases with Z-RTT and classic patients

In order to better understand the clinical differences between the different RTT variants, we compared the clinical score of the early-onset seizure variant patients with 128 classic and 25 Z-RTT MECP2-mutated patients [2]. In the 9 patients with CDKL5 mutation, the median value is 27, with a total score ranging from 22 to 29. In the 128 classic RTT the median value is 27, with a range from 13 to 39 (personal data). In the Z-RTT the median value is 13, with value ranging from 4 to 24 [2].

Statistical analysis showed that CDKL5 mutated patients have a severity score similar to that of Z-RTT

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### Table 2
Clinical features of the four previously reported patients.

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>Patient 1 Case 1 in Scala et al. 2005</th>
<th>Score</th>
<th>Patient 2 Case 2 in Scala et al. 2005</th>
<th>Score</th>
<th>Patient 3 Case 2 in Buontalenti et al. 2006</th>
<th>Score</th>
<th>Patient 4 Case 2 in Buontalenti et al. 2006</th>
<th>Score</th>
<th>Patient 5 Case 2 in Mari et al. 2005</th>
<th>Score</th>
<th>Patient 6 Case 2 in Grossi et al. 2007</th>
<th>Score</th>
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<td>12y</td>
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<td>c.2645_2646delCT</td>
<td>c.888_889delT; p.T358I</td>
<td>p.R555X/74</td>
<td>p.L879fsX264</td>
<td>c.234delG</td>
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<td>25°</td>
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<td>15° ent</td>
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<td>N.A.</td>
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<td>Constant; hand-mouth, clapping</td>
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<td>Constant; hand-mouth, clapping</td>
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</tr>
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<td>3</td>
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<td>Speech</td>
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<td>5 weeks</td>
<td>5 months</td>
<td>5 months</td>
<td>5 months</td>
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<td></td>
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<td>0</td>
<td>Mild</td>
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<td>Absent</td>
<td>0</td>
<td></td>
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<td>N.D.</td>
<td>2</td>
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<td>N.D.</td>
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<td>Scoliosis</td>
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<td>Kyphosis</td>
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<td>2</td>
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<td>Total score</td>
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<td>29</td>
<td>26</td>
<td>26</td>
<td>22</td>
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<td></td>
<td>22</td>
<td></td>
<td>22</td>
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<td>22</td>
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</tbody>
</table>

OFC: occipitotemporal circumference; GI: gastrointestinal; N.D.: Not detectable; N.A.: Not available.

(p > 0.05) for the following single signs: head, weight, height, hand use, sitting, gastrointestinal disturbances, breathing disorders, cold extremities and sphencter control. The early-onset seizure variant is worse than the Z-RTT for these signs: epilepsy (p = 0.000), stereotopic hand movements (p = 0.001), walking (p = 0.000), age of walk (p = 0.000), speech (p = 0.023), intellectual disability (p = 0.016) and age of regression (p = 0.016). O'Connell and parkinsonian apparatus is less involved in CDKL5 mutated patients than in Z-RTT (p = 0.003, p = 0.018, p = 0.007). The severity score is milder in the early-onset seizure variant than in classic RTT for: head (p = 0.001), cold extremities (p = 0.000), breathing disorders (p = 0.023), p = 0.000, kynurin (p = 0.001), kynurin (p = 0.021). It is worse for epilepsy (p = 0.000), speech (p = 0.025), age of walking (p = 0.043), age of regression (p = 0.006).

### 4. Discussion

In this paper we re-evaluated the phenotype of four previously described patients with mutations in CDKL5 gene and we identified five additional patients. We confirmed that the phenotype associated is clinically recognizable and corresponds to the early-onset seizure variant of RTT. We also compared the features of our group of patients with clinical data of the 34 different patients with CDKL5 point mutations previously described [7,12-18].

In accordance with previously reported cases, all our patients with mutation in CDKL5 gene experienced seizures during their lives [7,12-18].

In literature the only exception is one of the identical twin sisters reported by Weening who remains the most mildly affected patient with a diagnosis of autism. All patients present a drug resistant epilepsy with onset...
between the 1st and the 10th week of life [7,12–18]. The seizure disorder is polymorphic and is mainly characterized by complex partial seizures, infanticile spasms, myoclonic, generalized tonic-clonic seizures and tonic seizures. No unique EEGs pattern was identified, in fact it seems to vary with age and seizures type, as recently described by Babi-Buisson [18].

Concerning the regression stage and given the precarious timing of seizure onset, we point out that a regression period is not appreciable. However, in our patients the perinatal period is not really “normal”. In previously described patients detailed clinical information about the period preceding the onset of epilepsy is not available. Psychomotor development results severely impaired since the first clinical or neurological examinations, usually performed at the epilepsy onset. Severe neurological impairment is reported in all previously described cases: in only six cases is reported the ability to sit, in four cases the ability to stand up unsupported and in two cases the capacity to say a few words. Manual ability is severely impaired in all the affected individuals. In the majority of cases this can be ascribed to the constant presence of stereotypic hand movements, reported in all patients with CDKL5 mutations [7,12–18]. We also observed that the head circumference of the majority of our cases remains in the normal range. In previously reported cases, data about head circumference are not available in all patients, in 18 cases in which this information is reported, 5 present a head circumference within the normal range, while 13 show a deceleration of head growth (4 have postnatal microcephaly). This observation underlines that the deceleration of head growth is not a necessary criteria for this variant of RTT [7,12–18].

Similarly, autonomic dysfunctions does not represent an important sign in patients with CDKL5 mutation. In our patients breathing disorders are occasionally present, distal extremities are normotrophic not cold, gastroesophageal reflux is present only in early infancy and is easily treatable with anti-reflux agents. The presence of autonomic features is rarely described in literature. Evans excluded this characteristic in his patients, while among the 7 reported by Archer only 2 show autonomic dysfunction [7,13]. Brain magnetic resonance imaging is normal in all cases. Non specific alterations are reported in few cases.

It is very interesting that poor eye contact and absence of response to social interactions were evidenced in all patients, in contrast with the diagnostic criteria for variant RTT that suggested intense eye contact and/or eye pointing as a supportive criteria.

The statistical comparison of our 9 CDKL5-mutated cases with classic patients revealed the same median value, though it is interesting to underline the lower range of variation in the CDKL5 mutated patients compared with classic RTT. In classic RTT head circumfer-

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Diagnostic criteria for early-onset seizure variant of RTT.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Necessary criteria</td>
<td>Normal prenatal history</td>
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<tr>
<td></td>
<td>Irritability, drowsiness and poor sucking in the perinatal period before the seizures onset</td>
</tr>
<tr>
<td></td>
<td>Early epilepsy, with an onset between the first week and 5 months</td>
</tr>
<tr>
<td></td>
<td>Hand stereotypes</td>
</tr>
<tr>
<td></td>
<td>Severe impaired psychomotor development</td>
</tr>
<tr>
<td></td>
<td>Severe hypotonia</td>
</tr>
<tr>
<td>Supportive criteria</td>
<td>Infanticile spasm at onset or during the course of epilepsy</td>
</tr>
<tr>
<td></td>
<td>Poor eye contact and absence of response to social interactions</td>
</tr>
<tr>
<td></td>
<td>Absence of speech</td>
</tr>
<tr>
<td></td>
<td>Absence of hand skills</td>
</tr>
<tr>
<td></td>
<td>Absence of scoliosis</td>
</tr>
<tr>
<td></td>
<td>Normal head circumference at birth that remains normal or has a slight deceleration of growth</td>
</tr>
<tr>
<td></td>
<td>Normal weight and height</td>
</tr>
<tr>
<td></td>
<td>Rare neurovegetative dysfunction: gastrointestinal disturbances, breathing irregularities, cold extremities</td>
</tr>
</tbody>
</table>

ence, autonomic system (cold extremities, breathing disorders) and osteoarticular system (pes planus/genu valgus, scoliosis, kyphosis) are more severely involved. On the contrary, the scores of epilepsy, speech, age of walking and age of regression are higher in CDKL5 patients. As expected the median value for total score in Z-RTT is lower than early-onset seizure variant. Also in this cases, osteoarticular apparatus appears to be less involved in CDKL5 mutated patients than in Z-RTT.

Considering the great differences between the classical and the variant RTT forms highlighted through both the statistical and the descriptive approach, we suggest clinical criteria that will be of practical value in favoring the diagnosis of the early-onset seizure variant of RTT (Table 3). First of all we sustain that early epilepsy, with an onset between the first week and 5 months, is the key feature to identify patients likely to have a CDKL5 mutation. In addition, the hallmark of the disease is the presence of stereotypic hand movements typical of RTT, such as hand-mouthing, hand washing or clapping. Additional characteristics include normal prenatal history and a quite normal perinatal period, severe hypotonia, severely impaired psychomotor development and normal somatic growth. Other manifestations of early-onset seizures variant of RTT include poor eye contact and absence of response to social interactions, absence of speech, absence of hand skills and rarer neurovegetative dysfunctions such as gastrointestinal disturbances and breathing irregularities.

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References

Result 3.3

FOXG1 is responsible for the congenital variant of Rett syndrome.


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**FOXG1 Is Responsible for the Congenital Variant of Rett Syndrome**

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Rett syndrome is a severe neurodevelopmental disease caused by mutations in the X-linked gene encoding for the methyl-CpG-binding protein MeCP2. Here, we report the identification of FOXG1-truncating mutations in two patients affected by the congenital variant of Rett syndrome. FOXG1 encodes a brain-specific transcriptional repressor that is essential for early development of the telencephalon. Molecular analysis revealed that Foxg1 might also share common molecular mechanisms with MeCP2 during neuronal development, exhibiting partially overlapping expression domain in postnatal cortex and neuronal subnucleus localization.

