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Title

Multiplex Ligation-dependent Probe Amplification analysis of Copy Number Variants in mentally retarded patients

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Abstract

The aetiology of mental retardation and dysmorphism is still poorly understood in over half of the affected individuals. Recent studies have shown that genomic mutations such as Copy Number Variants (CNV) can be important factors predisposing to the highly heterogeneous clinical conditions observed in affected individuals. Genome−wide array−Comparative Genomic Hybridization (aCGH) is the best technology available so far for a first screening of CNVs in genomic DNA in patients and control individuals. However, CNVs detected by aCGH need validation by an independent method such as Multiplex Ligation-dependent Probe Amplification (MLPA). In this report we present the results of a MLPA analysis performed on 120 mentally retarded patients and their parents and 400 samples from a reference population. Patients who underwent MLPA were previously scored positive to aCGH.

Our results show that, overall, from the 150 CNVs identified by aCGH, 123 were confirmed by our MLPA analysis and overall account for 65 gains (size range: from 3,6 Kb to 3,0 Mb) and 58 losses (size range: from 2,2 Kb to 1,9 Mb). At least 16 CNVs are *de novo* (11 loss, 5 gain). Inheritance from a healthy parent could be established only for 83 CNVs. For 24 CNVs it was not possible to establish if they were *de novo* or *inherited* because one or both parents were unavailable for the analysis. In the majority of cases, a given CNV appear to be unique to a particular patient as far their chromosomal location and size is concerned. In contrast five

CNVs are shared each by two patients. One CNV_loss in chromosome 15q11.2 was shared by six unrelated patients.

To investigate on the possible pathogenic role played by CNVs in this phenotypically and genetically heterogeneous population of mentally retarded patients and thus select the strongest candidate CNVs we have applied a multi-step strategy. Briefly, we first excluded CNVs also occurring in the general population. To this end, we screened by MLPA 400 neurotypical geographically- and ethnically-matched individuals. Second only CNVs occurring as de novo events were considered. Third, we queried a large body of data stored in the literature and in several databases to exclude CNVs previously associated to mental retardation (MR) or described as phenotypically unexpressed polymorphisms in the general population. The best candidate CNVs selected as just described were then analyzed for their gene content and to assess by *in silico* functional annotation analysis the role of these genes in nervous system. This approach led in some cases to the identification of several CNV, genes and statistically significant ontologies related to nervous system thus supporting their pathogenic role. A result of special interest was the identification in one MR patient with a complex phenotype of a duplicated microRNA (hsa-miR-150) overlapping a CNV on chromosome 19. The functions and diseases associated to the individual target genes/mRNA regulated by this microRNA appear to be highly correlated to the clinical phenotypes displayed

by this patient and suggest the occurrence of a new MR syndrome caused by a duplicated microRNA.

In summary, the results of our study: (i) emphasize the importance of MLPA to exclude false positives generated by aCGH, (ii) describe the identification of novel CNVs and genes potentially implicated in MR. We believe that the results of this study will significantly contribute to the discovery of new microdeletion syndromes and a more precise correlation of the mutant genotype with the highly variable phenotype displayed by mentally retarded patients.

Introduction

Mental retardation (MR) or developmental delay is a pervasive condition, mainly defined as an incomplete or arrested development of the mind and often associated with malformations or multiple congenital anomalies and/or dysmorphic features, which is estimated to affect 2–3% of the population **(1)**, thus representing highest socioeconomic costs in developed societies (2). From a genetic point of view, MR can be considered as a complex disorder with an extremely high degree of genetic heterogeneity highlighted by the evidence that the underlying known defects span from single point mutation to large deletion or duplication at thousand of different loci.

The search for the genes involved in mental retardation is hampered by an extremely high degree of genetic and phenotypic heterogeneity and by the fact that most of these disorders occur sporadically in the population, mainly because the *de novo* nature of the mutations and because affected patients will seldom have offspring. It is not surprising that the majority of loci found to be associated with MR have been identified in X-linked conditions because of their characteristic inheritance patterns. Many other loci for MR have been mapped to autosomes by classical gene mapping, but despite these large efforts roughly 40-50 % of the genetic causes of MR still remains unknown. Given this background, whole genome scan methods, such as karyotype analysis, have been successfully used to investigate the MR and until now cytogenetic imbalances are the most frequently

identified causes of MR. More recently, a new high resolution whole- genome scan method called Array-CGH (comparative genomic hybridization) (3) has been implemented. Since its introduction in MR research, Array-CGH has proven to be a powerful and promising method that is revolutionizing cytogenetic diagnosis, at least doubling the detection rate of MR loci [4]. While highlighting many formerly unresolved cases, the array-CGH demonstrated the high degree of variability in Human genome in term of structural variation. It is now recognized that the genomes of any two individuals in the human population differ from one another with respect to the copy number of thousands [5]. As many of these structural variations, called CNVs could potentially represent risk factors, and some of them have already been linked to human diseases, they will have a great impact into the study of the genetic basis of complex diseases. On the other hand, such a complexity becomes a challenge for the cytogeneticist when interpreting array-CGH results performed in clinical diagnostic settings because there isn't yet a confident background about the biological significance for many of the CNVs [6]. In respect to MR, classification into benign or pathogenic CNVs greatly depends on whether they have been found in normal individuals or identified in patients as a *de novo* events. This information is thus crucial both for a correct diagnosis and for the understanding of the role of the genes involved.