In the classic form of Rett syndrome (RTT [MIM: 312700]), females are heterozygous for mutations in the X-linked MECP2 gene (MIM 300005) and the few reported males have an XXY karyotype or MECP2 mutations in a mosaic state. A number of variants have been described including the congenital, the early-onset seizures, and the preserved speech variant. Soon after the discovery of MECP2 as the RTT gene, we demonstrated that the preserved speech variant is allelic to the classic form. More recently, we and others showed that CDRKL5 (MIM 300203) is responsible for atypical RTT, namely the early-onset seizures variant.1,5 The congenital variant was initially described by Rolando in 1985,7,8 In this form, girls are floppy and retarded from the very first months of life. The majority of congenital variants do not bear MECP2 or CDRKL5 mutations,7,8 with only four cases being reported with MECP2 mutations.9–11

Using oligo array CGH, we recently identified a de novo 3 Mb interstitial deletion of chromosome 14q12.1 in a 7-year-old girl.12 She showed dysmorphic features and a Rett-like clinical course, including normal perinatal period, postnatal microcephaly, seizures, and severe mental retardation. The deleted region was gene poor and contained only five genes. Among them, FOXG1 (MIM: 164674) turned out to be a very interesting gene because it encodes a brain-specific transcriptional repressor. We analyzed this gene with a combination of both DHPLC and real-time quantitative PCR in a cohort of 53 MECP2/CDRL5 mutation-negative RTT patients, including seven classic, 21 preserved speech, seven early-onset seizures, one “forme fruste,” two congenital variants and 15 Rett-like cases. For real-time qPCR analysis, we designed primers and TaqMan probe complementary to a segment located in the middle of the single exon of the gene using Primer Express software (Applied Biosystem). Sequences of primers and probe (FAM labeled) are listed in Table S1 available online. We used an RNase P kit as an internal reference (VIC-labeled probe, Applied Biosystems) PCR was carried out as previously described.14 The starting copy number of the unknown samples was determined with the comparative Ct method, as reported by Livak.15 By DHPLC, we identified a different de novo FOXG1 truncating mutation in the two congenital variant patients. Real-time qPCR failed to identify any microdeletion in the 53 patients.

**FOXG1** encodes forkhead box protein G1, Foxg1 (formerly brain factor 1 [BF-1]), a transcriptional factor with expression restricted to fetal and adult brain and testis. Foxg1 interacts with the transcriptional repressor JARD1B and with global transcriptional regulators of the Groucho family. The interaction with these proteins is of functional importance for early brain development.16,17 Like MeCP2, Foxg1 also indirectly associates with the histone deacetylase 1 protein.1,12 Both mutations disrupted the protein at different levels (Figure 1). In case 1, a stop-codon mutation p.W255X (c.766G→A) impaired the DNA binding because of the disruption of the forkhead domain (Figure 1D, left). Case 2 showed a 1 bp deletion c.969delC (p.S323fsX325) causing the loss of JARD1B-interacting domain and the misfolding of the motif responsible for the Groucho binding (Figure 1D, right). Lastly, both FOXG1 mutations affected all the four brain fetal isoforms that lack the last 37 amino acids and have different C-terminal domains.18

The two mutated individuals, aged 22 (case 1) and 7 years (case 2), fulfilled the international criteria for RTT variants.19 Pregnancy, delivery, and auxological parameters at birth were normal. Neurological and behavioral neonatal evaluations were reported as normal, but at three

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months, an abnormal head-circumference growing was noticed in the patients. These patients appeared to weep incoherently, and they did not respond when called and were unable to lift their heads. Case 1 was never able to sit unaided and laid permanently in bed, whereas case 2 was barely able to sit. They were always apathetic and from 1 year of age, they showed peculiar (ery movements of the upper limbs and midline stereotypic activities, typical of Rett syndrome (Figure 2). They never acquired spoken language. Generalized convulsions appeared at 14 years in case 1 and at 2 1/2 years in case 2. Ever since cases 1 and 2 were 3 and 5 years old, respectively, an EEG showed features often found in Rett patients: a multifocal pattern with spikes and sharp waves and occasional paroxysmal activity. In both patients brain MRI showed corpus callosum hypoplasia, a finding which has already been reported in Rett. Currently, they show microcephaly (OFC of 49 cm in case 1, and 47 cm in case 2). They have occasional periods of deep breathing with exaggerated inspirations. Salivation, bruxism, scoliosis, and cold lower extremities as well as apneas are present in both patients who are currently fed by mouth.

These two girls show neurological and neurovegetative symptoms as well as somatic features consistent with a diagnosis of congenital Rett variant. It should only be noted that a retrospective assessment concerning the possible presence of a regression was not feasible. We attempted to compare their phenotype with the four other MEC2-mutated girls described as congenital variants. However, they have been reported with very little detail, thereby hampering a posteriori clinical re-evaluation according to the revised criteria. According to the new criteria, in the classic form, psychomotor development may have been delayed from birth; thus, a re-evaluation of these four patients would have lead to their reclassification as classic form. Alternatively, the disruption of either MeCP2 or FoxG1 may lead to a phenotype, namely the congenital variant, indistinguishable at the level of the clinical and instrumental investigations performed.

A translocation affecting fetal isoforms of FOXG1 was recently described in a 7-year-old girl. She had acquired microcephaly, ataxia, inability to sit and walk, and epilepsy in common with the present cases. Corpus callosum was absent, whereas in our cases, it was hypoplastic. Stereotypic hand activities were not mentioned, and tetraplegia was described. The clinical features of this patient have something in common with a Rett phenotype. The impairment of only fetal FOXG1 isoforms and the possible contribution of genes at the other two breakpoints of the complex rearrangement might explain the phenotypic differences.

The mouse ortholog FoxG1 has a restricted expression domain in the central nervous system coinciding with the emergence of the telencephalic structures of the brain. Its function has been extensively characterized and found to promote telencephalon development by sustaining proliferation of the progenitor pool and preventing

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premature cortical neural differentiation. In agreement, FoxG1 expression is found in the proliferating neuroepithelium starting from early development onward. This expression profile might explain the particular early onset of the neurological symptoms displayed by the patients. Despite its early expression in telencephalon development, in this study we found that FoxG1 expression is detectable in the differentiating cortical compartment in the postnatal stages, although at lower levels with respect to the early embryonic phases (Figure 3A). This expression profile overlaps with the described MeCP2 expression

![Image](https://via.placeholder.com/150)

**Figure 2.** Pictures of the Two Congenital RTT Patients
Case 1 (A156) is shown on the left; case 2 (A106) is shown on the right. They show peculiar jerky movements of the upper limbs frequently pushed in different directions accompanied by continuous flexion-extension, wringing movements of the fingers of the hands. The hands were brought together in hand-washing and hand-mouth stereotypic activities, which were intense and present all the time they were awake. Similar flexion-extension movements of the toes were noticed in the feet. The double scoliosis of case 1 is clearly evident, whereas the other girl maintained a straight vertebral column as often occurs in RTT in the first decade. Teeth grinding was present, and the tongue often protruded out from the mouth.

**Figure 3.** FoxG1 and MeCP2 Expression Domain in Postnatal Cortex and Neuronal Subnuclear Localization
(A and B) Expression analysis by in situ hybridization of FoxG1 and MeCP2 on P8 postnatal forebrain tissue. As shown in (A), FoxG1 expression is found in differentiating and mature cortical neurons in the definitive cortical plate (indicated by arrows in [A]) similar to the MeCP2 expression pattern (indicated by arrows in [B]). In (A'), the inset shows background staining with a sense cRNA for FoxG1 in the same in situ hybridization conditions used for (A) and (B).

![Image](https://via.placeholder.com/150)

1201V (days in vitro) primary hippocampal neurons. In (G), MeCP2 endogenous protein is accumulated in heterochromatic foci (indicated by arrows). As shown in (H), FoxG1–flag exogenous nuclear localization is excluded from heterochromatic puncta (indicated by arrowheads). As shown in (I), MeCP2 and FoxG1–flag co-localize in the nuclear compartment outside the heterochromatic foci. As shown in (J), Nuclear FoxG1–flag localization is detected in a differentiated β-III-tubulin-positive neuron. The following abbreviations are used: cc, cerebral cortex; se, septum; str, striatum; and v, vermis.
domain in cortical tissues, in differentiating and mature neurons (Figures 3A and 3B). FoxG1 homozygote-mutant mice die shortly after birth with severe brain defects.24-26 Unfortunately, the severe compromised development of FoxG1 mutant telencephalon has prevented the analysis of its function in more differentiated neurons. At the single cell level, FoxG1 localizes in the nuclear compartment but is excluded from the MeCP2-positive heterochromatic foci both in nonneural and primary neurons (Figures 3C-3J). These findings suggest that, differently from MeCP2, FoxG1 is not a transcriptional repressor stably associated with heterochromatin. However, both proteins have a large colocalization domain in other nuclear compartments (Figure 3I).

Overall, these data suggest that FoxG1 may exert some additional functions in differentiating and mature neurons, thus sharing similarities with those described for MeCP2. These findings may provide some biological evidence for the development of similar clinical manifestations in disorders affecting the two genes. However, it is also possible that the two transcriptional regulators act on different stages of the process that leads to proper cortical development, from early cell-fate decisions to later circuit connectivity and dendritic development.

FoxG1 shares some interesting analogies with MeCP2 in its molecular functions, raising the question whether the two protein networks may interact in some circumstances and on selective common targets. Future studies will address this intriguing hypothesis. Recently, heterozygous FoxG1+/− mice were found to display subtle defects including a reduction in size of the corpus callosum together with specific patterning defects.25,27 Furthermore, heterozygous FoxG1+/− mice exhibit learning deficits based on fear-condition behavioral tests associated with a loss of postnatal neurogenesis in the hippocampus.27 These mice represent a very interesting animal model for further investigation about how FoxG1 haploinsufficiency may impact on brain development and neuronal maturation and function. In conclusion, we demonstrated that FoxG1 is a previously unrecognized gene responsible for variant Rett syndrome. It is worth noting that in the revised criteria for Rett syndrome the female sex is no longer present as inclusion criteria.19 This seemed to open the door to the discovery of an autosomal gene.

### Supplemental Data

One table listing primers and probes for real-time qPCR is available at [http://www.anhkg.org/](http://www.anhkg.org/).

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### Web Resources

The URLs for data presented herein are as follows:

- **Italian Rett database and biobank**: [http://www.biobank.unisi.it/](http://www.biobank.unisi.it/)

### References

Novel FOXG1 mutations associated with the congenital variant of Rett syndrome.


Novel FOXG1 mutations associated with the congenital variant of Rett syndrome


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Novel \textit{FOXG1} mutations associated with the congenital variant of Rett syndrome.

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ABSTRACT

BACKGROUND: Rett syndrome is a severe neurodevelopmental disorder representing one of the most common genetic causes of mental retardation in girls. The classic form is caused by MECP2 mutations. In two patients affected by the congenital variant of Rett we have recently identified mutations in the FOXL1 gene encoding a brain-specific transcriptional repressor, essential for early development of the telencephalon. METHODS: Sixty MECP2/CDKL5 mutation-negative European Rett patients (classic and variants), 45 patients with encephalopathy with early-onset seizures and 4 atypical Rett patients were analyzed for mutations in FOXL1. RESULTS: Mutations have been identified in 4 patients, independently classified as congenital Rett variants from France, Spain and Latvia. CONCLUSIONS: Clinical data have been compared with the two previously reported patients with mutations in FOXL1. In all cases hypotonia, irresponsiveness and irritability were present in the neonatal period. At birth head circumference was normal while a deceleration of growth was recognised soon afterwards, leading to severe microcephaly. Motor development was severely impaired and voluntary hand use was absent. In contrast with classic Rett, patients showed poor eye contact. Typical stereotypic hand movements with hand-washing and hand-mouthing activities were present continuously. Some patients showed abnormal movements of the tongue and jerky movements of the limbs. Brain MRI showed corpus callosum hypoplasia in most cases, while epilepsy was a variable sign. Scoliosis was present and severe in the older patients. Neurovegetative symptoms typical of Rett were frequently present.
INTRODUCTION

Rett syndrome (RTT) is characterized by a serious and global developmental disorder affecting the central nervous system. First described by Andreas Rett 40 years ago, the syndrome has been the object of extensive investigations, revealing a wide spectrum of clinical phenotypes including the classic form, the early-onset seizure variant, the Zappella variant (Z-RTT), the congenital variant, the "forme fruste" variant and the late regression variant. Mutations in the MECP2 gene, located in Xq28, are responsible for 95% of classic RTT and for 50% of Z-RTT; while the early-onset seizure variant results from mutations in the CDKL5 gene, in Xp22. 3 We have recently used a candidate gene approach to demonstrate that the FOXG1 gene, located in 14q12, is responsible for the congenital variant of Rett syndrome. 4 In this variant, initially described by Rolando in 1986, the affected girls present the same clinical features as in classic RTT but in addition they are floppy and retarded from the very first months of life. 6

Here, we report the identification of FOXG1 mutations in four additional congenital RTT girls, through mutation screening of a cohort of 107 European patients: 60 RTT patients (classic and variants), 43 patients with epileptic encephalopathy and 4 RTT-like patients. Clinical data of these four patients were compared with the two previously reported girls in order to improve the characterization of the phenotype associated with FOXG1 mutations. 5

MATERIALS AND METHODS

Patients and phenotype definitions.

We collected a cohort of 107 European patients (56 patients from France, 49 from Spain and 2 from Latvia) with the following clinical classification: 60 RTT girls (33 classic, 16 congenital, 7 with early onset seizures, 2 late regression, 1 Z-RTT and 1 "forme fruste"), 43 patients with encephalopathy with early onset seizures (40 females and 3 males) and 4 RTT-like patients (1 female and 3 males with microcephaly, hand stereotypes and autistic features). Patients with classic and variant RTT were diagnosed according to the international criteria. 7 RTT-like cases are patients who show some RTT clinical features but who do not fulfill all diagnostic criteria for classic or variant RTT. Phenotypic scores have been calculated using the severity score system previously reported by Renieri et al. 7 All patients have been tested negative for MECP2 and CDKL5 mutations by a combination of DHPLC and MLPA/qPCR analysis.

Molecular analysis.

Blood samples were obtained after informed consent. DNA was extracted from peripheral blood using a QIAamp DNA Blood Kit (Qiagen). To determine the appropriate DNA concentration, we used the OD260/280 method on a photometer. 8 DNA samples were screened for mutations in FOXG1 gene using Transgenomic WAVE Denaturing High Performance Liquid Chromatography (DHPLC). The entire coding portion of FOXG1 was analyzed as previously reported. 3 PCR products resulting in abnormal DHPLC profiles were sequenced on both strands using PCR primers with fluorescent dye terminators on an ABI PRISM 310 genetic analyzer (PE Applied Biosystems, Foster City, CA, USA).

Clinical score.

For each patient a severity score was assigned through the evaluation of 22 different clinical signs. 2 Results were compared with the severity score of 128 classic and 25 Z-RTT patients with mutation in the MECP2 gene and 9 patients with the early onset seizures variant of RTT with a CDKL5 mutation. 2, 9 Statistical analysis was performed using the median test.

RESULTS

Molecular analysis.
A mutation in the FOXG1 gene was identified in four patients clinically classified as congenital RTT. The de novo origin of the mutation was confirmed in three cases (case 2-4), while in one case (case 1) parents were not available. Cases 3 and 4 bear the truncating mutations p.S185fsX454 (c.551_552insC) and p.Y208X (c.624C>G). The remaining two cases have missense mutations that lie within the forkhead domain and affect residues highly conserved in different species (including *Tetraodon nigroviridis* - CAF93027; *Xenopus tropicalis* - NN_001116933; and *Xenopus laevis* - NP_001079165); p.N227K (c.681C>G) in case 1 and p.F215L (c.645T>C) in case 2.

**Clinical description.**

Case 1 (#1091), is a female from Latvia, presently aged 17 years; she is in the fourth stage of RTT with spastic paraparesis (Tab. 1; Fig 1b). Since the age of 16 months she has grown up in an orphanage and her parents are presently unavailable. The girl was her mother’s first child; born after a normal pregnancy; the birth weight was 3900g with Apgar score 8/9. Psychomotor delay was appreciated from the age of 6-7 months. The time of occurrence of the first seizures is unknown. The girl has been examined only once, at the age of 13 years and 2 months.

Case 2 (#RTT00967), is a female from France, presently aged 8 years and 6 months (Tab. 1, Fig. 1c). She is the second child of a non consanguineous couple; another child was born afterwards. She was born at 39 weeks after a normal pregnancy; auxological parameters at birth were normal. In the neonatal period sleep disturbance and severe distress (crying) were noticed. She was referred to a clinical unit at six months because of psychomotor retardation. At this age, examination revealed severe hypotonia with sleep disturbances and no dysmorphic features except strabismus and hypermetropia. A reevaluation at two years showed normal weight and height (height 81 cm, weight 12 kg), while OFC was at -2SD (45 cm), developmental delay, hypotonia, very poor social contact, manual stereotypies and dystonia of the extremities. At six 1/2 years, she could only stand up with support and did not walk; she could hold an object (feeding bottle) with a simple grasp. She showed trunk rocking and tongue chewing. On last examination, at 8 years, the clinical phenotype was unchanged; she was only able to pronounce two disyllabic words (mama, papa) and brain MRI showed microcephaly with abnormal development of the frontal lobes without gyration defect.

Case 3 (#RTT1158), is a female from France, presently aged 3 years (Tab. 1). The patient is the first child of non consanguineous parents from Benin, without a relevant family history. She was born at term and showed normal auxological parameters. She developed severe microcephaly at an early stage. Brain MRI performed in the first months showed isolated ventricular dilatation. Subsequently, she developed severe epileptic encephalopathy. In addition she had a disturbed sleep pattern from two years. When last examined, she was able to hold her head steadily but showed asymmetric spastic tetraplegia and scoliosis. Brain MRI performed at two years showed delayed myelination with hypoplasia/hypomyelination of the corpus callosum.

Case 4 (60719368) is a female from Spain, presently aged 3 years and 2 months (Tab. 1, Fig. 1a). The mother has a normal boy of 9 years and has had 3 spontaneous abortions. At birth she showed very severe hypotonia and normal auxological parameters. Subsequently microcephaly became evident. At 4 months OFC was at -2SD, at 9 months at -3.5, and since the age of 2 years at -5SD. She developed hand stereotypes at 12 months: she used to bring her hands to the mouth and pat her fingers on her lips. Protruding tongue movements have been constant from the age of 4 months.

**Clinical comparison of the six patients with FOXG1 mutations.**

We compared the clinical picture of these four additional patients with a FOXG1 mutation to the two previously reported. These six patients, with age ranging from 3 to 22 years, present a distinct clinical phenotype (Tab. 1).
In order to recognize the clinical differences among the RTT phenotypes, we compared the clinical scores of four patients with mutations in FOXG1 for whom we could obtain complete clinical information (patients 3-6) to the scores of classic and Z-RTT patients with a MECP2 mutations and those of early-onset seizure variant patients with CDKL5 mutations. In patients with a FOXG1 mutation the clinical score median value is 31.5, with total score ranging from 28 to 38. Among the classic RTT cases the median value is 27, with a range from 13 to 39. 2 In the 9 patients with CDKL5 mutation, the median value is 27, with total score ranging from 22 to 30. 3 In the Z-RTT the median value is 13, with values ranging from 4 to 24. 2

**DISCUSSION**

This work confirms that the FOXG1 gene is responsible for the congenital variant of RTT. In fact, FOXG1 mutations have so far been identified only in patients originally classified by different clinical centres as affected by this variant of RTT.

The overall clinical phenotype is characterized by normal pregnancy and delivery, normal auxological parameters at birth followed by hypotonia, irresponsiveness and irritability in the neonatal period. Deceleration of head growth represents one of the most important diagnostic signs: in the four cases for whom the OFC had been recorded in the first months of life, microcephaly was evident already before the fourth month. Apparently microcephaly is more severe than in classic RTT (mean -4.32sd compared with a mean of -2.4 sd in classic RTT). It is also interesting to note that all 5 patients bearing a rearrangement on chromosome 14q12 involving FOXG1 presented with microcephaly; in the first reported case the onset was not defined; 10 in three patients microcephaly was evident between 5 to 8 months; 11, 12, 13 while in the fifth patient head circumference was below the 3rd centile at the age of 11 months. 14

In the two girls previously described with FOXG1 mutations, a retrospective assessment as to whether there had been a period of normal development was not feasible although the parents noted a delay only at 3 months. In patient 3, who presented with the earliest onset, a real regression period was not identifiable, while the other three cases (patients 1, 2 and 4) presented a regression before 6 months of life, more precocious than in classic RTT, in accordance with the diagnosis of the congenital variant. Motor development is severely impaired: no girl with a mutation could either walk with support or speak, although case 2 and case 6 can stand up with assistance (Fig 1c). Voluntary hand use was absent in the majority of patients (3/5) and poor in two (case 2 and case 4). Poor eye contact and absence of response to social interaction were evident in 5 out of 6 patients, in contrast with classic RTT where eye contact is intense and this feature represents a supportive criterion for diagnosis.