A critical aspect of aCGH is that it requires confirmation of CNVs by an independent method such as Multiplex ligation-dependent probe amplification (MLPA) [7]. MLPA is a new technique suitable for measuring sequence dosage, allowing large number of samples to be processed simultaneously, thus reducing significantly laboratory work. In this study we have used MLPA to re-assess a large sample of MR patients previously tested by aCGH.

Materials and methods

Patients

DNA samples

From initial aCGH screening of 1200 patients (see above) 150 CNVs borne by 120 patients were selected after the exclusion of: (i) CNVs also reported in the Database of Genomic Variants (http://projects.tcag.ca/variation) (general population) and (ii) CNVs described as pathogenic according to the literature. We have also used 400 DNA samples from ethnically/geographically-matched subjects recruited among the healthy volunteer blood donors.

MLPA analysis

Principle of the method and main analytical steps

Multiplex Ligation-Dependent Probe Amplification (MLPA) is an application that can be used for the detection of multiple chromosomal aberrations consisting of gene and genomic dosage variations such as Copy Number Variants. The main steps of this procedure are summarized hereafter (figure 1):

1. DNA sample is heated so that it denatures. The MLPA probes, each consisting of two oligonucleotides, hybridize to the sample DNA.

2. When two oligonucleotides of each probe are hybridized to their target sequence, they are connected by enzymatic ligation to form one complete probe.

3. During the PCR reaction, only complete probes are amplified exponentially

4. Amplification products are separated and quantified using ABI3130 capillary electrophoresis and Gene Mapper software (Applied Biosystems).

Designing synthetic MLPA probes

MLPA probes (fugure 2) consist of two oligonucleotides: the Left Probe Oligonucleotide (LPO) and the Right Probe Oligonucleotide (RPO) (Figure 2, part 7 and 8). Each oligonucleotide in turn usually contains 2 parts. The LPO contains the recognition sequence of the Forward PCR primer at its 5' end (part 1) and the left hybridizing sequence (LHS) at its 3' end (part 3). The RPO contains a right hybridizing sequence (RHS, part 4) at its 5' end and the sequence recognized by the reverse primer at its 3'end (part 6). The primer sequences are used in the amplification of the probe during the PCR reaction.

For the analysis of CNV detected in mentally retarded patients and controls, the MLPA probes were designed and built for each selected CNV. Two-four probes per locus were designed according to the protocol available at MRC Holland website (http://www.mrc−holland.com/pages/indexpag.html). For each CNV two probes were designed near the internal CNV boundaries, based on aCGH results. A Blast http://genome.ucsc.edu/cgi-bin/hgGateway) analysis was instead

used to ensure that probes hybridize to the suitable genomic regions. For every multiplex reaction, probes for three control loci known to be represented only once per haploid genome, was added in the multiplex reaction.

Statistical analysis of the electropherogram-related data

In order to efficiently process MLPA data, a spreadsheet was generated in Microsoft Excel. First, data corresponding to each sample (patient and control DNA) were normalized by dividing each probe signal's strength value (the area of each peak) by the average signal strength of 10 control probes to generate for each peak a Relative Peak Area (RPA). The RPA for each probe in a patient sample was then compared to the RPA obtained from a control sample by dividing the patient RPA by the control RPA for each peak. This value was then used to define the following categories according to Calì et al. [8]: (i) "normal" for values in the range 0.75-1.25 (these values correspond to more than three standard deviations above and below the mean of the normalized data); (ii) "deleted" for values smaller than 0.60, and (iii) "duplicated" for values larger than 1.40 (these values correspond to more than five standard deviations above and below the mean of the normalized data). As a quality test for the probes, we computed the standard deviation (SD) of the normalized signals for the ten control probes for both patients and control individuals. MLPA analysis was repeated for samples yielding a SD value exceeding the threshold value of $\sigma t = 0.05$. This value

corresponds approximately to the mean value of the standard deviations of the normalized data relative to the ten control probes for all individuals (both patients and control).

In silico analyses

We referred to several international databases which collect information on CNVs and overlapping genes:

- Database of Genomic Variants (http://projects.tcag.ca/variation)

- PubMed (http://www.ncbi.nlm.nih.gov/pubmed)

- OMIM (http://www.ncbi.nlm.nih.gov/omim/)

- Entrez Gene (http://www.ncbi.nlm.nih.gov/gene/)

For statistical functional annotation analyses the following databases and implemented software were used:

- Ingenuity Pathways Analysis (IPA) http://www.ingenuity.com/products/analysis_services.html

- Gene Ontology (GO) http://www.geneontology.org/

Mapping of microRNA in CNVs was achieved using the UCSC Genome Browser (http://genome.ucsc.edu/) using the Build 36 version of the human genome. Genes targeted by microRNAs and ontologies and diseases associated to the targeted genes were identified using the miR-Ontology Database (http://ferrolab.dmi.unict.it/miro/).