Stereotypic hand movements are typical as in classic RTT with hand-washing and hand-clapping activities intense and continuous. In addition, all girls showed constant movements of thrusting of the tongue. These repetitive movements are not so typical of classic RTT, where ineffective chewing movements (seen in one of our patients) are found rather than thrusting of the tongue. Similar tongue protruding movements were present in the girl with the 14q12 deletion, who led to the identification of FOXG1 mutations in the first patients with congenital RTT. 12 It is also interesting to note that in 4/5 patients jerky movements are often seen in the upper limbs, that are frequently pushed in different directions, while in classic RTT such movements are rarely reported.

In this small cohort of patients, epilepsy was a variable sign: two girls, aged three years and six and a half years respectively, have never presented with epileptic seizures (case 2 and case 4); in four patients epilepsy was present with an onset between 14 and 30 months. In two the epilepsy was well controlled by antiepileptic drugs (patients 3 and 6) while, in remaining two, seizures recurred despite therapy.

Neurological and neurovegetative symptoms are consistent with a diagnosis of RTT: constipation is reported in 4/6 patients; breathing abnormalities in 4/6; cold extremities in 3/6; bruxism in 4/6; salivary in 4/6. Skeletal alterations such as scoliosis and kyphosis, genu valga and pes planus are severe in the older cases.
Brain MRI showed corpus callosum hypoplasia in four patients, and this has been excluded in only one of the remaining two cases. Moreover, complete agenesis of the corpus callosum has been identified in patients with chromosomal rearrangements involving FOXG1. All these findings accord with the phenotype of heterozygous Foxg1−/− mice, showing a corpus callosum defect.

Our results contribute to the clarification of the phenotype associated with FOXG1, confirming its role in the RTT spectrum. In particular, they seem to be associated with the most severe end of this spectrum. In fact, the median of the total clinical score in this group of patients is higher in comparison with patients affected by classic or early onset seizure variant of RTT.

It is worth noting that all the patients reported to date with FOXG1 mutations are female. This is probably due to an ascertainment bias and we expect that further mutations will be identified in male cases, given that FOXG1 is an autosomal gene.

In conclusion, we suggest that FOXG1 gene mutation analysis should be performed in female and male patients showing RTT features but lacking the typical early normal period due to the precocious onset of symptoms. Besides other features typical of classic RTT, major signs possibly indicating a FOXG1 mutation are severe psychomotor delay with inability to walk, severe postnatal microcephaly evident before the age of 4 months, poor eye contact, tongue stereotypes and jerky movements of limbs and corpus callosum hypoplasia.

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Competing interest. None

Patient consent. Parental/guardian consent obtained

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| Table 1. Clinical features of patients with FROZI mutations |
|---------------------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|
| **Age/Sex**                      | Patient 1 (1091)   | Score              | Patient 2 (RTT0066) | Score              | Patient 3 (RTT01158) | Score              | Patient 4 (RTT07818) | Score              |
|                                 | 33/23m/Female      |                   | 33/23m/Female       |                   | 33/23m/Female       |                   | 33/23m/Female       |                   |
| **Inheritance**                 | parents not       |                   | parents DNA         |                   | parents DNA         |                   | parents DNA         |                   |
|                                 | available          |                   | requested           |                   | requested           |                   | requested           |                   |
|                                 | de novo            |                   | de novo             |                   | de novo             |                   | de novo             |                   |
| **Mutation**                    | e.681C>G;          |                   | e.681C>G;           |                   | e.651C>G;           |                   | e.675C>G;           |                   |
|                                 | p.32271K           |                   | p.3214               |                   | p.3013               |                   | p.3023               |                   |
| **ORC at birth**                | N.A.               |                   | (28,5 cm)           |                   | (28,5 cm)           |                   | (28,5 cm)           |                   |
| **Weight at birth**             | (3,9 kg)           |                   | (3,9 kg)            |                   | (3,9 kg)            |                   | (3,9 kg)            |                   |
| **Length at birth**             | (49 cm)            |                   | (60 cm)             |                   | (60 cm)             |                   | (60 cm)             |                   |
| **Perinatal signs**             | N.A.               |                   | intermittent        |                   | intermittent        |                   | intermittent        |                   |
|                                 | intractable        |                   | vomiting            |                   | vomiting            |                   | vomiting            |                   |
|                                 | sneezing            |                   | constipation         |                   | constipation         |                   | constipation         |                   |
|                                 | constipation        |                   | constipation         |                   | constipation         |                   | constipation         |                   |
|                                 | intractable        |                   | constipation         |                   | constipation         |                   | constipation         |                   |
| **Age of regression**           | 6 months           |                   | 6 months            |                   | 6 months            |                   | 6 months            |                   |
| **Present ORC**                 | (46 cm)            |                   | (46 cm)             |                   | (46 cm)             |                   | (46 cm)             |                   |
| **Present weight**              | (3,9 kg)           |                   | (3,9 kg)            |                   | (3,9 kg)            |                   | (3,9 kg)            |                   |
| **Hand stereotypes**            | intermittent hand-mouth, washing | | intermittent hand-mouth, washing | | intermittent hand-mouth, washing | | intermittent hand-mouth, washing | | intermittent hand-mouth, washing |
| **Age of stereotypy onset**     | N.A.               |                   | 12 months           |                   | 12 months           |                   | 12 months           |                   |
| **Sensory movements**           | N.A.               |                   | yes                 |                   | yes                 |                   | yes                 |                   |
| **Walking**                    | not acquired       |                   | 2                   |                   | 2                   |                   | 2                   |                   |
| **Age of walking**              | never              |                   | 2                   |                   | 2                   |                   | 2                   |                   |
| **Speech**                     | not acquired       |                   | 2                   |                   | 2                   |                   | 2                   |                   |
| **Level of speech**             | absent             |                   | 2                   |                   | 2                   |                   | 2                   |                   |
| **Level of phrases**            | absent             |                   | 2                   |                   | 2                   |                   | 2                   |                   |
| **Epilepsy**                   | not controlled     |                   | 0                   |                   | 0                   |                   | 0                   |                   |
|                                 | by therapy         |                   | 2                   |                   | 2                   |                   | 2                   |                   |
| **Seizure onset**               | N.A.               |                   | 17 months           |                   | 14 years            |                   | 30 months           |                   |
| **Sleep disturbances**          | yes                |                   | yes                 |                   | yes                 |                   | yes                 |                   |
| **Eye contact**                | poor               |                   | yes, severe         |                   | yes, severe         |                   | yes, severe         |                   |
| **GI disturbances**            | moderate constipation | 2 severe, reflux, constipation | 2 constipation  | 1 constipation  | 1 constipation  | 1 constipation  | 1 constipation  |
| **Breathing disorders**         | N.A.               |                   | 0                   |                   | 0                   |                   | 0                   |                   |
| **Oedema**                     | no                 |                   | 0                   |                   | 0                   |                   | 0                   |                   |
| **Tongue protruding movements** | N.A.               |                   | yes                 |                   | yes                 |                   | yes                 |                   |
| **Arms**                       | no                 |                   | yes, teeth chattering |                 | yes, teeth chattering |                 | yes, teeth chattering |                 |
| **Seizures**                   | N.A.               |                   | yes                 |                   | yes                 |                   | yes                 |                   |
| **Sedation**                   | yes                |                   | yes                 |                   | yes                 |                   | yes                 |                   |
| **Other**                      | no                 |                   | yes, mild           |                   | yes, mild           |                   | yes, mild           |                   |

**Total score:** 28  32  38  31
SUMMARY TEXT BOX

- *FOXI1* gene, located in 14q12, is responsible for the congenital variant of Rett.
- Mutation analysis should be performed in female and male patients showing Rett features but lacking the typical early normal period due to the precocious onset of symptoms.
- Major signs possibly indicating a *FOXI1* mutation are severe psychomotor delay with inability to walk, severe postnatal microcephaly evident before the age of 4 months, poor eye contact, tongue stereotypes, jerky movements of limbs and corpus callosum hypoplasia.
REFERENCES


LEGENDS TO FIGURES

Figure 1. Pictures of three of the new patients with FOXG1 mutations. On the left, in the upper part case 4, in the bottom, case 1; on the right case 2. Note in patient 4 the constant and intense hand-mouth stereotypic activities. The severe microcephaly of case 1 is clearly evident. Patient 2, presently aged 6 years and 6 months, is able to stand up only with support but she can not walk. Parental guardian consent have been obtained.

Figure 2. FOXG1 Mutations and Alterations of the Functional Domains
Schematic representation of FoxG1 protein. The three main functional domains are shown: the DNA binding fork-head domain in light blue, the Groucho-binding domain in violet, and the JARID1B binding domain in red. The numbers at the top refer to the amino acid positions. The frameshift and stop mutations are showed below by zigzag red lines. The missense mutations are indicated at the top by red lines. The asterisks indicate the two mutations previously reported in Ariani et al. 3
Result 3.5

The Italian XLMR bank: a clinical and molecular database.


Databases

The Italian XLMR Bank: A Clinical and Molecular Database


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Communicated by Janie Curtin

Mental retardation (MR) is a nonprogressive condition characterized by a significant impairment of intellectual capabilities with deficit of cognitive and adaptive functioning and onset before 18 years. Mental retardation occurs in about 2 to 3% of the general population and it is estimated that 25 to 35% of the cases may be due to genetic causes. Among these “genetic” MR, 25 to 30% are probably due to mutations in a gene on the X chromosome (X-linked mental retardation, XLMR). Given the genetic heterogeneity of XLMR, the availability of a considerable number of patients with accurate phenotypic classification is a crucial factor for research. The X-linked Mental Retardation Italian Network, which has been active since 2003, has collected detailed clinical information and biological samples from a vast number of MR patients. Collected samples and clinical information are inserted within the XLMR bank, a comprehensive molecular and clinical web-based database available at the address http://xlmr.unisi.it. The database is organized in three distinct parts: Part I and II contain several electronic schedules to register information on the family, the phenotypic description, the photographs, and a 20 sec movie of the patient. Part III allows the registration of molecular analyses performed on each case and clinical data are usable via password-protected access. Clinical and molecular centers interested in joining the network may request a password by simply contacting the Medical Genetics of the University of Siena. The XLMR bank is an innovative biological database that allows the collection of molecular and clinical data, combines descriptive and iconographic resources, and represents a fundamental tool for researchers in the field of mental retardation. Hum Mutat 28(1), 13–18, 2007. © 2006 Wiley-Liss, Inc.

Key Words: XLMR; X-linked mental retardation; molecular database; clinical database; XLMR Italian Network

Introduction

Mental retardation (MR) is a nonprogressive condition characterized by a significant impairment of intellectual capabilities with deficit of cognitive and adaptive functioning and onset before 18 years (Chelly and Mundel, 2001). General intellectual functioning is defined by the intelligence quotient (IQ) and adaptive functioning refers to how individuals deal with common life demands. Mental retardation occurs in about 2 to 3% of the general population (Gecc, 2004) and represents an important socioeconomic and medical issue, given that MR patients need a continuous support from families and healthcare operators. It is estimated that a percentage of MR cases between 25% and 35% may be due to genetic causes. On the basis of the IQ value, mental retardation may be classified in four categories of severity: mild (IQ 50–70), moderate (IQ 35–50), severe (IQ 20–35), and profound (IQ<20). Moreover, MR may be present in association with other clinical manifestations (syndromic MR) or may be isolated (nonsyndromic MR) (Mulley et al., 1992). Males are more frequently affected than females, with an excess of about 30% of male cases explained by the presence of several causative genes on the X chromosome responsible for the X-linked cases of MR (XLMR) (McLaren and Beyson, 1987; Baird and Sadovnick, 1985; Neri and Chiurazzi, 1999). At present, 27 genes for nonsyndromic and 45 genes for syndromic XLMR are known (Renieri et al., 2005; Bauters et al., 2005). Some genes are responsible for both syndromic and non-syndromic XLMR and most of them are responsible for a very low percentage of cases, comprising between 0.1% and 1%. The large number of known XLMR genes and the fact that no major genes exist makes it difficult to offer a molecular diagnosis to MR patients. Given the genetic heterogeneity of XLMR, the availability of a considerable number of patients with accurate phenotypic classification is a crucial factor for research. The X-linked Mental Retardation Italian Network includes 12 laboratories and 27 clinical centers and has been active since 2003, collecting MR patients with detailed clinical information according to common criteria and analyzing known XLMR genes.

The Supplementary Material referred to in this article can be accessed at http://www.interscience.wiley.com/pages/3059-7794/suppmat.

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The members of the XLMR Italian Network are listed in Appendix A.

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It collects both sporadic and familial mentally retarded male patients negative for Fragile X Mental Retardation Syndrome (FRAXA), chromosomal and subtelomeric rearrangements. Biological samples are conserved in a biobank located at the Medical Genetics Laboratory of the University of Siena and at the Genetic Institute of the Università Cattolica del Sacro Cuore in Rome. Collected samples and clinical information are inserted in a web-based database available at the address http://almonini.unisi.it through a password-restricted access. All centers interested in joining the Network can contact the Medical Genetics of the University of Siena.

We describe here the “XLMR bank,” a comprehensive web-based database that includes accurate clinical and molecular data on the MR patients present in the biobank. Samples and clinical data are available upon request for specific studies. The XLMR bank represents an important tool for researchers involved in the discovery of the molecular genetic bases of autosomal and X-linked MR and the clarification of the pathogenic processes that underlie the disease.

THE XLMR BANK

The XLMR bank is available at the web address http://almonini.unisi.it and it is maintained and updated on a server at the University of Siena. The database was created using Access software (Microsoft Office; Microsoft; www.micrsoft.com) for data management. The website is written in Virtual Basic script (VBScript) and it takes advantage of a Microsoft Internet Information Server (IIS) with Active Server Pages (ASP) technology.

Database Structure

The home page of the XLMR bank (http://almonini.unisi.it/home-page.asp) contains general information about the project. In particular, it explains the aims and the overall organization of the project (clinical centers and laboratories involved, patients enrolled, and genes analyzed). Using the “Search” option, external users can visualize the complete list of patients and pedigree and provisional or definitive diagnosis for each patient (Fig. 1).

The overall design of the XLMR bank database includes three distinct parts with password-restricted access (Supplementary Figure S1; available online at http://www.interscience.wiley.com/jpages/0165-5797/suppmat). Each part is organized in several electronic schedules, to collect detailed clinical and molecular data. Part I includes forms filled by the clinical center that has visited the patient and collected the biological samples (Fig. 2). The forms are divided in the following specific sections: 1) private data; 2) family history (pedigree, clinical data, and relatives’ photograph); 3) anamnestic form (clinical history of the patient, IQ value, adaptive functioning evaluation and behavioral tests, laboratory results for standard cytogenetic analysis, investigation of subtelomeric rearrangements, and fragile-X test); 4) clinical data of the patient (clinical features, morphologic examination, neurological data, JPEG format photographs, and 20 sec movie.

![Figure 1](https://www.interscience.wiley.com)
whenever relevant); 3) privacy (name and address of the physician that retains the informed consent); 4) diagnosis (provisional diagnostic hypothesis); 5) trait description, using conventional dysmorphology dictionary (from London Dysmorphology [LEDM] and Poseum databases); 6) shipped biological samples (collected samples and biobank address). To guarantee confidentiality, each sample in the database is uniquely identified by a code of three numbers separated by an hyphen: a progressive number generated by the system that characterizes the family and is the same for each family member and two numbers indicating pedigree position of the subject (Fig. 1).

Part II is completed by biobank curators and it includes information on the source and the type of stored biological samples (Fig. 2). Currently, the following biological samples are stored: DNA, plasma, and lymphocytes and lymphoblastoid cell lines in dimethyl sulfoxide (DMSO) medium.

Part III includes information on laboratory tests performed on each sample (Fig. 4). This part is completed by the laboratories that join the Network and execute the molecular analyses. The laboratory tests currently performed are listed in Table 1.

Database Accessibility and Clinical and Molecular Data Management

The database is organized on five levels of admittance, a public level and four different participant levels, accessible through a password only to centers belonging to the "X-linked Mental Retardation Italian Network" (Supplementary Figure S1). The first level is freely available to external users going to the "Search" option. External users can freely visualize the pedigree of each case. Specific information is accessible only for participants through a "Log in" page, which includes detailed clinical data, location, and preservation of biological samples (DNA, lymphoblastoid cell lines, plasma, urine samples, etc.) and molecular data. A second level password is assigned to clinical centers that visit the patient and decide to insert the case into the XLMR bank. Clinical centers collect biological samples and send them to the laboratories qualified for storage. A laboratory qualified for storage has to guarantee the establishment and maintenance of lymphoblastoid cell lines. Using the second level password, clinical centers can visualize and modify clinical data limited to patients they have inserted and they can consult but not modify their molecular data. A third level password allows to clinical centers to visualize clinical and molecular data of all patients in the bank. Laboratories that perform molecular analyses have a fourth level password that allows them to complete Part III of the database. Each laboratory can visualize molecular information inserted by other laboratories. The fifth level password is the administrator level. Only bank curators have this access, which allows users to visualize and modify all the information maintained in the database, including nomenclatural data.

Security and Quality Assurance

The database has been constructed in compliance with the guidelines of the Italian Society of Human Genetics and Telethon
The database design ensures patient confidentiality, according to international criteria [Godard et al., 2003]. Moreover, the informed consent has been written explicitly stating all aspects of stored samples and personal data management [Godard et al., 2003]. The XLMR bank is available to all users who contact bank curators and fill in and sign a specific form asking to join the Network on X-Linked Mental Retardation and to receive a new password. The Network members agree to follow the rules of the bank and commit themselves not to distribute the password and the information maintained in the database. Anonymity of the samples is assured in each step of the research. Only the bank curator has access to the whole information content of the database, including personal data. Neither other members of the Network nor external investigators wishing to take advantage from stored samples can access patients' personal data (anographical data, photographs, movies, etc.) and consequently they are not able to identify individuals. Only clinicians with a second level password can visualize personal data limited to patients they enrolled in the database. Both genomic DNA and lymphoblastoid cell lines (or primary lymphocytes for future cell line establishment) are stored for all probands and for available relatives. DNA aliquots for standard analyses by different network laboratories are prepared immediately upon DNA extraction. Subsequently, two aliquots of at least 400 µg each are prepared and they are stored in −20°C freezers for long-term conservation. Regarding cell cultures, six distinct aliquots for each lymphoblastoid cell line are stored (four aliquots in the −135°C freezer and two in the liquid nitrogen Dewar). Cells are frozen at passage 2 in 1.5–2 × 10^6 cells/ml aliquots. To prevent the accidental damage of samples, storage of biological materials is organized in different −20°C or −135°C freezers.

**Database Functions**

Users that visit the online database can search for a patient by simply looking through the list of registered cases. Alternatively, the database may be explored using the 'Search' option available after login. The search page allows to browse the database selecting for any field the user wants to search for (Supplementary Figure S21). It is possible to search by a single field or to combine two or more fields. As a result of the search, the user will visualize the list of patients that present the selected feature(s). Information on the selected patients will be available according to the level of the login password.

**DISCUSSION**

The primary focus of the XLMR bank is to collect a wide number of both syndromic (unknown syndromes) and nonsyndromic mental retardation cases. Familial cases with an X-linked pattern of transmission are preferred. However, the bank collects...
TABLE 1. Laboratory Tests Currently Executed by the XLMR Italian Network Members

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<th>Mutation analysis</th>
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An innovative feature of this biological database is the accuracy of clinical data collection using a combination of descriptive and iconographic tools: text areas, JPEG format photographs, and 20 sec movies. The availability of a detailed description will allow a better definition of the phenotype, since patients with the molecular defect will be identified; this may help researchers in the characterization and definition of new syndromes.

Given that part of the laboratories involved in the project have expertise in lymphoblastoid cell lines culture and DNA storage procedures, a characteristic of the biobank described here, is the absence of a main center for the storage of biological samples. All participating laboratories able to establish lymphoblastoid cell lines may propose to store some of the samples.

Requirements that a laboratory candidate for biobanking activities must guarantee are those established by the referral guidelines [Goddard et al., 2003]. In our opinion these collaborative efforts permit a better use of resources due to a distribution of laboratory costs and operators working time. After the first 2 years of activity, approximately 70% of biological samples are stored in Siena and 30% in Rome.

A truly collaborative effort has been made to collect detailed molecular data about each patient. For this aim, mutation analysis is performed on all patients for all genes in study. Linkage analysis for families with at least three affected individuals.
in two generations and exclusion/compatibility mapping for smaller families are performed. Finally, we perform array-based comparative genomic hybridization (CGH) in all enrolled patients to investigate segmental submicroscopic anomalies.

The XLMR bank is an ongoing dynamic database. New clinical centers and laboratories can join the Network at any time just by requesting an appropriate level password from the bank curator. This flexibility allows to meet the demands deriving from scientific knowledge.

Each newly identified mental retardation gene could be added to the list of the molecular tests performed, as soon as a laboratory becomes available. At the same time, the members of the Network may decide to stop the analysis of genes for which the testing is of unproven utility according to the scientific community.

A clinical center may ask for the appropriate level password just to include a single family or a single patient in the database. This allows a more comprehensive collection of interesting cases that have been seen also in small clinical centers spread over Italy.

To our knowledge the XLMR bank is a unique resource that combines molecular data with accurate clinical information. The XLMR bank with the biobank service and the collection of detailed clinical information represents an important tool for both researches in the field of mental retardation. The availability for the scientific community of biological samples and clinical information from our bank may help in the identification of new mental retardation causative genes and in the delineation of new syndromes.

ACKNOWLEDGMENTS

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APPENDIX: A

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REFERENCES


Result 3.6

Searching for new XLMR genes

Unpublished results
3.6.1 Case 1

We report a three generation family in which an X-linked semi-dominant trait seems to segregate (Fig.7). Two male patients show microcephaly and mental retardation, while six females show isolated microcephaly. Analysis of 7 XLMR known genes (MECP2, ARX, FMR2, NLGN3, NLGN4, PQBP1 and AGTR2) in the context of the Italian XLMR network (http://www.biobank.unisi.it) and has been previously performed with negative results. Moreover, X-inactivation status resulted balanced in DNA isolated from blood cells of the mother of the two affected males. We also excluded the presence of deletions/duplications throughout the genome by array-CGH with 25 kb resolution (Agilent Human Genome CGH Microarray 105A). Following the hypothesis of an X-linked disorder, linkage analysis was then performed with markers spanning the entire X-chromosome. Moreover SNP array with 10kb resolution (Affymetrix Genome-Wide Human SNP Array 6.0) has been executed to investigate the presence of smaller imbalances.

Figure 7: Pedigree of the family.
Materials and methods

DNA analysis

Blood samples were obtained after informed consent. DNA was extracted from peripheral blood using the QIAamp DNA blood kit (Qiagen). The CASK coding region was entirely analysed using primers and PCR conditions given in Table 7. PCR products were sequenced on both strands by use of PCR primers with fluorescent dye terminators on an ABI Prism 310 genetic analyser (PE Applied Biosystems, Foster City, CA, USA).

Markers for linkage analysis were amplified by polymerase chain reaction (PCR). A set of 16 markers spaced at 0.4–5 cM over the entire X chromosome were used. The forward primers were fluorescently labelled with Fam, Vic, Pet or Ned. PCR conditions were optimized for individual primer pairs in a 9600 thermocycler (Applied Biosystems). The programs used were 95°C for 12 min, followed by 35 cycles of melting at 94°C for 15 s, annealing at the optimal temperature for 15 s, and then extension at 72°C for 30 s. A final extension was performed at 72°C for 4 min. PCR products were run on an ABI 377 sequencer (Applied Biosystems). Exclusion mapping was used to identify candidate regions on the X chromosome under the following assumption: alleles not shared between the two affected brothers were excluded; alleles shared between the affected brothers and females with microcephaly were considered good candidates.

SNP array

The proband was tested using the Affymetrix Genome-wide Human SNP 6.0 Probe datasheets are found at http://www.affymetrix.com/product/arrays/index.affx. Briefly, genomic DNA (250ng) was digested with StyI and NspI (New England BioLabs),
adaptor-ligated, and PCR-amplified using a generic primer that recognizes the adaptor sequence. PCR conditions were optimized for each array, and the purified PCR products were fragmented with DNase I, biotin-labeled, and hybridized to arrays according to the manufacturer's recommendations. Intensities of the probe hybridization were analyzed using Affymetrix GeneChip Operating Software version 4.1. The Affymetrix Genotyping Console 2.1 with Birdseed genotype calling algorithm was used for genotyping, and CN analysis was carried out with CNAT 5.0 using default configurations. The Affymetrix segment-reporting tool was used to define the magnitude, position, and size of regions of CN gain or loss.

Results

Clinical description

The proband (III-7, Fig. 7) was born pre-term (7° month of gestation). Birth weight was 1700 gr. and OFC was 26 cm (10° percentile). At 5 months of age, microcephaly (OFC 33 cm; <3° percentile) was noticed. An ophthalmologic examination performed at that time was negative. At 4 years the child was hyperactive, he had attention deficit disorder and showed auto aggressive episodes and language delay. At 10 years he had movement coordination difficulties, which affected both deambulation and manual abilities. He was first examined by our clinicians at the age 29 years (Fig. 8). He showed microcephaly (OFC 42.5 cm; <3° percentile), hypotelorism, height of 182 cm (90° percentile), weight of 50 Kg, moderate-severe mental retardation and language delay. He was unable to read but he was able to write his name. He was attending a community for
disabled people and he practiced several sports. Brain MRI showed a global reduction of brain volume, especially in the supratentorial region, thin brain stem, normal corpus callosum, undergrowth of frontal lobes and temporal pachygiria. Cardiac ultrasound revealed a mild tricuspid valve insufficiency. Auditory evoked potentials showed normal hearing. Ultrastructural examination of the hair was negative.

The younger brother (III-9, Fig. 7) was born at term. Birth OFC was 26.5 cm (<<3° percentile), birth weight was 2090 gr. (<3° percentile), birth length was 43 cm (<3° percentile). At birth cleft lip/cleft palate was noted. He walked at 18 months and he said his first words at 3 years of age. Parents referred that he had been always hyperactive, and sometimes showed an auto and etero-aggressive behavior. An ophthalmologic examination performed at 3 years of age was negative. At the time of the first examination by our clinicians, he was 20 years old (Fig. 8). He showed microcephaly (OFC 41 cm; <<3° percentile), hypotelorism, severe mental retardation, height of 160 cm (<3° percentile) and language delay. He was not self-governing, he could not read or write. He practiced several sports. Cardiac ultrasound revealed a mild tricuspid and pulmonar valve insufficiency. Auditory evoked potentials showed sensoryneural hearing loss of moderate degree for medium-high frequencies. Ultrastructural examination of the hair was negative.
Figure 8: Patients’ pictures at the time of the last examination (III-7 was 29 years old and III-9 was 20 years old). Receding forehead is present in both brothers. III-9 shows the surgical scar for cleft lip/cleft palate.

Clinical investigation of healthy females revealed the presence of isolated microcephaly in patients’ sister (III-8; OFC 50,5; <3° percentile), their mother (II-5; OFC 48,5; <3° percentile), their two maternal aunts (II-2: OFC 52,5; <10° percentile and II-4: OFC 52; <3° percentile) and two of their female cousins (III-3: OFC 50,5 <3° percentile, and III-6: 52; <3° percentile).

**Molecular analysis**

The analysis through SNP array 6.0 didn’t reveal any pathogenic chromosomal rearrangement. By linkage analysis we found four regions that segregates in all family members with microcephaly (Fig. 9). Among them,
one includes 3 markers (DXS8090, DXS8012, DXS1003) (Fig. 9). This region spans 9.5 Mb and contains 37 genes (Fig. 10). Bioinformatic analysis indicated CASK (NM_003688) as a good candidate gene for microcephaly. However, sequence analysis of CASK coding region in all patients did not revealed any pathogenic point mutation.

Figure 9: Pedigree of the family, and genotype of each subject for the tested microsatellites. The red boxed markers segregate in all family members with microcephaly. Haplotypes segregating with microcephaly are shown with black boxes. Result of marker DXS8096 are missing due to technical problems.
Figure 10: Gene content of the 9.5 Mb region which segregates in the linkage analysis. In the region are present 37 genes (UCSC Genome Browser; http://genome.ucsc.edu). Red circles indicate the already known XLMR genes and CASK, which has selected as candidate gene responsible for microcephaly.
Table 7: Primer sequences on *CASK* gene (Piluso et al; 2008)

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<td>gatttccactcttgcttg</td>
<td></td>
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<tr>
<td>26</td>
<td>F</td>
<td>ttactttccaattgctggat</td>
<td>174</td>
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<tr>
<td></td>
<td>R</td>
<td>getgtgaccttacacgatgatttgg</td>
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<td>27</td>
<td>F</td>
<td>acgatcctactagattcatctcttt</td>
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<tr>
<td></td>
<td>R</td>
<td>taacaagagagcttttccacaaat</td>
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</table>
3.6.2 Case 2 (#86)

We report a family in which an X-linked inheritance was supposed (Fig. 11). The proband (III-8) and two maternal uncles (II-6, II-7) are affected by MR (Fig 11). The presence of specific features, such as microcephaly and short stature, previously suggested to investigate \( \text{PQPB1} \) gene for point mutations. The analysis resulted normal. X-inactivation status resulted balanced in DNA isolated from blood cells of the mother of the proband (II-8). A Kariotype with 350-500 bands resolution, FRAXA analysis and subtelomeric rearrangements investigated by MultiFISH resulted normal. According to the hypothesis of an X-linked inheritance, we performed a linkage analysis using markers spaced on the entire X chromosome. Moreover SNP array with 10kb resolution (Affymetrix Genome-wide Human SNP array 6.0) has been executed on DNA of the proband (III-8) to investigate the presence of smaller imbalances.

\[ \text{Figure 11: Pedigree of the family. Shaded symbols indicate individuals with mental retardation and open symbols indicate individuals who are unaffected. The arrow indicates the proband.} \]
Materials and methods

See Materials and Methods of Result 3.2.1

Results

Clinical description
The child experienced mild respiratory distress at birth without other complications. At last evaluation (4.5 years), he showed growth delay (height, weight and head circumference below the 3rd centile), peculiar facial and physical features including bilateral palpebral ptosis, long eyelashes, syndactyly between the III-and IV toe with V toe hypoplasia, short third toe and nail dysplasia. Mental abilities assessment with WISC-R revealed mild mental retardation (IQ of 59), the patient showed hyperactivity and attention deficit disorder, a wrist RX revealed delayed bone age (bone age of 2 years), brain MRI showed asymmetry and the EEG was normal.

Molecular analysis
The analysis through SNP array 6.0 did not reveal any pathogenic chromosomal rearrangement. Only the DNA sample of the proband (III-8) and one maternal uncle (II-7) were available. By linkage analysis no significant two-point LOD scores were obtained with any markers. However, three regions segregates in the two MR family members (Fig. 12).
Figure 12: Pedigree of the family, and genotype of each subject for the tested microsatellites. The red boxed markers segregate in all family members with MR. Haplotypes segregating with MR are shown with black boxes. Result of marker DXS8096 are missing due to technical problems.

The largest region includes 4 markers (DXS8090, DXS8012, DXS1003, DXS1199) (fig. 12). The region, which spans for 17 Mb, contains 138 genes. Gene content analysis of the region indicates the presence of 31 known causative disease genes. Among them, we found 18 already known XLMR genes: OTC, TSPAN7, BCOR, ATP6AP2, MAOA, NDP, ZNF41, ZNF674, ZNF81, PORCN, SYN1, FTSJ1, HUWE1, JARID1C, HADH2, SMC1A, SHROOM4, and the already screened PQBP1130,135-146,147 649,148-151 (Tab. 8).
Table 8. Known XLMR genes present in the region that segregate in the affected family members

<table>
<thead>
<tr>
<th>OMIM</th>
<th>Gene</th>
<th>Condition</th>
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<tr>
<td>300096</td>
<td>TSPAN7</td>
<td>MIRX68</td>
<td>Zemni R., 2000</td>
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<td>300461</td>
<td>OTC</td>
<td>Ornithine transcarbamylase</td>
<td>Maddalena A., 1988</td>
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<td>300485</td>
<td>BCOR</td>
<td>OFCD, Lenz microphthalmia</td>
<td>NG D., 2004</td>
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<td>300666</td>
<td>ATP6AP2</td>
<td>XMRE</td>
<td>Ramser J., 2005</td>
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<td>309860</td>
<td>MAOA</td>
<td>Brunner syndrome</td>
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<td>300668</td>
<td>NDP</td>
<td>Norrie disease</td>
<td>Berger W., 1992</td>
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<td>300673</td>
<td>ZNF674</td>
<td>MIRX92</td>
<td>Lugtenberg D., 2006</td>
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<td>314996</td>
<td>ZNF41</td>
<td>MIRX89</td>
<td>Sloichet S.A., 2003</td>
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<td>313440</td>
<td>SYN1</td>
<td>Epilepsy/macrocephaly</td>
<td>Garcia C.C., 2004</td>
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<td>314998</td>
<td>ZNF81</td>
<td>MIRX45</td>
<td>Kleefstra T., 2004</td>
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<td>300499</td>
<td>FTSJ1</td>
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<td>Ramser J., 2004</td>
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<td>Freude K., 2004</td>
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<td>300651</td>
<td>PORCN</td>
<td>Goltz</td>
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<td>300463</td>
<td>PQBP1</td>
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<td>Renpenning syndrome</td>
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<td>300679</td>
<td>SHROOM4</td>
<td>Stocco dos Santos</td>
<td>Hanges O., 2006</td>
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<td>314690</td>
<td>JARID1C</td>
<td>Jardinic-related</td>
<td>Jensen L.R., 2006</td>
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<td>300697</td>
<td>HUWE1</td>
<td>MIRX17, 31</td>
<td>Froyen G., 2008</td>
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<td>300040</td>
<td>SMC1A</td>
<td>X-LINKED Cornelia de Lange</td>
<td>Musio A., 2006</td>
</tr>
</tbody>
</table>

Mutations in genes indicated in red have been associated to some clinical features also present in our patient.
3.6.3 Case 3 (#87)

We describe a family with 3 brothers affected by MR (III-2, Fig. 13) with possible X-linked inheritance. The standard karyotype was normal. Molecular test for FRAXA and MultiFISH analysis to search for subtelomeric rearrangements were negative. Only the DNA of the proband (III-4) was available and therefore we could not perform a linkage analysis on chromosome X. The DNA of the patient was investigated for chromosomal rearrangements by SNP array with 10kb resolution (Affymetrix Genome-Wide Human SNP Array 6.0).

Figure 13: a) Pedigree of the family. Shaded symbols indicate individuals with mental retardation and open symbols indicate individuals who are unaffected. b) Pictures of the proband (III-4) and one of the two affected brothers (III-5)
**Materials and methods**

See DNA analysis and SNP array 6.0 in *Materials and methods* of results 3.2.1

**Real-time**

The TaqMan probe and primers were designed using the Primer Express software (Applied Biosystems,[https://products.appliedbiosystems.com](https://products.appliedbiosystems.com)), following the criteria indicated in the program. We designed the SHC3 probe to be complementary to a segment located in the middle of exon 9. SHC3 forward primer: 5’-GGT GAG AGG CAA GGT GAG ATG-3’; SHC3 reverse primer: 5’-AAG TGT CTT TTG TGT TTC TC-3’. As concern the intragenenic deletion, primers and probe were designed in the exon 2 of CNTNAP2 gene, which found deleted by array analysis. CNTNAP2 forward primer: 5’-.GCC ACT TGT CTC TGG ACT CC- 3’; CNTNAP2 reverse primer: 5’- CCC ATG TGG CTT TCA G-3’. PCR was carried out using an ABI prism 7000 (Applied Biosystems) in a 96-well optical plate with a final reaction volume of 50 µl. A total of 100 ng (10 µl) was dispensed in each of the four sample wells for quadruplicate reactions. Thermal cycling conditions included a prerun of 2 min at 50°C and 10 min at 95°C. Cycle conditions were 40 cycles at 95°C for 15 sec and 60°C for 1 min according to the TaqMan Universal PCR Protocol (ABI). The TaqMan Universal PCR Master Mix and Microamp reaction tubes were supplied by Applied Biosystems. The starting copy number of the unknown samples was determined using the comparative Ct method as previously described.\(^\text{152}\)
Results

Clinical description
The patient is a 29 years old male (III-4). Pregnancy and delivery are reported normal. He showed psychomotor delay and epilepsy. At the last evaluation he showed severe delay, flat occiput, downslanting palpebral fessures, clynodactyly of the fifth finger bilaterally. The EEG and the CT scan of the brain are normal.

Molecular analysis
SNP array 6.0 revealed the presence of two deletions in the DNA of the patient (III-4): 1) deletion of 3.7 Mb on 9q21.33-q22.2 containing 16 genes and (Fig. 14) 2) an intragenic deletion in the CNTNAP2 gene located on 7q35 (fig.14 a.b).
Figure 14: Molecular results. a) Chromosome view displays chromosome 9 along with the accompanying data graphs and annotation tracks indicating loss of heterozygosity (LOH), Log ratio and Copy Number state, overlapping genomic variant and ref seq of genes. b) Gene content of the 3.7 Mb deleted region. In the region are present 37 genes (UCSC Genome Browser: http://genome.ucsc.edu).
In order to extend the analysis to all family members, we requested their DNA samples (II-1, II-8, III-1, III-2, III-3, III-5, III7) and we performed Real Time qPCR with specific probes for the two rearrangements. Regarding the largest deletion, by the use of a qPCR probe designed on SHC3 gene, we confirmed the rearrangement in the proband but we could not find it in any other family member. As concern the intragenic deletion, using a probe specific for CNTNAP2, we confirmed the proband deletion that was also present in the DNA of: healthy mother (II-8), one affected brother (III-5), and one healthy brother (III-7) (Fig 15 a, b).
Figure 15: Real Time quantitative validation experiment. SHC3 (a) and CNTNAP2 (b) ddCT ratios and standard deviations obtained for all family members (Fig.13a) and control sample (C). a) The proband (III-4) shows a ddCT ratio of about 0.6, indicating the presence of a single copy of SHC3 (deletion), while the other family members and the control show a ddCT ratios about 1, indicating a double copy of the gene (normal). b) The proband (III-4), the mother (II8), an affected brother (III-5) and an healthy brother (III-7) shows a ddCT ratio of about 0.5, indicating the presence of a single copy of CNTNAP2 (deletion), while the father (II-1), an affected brother (III-2) and an healthy brother (III-3) and the control (C) show a ddCT ratios about 1, indicating a double copy of the gene (normal).
3.6.4 Case 4 (#164)

We described a family collected in Italian XLMR network (http://www.biobank.unisi.it) with two brothers with MR. Another family member (III-1) is reported as affected by mild MR. In the patient III-5 karyotype, molecular tests for Fragile X and PTEN mutations are normal.

Figure 16: a) Pedigree of the family. Shaded symbols indicate individuals with mental retardation and open symbols indicate individuals who are unaffected. b) Picture of the patient III-5
**Materials and methods**

*See the Materials and methods of Result 3.2.1*

**Results**

**Clinical description**

Patients are two brothers, the older is 9 years old and the younger is 6 years old. Pregnancy and delivery were normal for both. They showed a normal psychomotor development in the first 2 years of life when they started to present behavioural disturbances. The older brother (III-5, Fig. 16) present hyperactivite and attention deficit disorder, flapping of the upper limbs and echolalia. His growth is normal, he shows pointed chin and single palmar crease bilaterally. The younger brother (III-6) started showing autistic behaviour at two years of age. At the last evaluation the language was absent, growth parameters were normal, he showed hypertelorism and frontal bossing. Karyotype, molecular tests for Fragile X and PTEN mutations are normal.

**Molecular analysis**

The analysis through SNP array 6.0 on the DNA of one of the two affected brother (III-5) did not revel any pathogenic chromosomal rearrangement. Only the DNA sample of the brothers (III-5, III-6) were available. Two regions segregates in the two MR brothers (Fig. 17): a 5 Mb region including two markers (DXS1036, DXS8090) and a 31 Mb region including six markers (DXS1212, DXS1047, DXS1227, DXS8043, DXS1193, DXS1073). (fig.17).
The smaller region contains 9 genes. Among them, there is a known XLMR gene: **DMD** (300373). The other region contains 175 genes including 15 known XLMR genes: **SLC9A6, ARHGEF6, SOX3, FMRI, FMR2, IDS, MTM, RPL10, SLC6A8, ABCD1, L1CAM, MECP2, FNLA, GDII, IKBKG**. (Tab. 9)
Table 9. Known XLMR genes present in the larger region that segregate in the affect family members

<table>
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<tr>
<th>OMIM</th>
<th>Gene</th>
<th>Condition</th>
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<tr>
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<td>ARHGEF6</td>
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<td>313430</td>
<td>SOX3</td>
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<td>309660</td>
<td>FMR1</td>
<td>Fragile XA</td>
<td>Kremer E. J., 1991</td>
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<td>309900</td>
<td>IDS</td>
<td>Mucopolysaccharidosis type II</td>
<td>Sukegawa K., 1992</td>
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<td>309548</td>
<td>FMR2</td>
<td>FRAXE</td>
<td>Barnicoat A. J., 1997</td>
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<td>300415</td>
<td>MTM</td>
<td>Myotubular myopathy</td>
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<td>300036</td>
<td>SLC6A8</td>
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<td>Selomons G. S., 2001</td>
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<td>300371</td>
<td>ABCD1</td>
<td>Adrenoleukodystrophy</td>
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<td>308840</td>
<td>L1CAM</td>
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<td>300005</td>
<td>MECP2</td>
<td>Rett</td>
<td>Amir R. E., 1999</td>
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<td>300017</td>
<td>FLNA</td>
<td>X-linked periventricular heterotopia</td>
<td>Fox J. W., 1998</td>
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<td>300104</td>
<td>GDII</td>
<td>MRX41,48</td>
<td>D'Adamo P., 1998</td>
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<td>300248</td>
<td>IKBKG</td>
<td>Incontinentia pigmenti</td>
<td>Smahi A., 2000</td>
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Mutations in genes indicated in red have been associated to some clinical features also present in our patient.
4. DISCUSSION AND FUTURE PERSPECTIVES
4. Discussion and future perspectives

MR is a highly heterogeneous condition with a variety of contributing factors, both genetic and environmental. It represents one of the most difficult challenges faced today by clinicians, genetics as well as social system. The identification of genes responsible for MR has a crucial implication for prevention and treatment. The number of genes involved in MR has grown rapidly in the recent years. However, much work remains to be done, as it is estimated that the cause of MR is still unknown in up to 60% of the cases.

My Ph.D. research project has been focused on searching for new genes involved in MR. During the first three years of the course, my activity was mainly dedicated to a syndromic of MR, Rett syndrome (OMIM), that represents one of the most common causes of intellectual disability in females. This research has leaded to the identification of the first autosomal gene involved in RTT spectrum: FOXG1 (OMIM 164874) (Result 3.3). This discovery has been made possible by two factors: i) availability of a biobank of RTT samples with detailed clinical and molecular characterization (http://www.biobank.unisi.it) (Result 3.1); and ii) employment of array-CGH technology. Initially, the biobank allowed us to rapidly search for a well characterized group of mutation-negative RTT patients (http://www.biobank.unisi.it) Array-CGH analysis performed in one of these patients with a RTT-like phenotype permitted us to identify a 3 Mb deleted region on 14q12. By “candidate gene approach”, we could selected FOXG1 and, performing mutation analysis in the entire collection of negative RTT patients, we identified the first two patients classified as congenital variant cases (Result 3.3). Subsequently, the establishment of an
international collaboration allowed us to collect a larger cohort of RTT patients and to identify four additional FOXG1 mutated cases (Result 3.4). These results gained important insight into the clinical phenotype associated with FOXG1 mutations and confirmed the role of the gene in the most severe form of RTT.

This successful story confirms the importance of array-CGH as tool for gene discovery. In fact, the identification of chromosomal imbalances in specific patients has already proved to be a successful way to identify the implicated genes and to gain insight in the pathogenesis of different genetic conditions 127-132. Further development and refinement of array-CGH, leading to more extensive coverage of the genome, will enhance our abilities of mapping genes underlying several genetic conditions.

The use of the Italian RTT biobank has been also fundamental for the identification of five new cases with mutations in CDKL5, the second gene involved in RTT. The detailed clinical classification has permitted to clarify the phenotype and to elaborate diagnostic criteria that will address the molecular analysis (Result 3.2).

During the last year of the Ph.D. course, my research activity in the laboratories of Human Genetics, Radboud University in Nijmegen (The Netherlands), was dedicated to the search new XLMR genes. The availability of a biobank (http://www.biobank.unisi.it) of samples collected through an Italian Network, facilitated the selection of patients to include in the analysis (Result3.6). In order to discover new XLMR genes, we followed two strategies :i) linkage analysis, using a collection of 16 STR markers evenly spaced across the X chromosome; ii) analysis of CNVs by whole-genome SNP microarray with resolution of 10 kb.

In Case 1, linkage analysis revealed four regions segregating in all family members with microcephaly. Interestingly, the larger region contains
a good candidate for patients phenotype: CASK (300172). CASK encodes for a calcium/calmodulin-dependent serine protein kinase that belongs to the membrane-associated guanylate kinase (MAGUK) family. Members of this family target to neuronal synapses and regulate trafficking, targeting and signaling of ion channels. CASK has been proposed to be a ‘pseudokinase’ and it functions as part of large signalling complexes in both pre- and postsynaptic sites. Recently, CASK has been found mutated in two patients mental retardation and microcephaly with pontine and cerebellar hypoplasia. Functional data confirm CASK as an excellent candidate gene, as Cask mouse mutants have small brain, abnormal cranial shape and cleft palate.

We thus performed CASK mutational screening in our family, but we could not find any pathogenic point mutations (Result 3.6.1). However, we can not exclude the presence of deep intronic or promoter mutations in CASK. Moreover, we did not investigate the presence of intragenic CASK rearrangements by techniques such as multiplex qPCR, MLPA or target microarray. During the last years, increasing use of these techniques demonstrated that a significant fraction of disease causing mutations is represented by small deletions/duplications not detectable by PCR based methods.

Alternatively, CASK is not involved in MR family and other candidates have to be considered. In fact, the region is quite large (9.5 Mb) and contains 37 genes including 7 known XLMR genes: OTC, TSPAN7, ATP6AP2, BCOR, MAOA, NPD, ZNF674.

Other candidates can be selected on the basis of their function and/or expression.

However, the “candidate gene approach” is not always the successful strategy to identify new disease genes. In several cases less obvious
candidates have proved to be the cause of the disease, such as MECP2 in RTT syndrome. In these cases, only a systematic gene screening approach has led to gene identification.

In Case 2 and 4, linkage analysis showed very large candidate regions containing a high number of genes, making difficult the selection of interesting genes (Result 3.6.2, 3.6.4). We will start with the analysis of genes already known to be involved in XLMR forms with additional clinical features overlapping with our case (Result 3.6.2, 3.6.4).

In Case 3, SNP array identified two rearrangements: one intragenic deletion in CNTNAP2 (7q35) and one deletion of 3.8 Mb on 9q21.33-q22.2. CNTNAP2 could represent a good candidate since it encodes a member of the neurexin family and is associated to cortical dysplasia-focal epilepsy syndrome. Our patient shows psychomotor delay, epilepsy and dysmorphic features (Result 3.6.3). In the other region, SHC3 represents an interesting gene since it is highly expressed in brain and is involved in BDNF signalling. However, the use of Real Time qPCR assays designed on the two regions revealed that both rearrangements do not segregate with MR in the family (Result 3.6.3), making unlikely their involvement in the disease.

In XLMR cases, the obtained results represents preliminary findings that will address future projects aimed at identifying the causative genes. These projects will require time and, possibly, collaborative efforts that will allow the sharing of technological platforms.

A recent alternative strategy for XLMR gene discovery is represented by large-scale systematic resequencing of X chromosome. This approach has recently allowed the discovery of nine new genes implicated in XLMR confirming the power of this strategy. However, the study by Tarpey et al. also highlights pitfalls and challenges of this approach. In fact, the
authors unexpectedly found that at least 1% of the X chromosome genes have truncating mutations in the normal population. Therefore, identification of protein-truncating variants requires careful evaluation and can not be regarded as strong evidence of disease causation on its own. Another problem is represented by the assessment of pathogenicity of missense sequence variants eventually identified. To this aim, Tarpey et al. adopted strategies based on the conservation of the altered amino acid and the number of missense variants in each gene. However, the discriminative power of such approaches may be limited. A key test is whether there is a difference in the prevalence a putative causative change between affected and control individuals. However, the size of the sample sets required could hamper this kind of studies in MR.

The annotated genome sequence and the improvement of technological platforms has greatly increased our possibility to discover the genetic causes of MR. The identification of the genes responsible for MR is an essential step not only for the definition of the pathogenic mechanisms involved in MR but also for the identification of pathways essential for normal brain development and functioning. Establishing the genetic causes in patients with MR is fundamental for improving clinical management, defining the prognosis, and facilitating genetic counselling for the families. In addition, this knowledge will be essential for planning potential therapeutic strategies for MR.
5. REFERENCES
5. References


115 Motazacker MM, Rost BR, Hucho T *et al.* A defect in the ionotropic glutamate receptor 6 gene (GRIK2) is associated with autosomal recessive mental retardation. *Am J Hum Genet* 2007; **81**: 792-798.


118 Kerchner GA, Nicoll RA: Silent synapses and the emergence of a postsynaptic mechanism for LTP. *Nat Rev Neurosci* 2008; **9**: 813-825.


Maddalena A, Spence JE, O’Brien WE, Nussbaum RL: Characterization of point mutations in the same arginine codon in


Curriculum Vitae

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Last name: Artuso

Date of Birth: 22/08/80

Place of Birth: Gioia del colle (BA)

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Professional experience:

November 2005 - today: worked as Ph.D student in the Research Project for the identification and characterization of new mental retardation genes in particular regarding genes involved in Rett syndrome under the supervision of prof. Alessandra Renieri, Medical Genetics, University of Siena.
LIST OF PUBLICATIONS

The Italian XLMR bank: a clinical and molecular database.

2q24-q31 deletion: report of a case and review of the literature.

Italian Rett database and biobank.


Mutations in FN1 cause glomerulopathy with fibronectin deposits.

FOXG1 is responsible for the congenital variant of Rett syndrome.

Autosomal dominant Alport syndrome: molecular analysis of the COL4A4 gene and clinical outcome.

Early-onset seizure variant of Rett syndrome: Definition of the clinical diagnostic criteria.
Brain Dev. 2009 Apr 9. [Epub ahead of print]

Novel FOXG1 mutations associated with the congenital variant of Rett syndrome.
Acknowledgements

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A thank to Mirella for helping me taking my first steps in the laboratories many years ago, to Ilaria and Francesca that as senior investigators have taught me how to become a senior investigator myself. I would like to thank my a friend and colleague Eleni for the reciprocal aid in order to survive the latest days. A thank to all my collegues, they taught me how to work with a smile. A special thank is reserved to Alessio, the only Y-chromosome still alive after 4 years. Given his resistance, it can’t be so bad working with 30 women!

I would like to thank Prof Hans vanBokhoven for his kind collaboration and for making my feel at home even in The Netherlands.

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