Results and Discussion

General features of CNVs confirmed by MLPA

Overall, the 123 selected CNVs consist of 65 gains (ranging from 3,6 Kb to 3,0 Mb) and 58 losses (ranging from 2,2 Kb to 1,9 Mb). 11 CNVs are gene deserts, i.e., containing no genes. The chromosomal location of these CNVs are shown in Figure 3. 16 CNVs are *de novo* (11 losses and 5 gains) (see Figure 4). 83 CNVs are inherited from a healthy parent, whereas for 24 CNVs it was not possible to establish if they were *de novo* or *inherited* because one or both parents were unavailable for the analysis. In many instances, a given CNV resulted specific to a given patient, whereas each of 5 CNVs was shared by two unrelated patients, and only 1 CNV resulted shared by six unrelated patients (see figure 5).

What can we say about the pathogenic role of these CNVs? A first important consideration concerns the phenotypic heterogeneity of the patients we have studied by aCGH/MLPA. Indeed, beside the intellectual deficit that is present in all patients (though the IQ appeared significantly variable across the subjects), the population of MR individuals that we have studied displays other phenotypes and endophenotypes that are only present in subgroups of patients. These latter phenotypes include: behavioral disturbances, epilepsy and EEG anomalies, dysmorphisms just to mention few relevant items. Then, *a priori* one expects a wide spectrum of etiological factors (genetic, environmental) to be potentially involved in the expression of these phenotypes and the variable degree of MR also not

necessarily involving the CNV borne by the patient. Consistent with this prediction, our results show that not only very few patients share the same deleted/duplicated genomic region and genes, also the analysis of biofunctions performed using Gene Ontology (GO) (results not shown) on all genes overlapping all CNVs detected in all MR patients did not yield statistically significant GO terms (no gene enrichment). In other words overall the genes overlapping CNVs appear to be associated to a wide range of biological processes and molecular functions. Another problematic aspect of this study concerns the assessment of the pathogenic role of the inherited CNVs for which no phenotype is seen in the parents. Consequently, the genes overlapping the *de novo* CNVs represent a set of more promising candidates for their involvement in MR. A strong argument in favour of the potential pathogenic role of the CNVs detected in MR patients we have studied is provided by the observation that about 65 % of the 123 CNVs were not found in an ethnically/geographically-matched population of 400 neurotypical individuals also analyzed by us with MLPA in this study. Another consideration concerns the number of genes involved in a CNV. Generally speaking, a larger number of genes duplicated or deleted suggest a higher chance of a pathogenic role for a given CNV. However, in this case it is difficult to say whether all genes in that CNV contribute to the expression of the phenotype. In contrast, for CNVs involving one (or very few) gene it should be easier to call that gene as a candidate for MR in a particular patient. Finally, a recurrent CNV

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among some MR patients (we have found several cases of this type in our study, see above) was also taken into account in our evaluation of the potential pathogenic role of CNVs.

All the above considerations were translated in an algorithm that we have used to select the best candidates among the many CNV identified in patients (see flowchart in fig. 6).

A recurrent CNV on chromosome 15q11.2

The only CNV shared by six patients consists of a deletion on chromosome 15q11.2 whose size ranges from 20,307,869 bp to 20,773,190 bp removing the TUBGCP5, NIPA1, NIPA2, and CYFIP1 genes (see figure 5). This deletion partly overlaps the Prader–Willi/Angelman (PWS/AS) syndrome region in all six patients, but the deleted patients do not display the phenotypic traits typical of PWS/AS. This deletion has been already described in nine MR patients who also share with our patients several clinical features, including delayed motor and speech development, dysmorphisms and behavioural problems (ADHD, autism, obsessive–compulsive behaviour) [9] (see Table 1 for a summary the clinical features present in the six patients).

This deletion was not found in 400 normal individuals tested by us with MLPA and by the 350 controls tested (see Figure 7) in the study of Doornbos et al. [9]. Another interesting finding was that all six patients also share astigmatism, a sign that was not reported in the study of Doornbos et al. [9]. This latter discrepancy between the two studies may be simply explained by

the fact that in the study of Doornbos et al. [9] astigmatism was not evaluated. The four genes overlapping the deletion on chromosome 15q11.2 are: TUBGCP5, NIPA1, NIPA2, and CYFIP1 genes. Below we report a brief description on the function of and other information (knockout mouse model) on these genes in relation to the clinical phenotype seen in the six patients.

TUBGCP5 (Tubulin complex-associated protein 5), or GCP5, is a gene that contains 23 exons and spans 46.8 kb. The gene is ubiquitously expressed, but the highest expression is found in the subthalamic nuclei of the brain (KIAA1899 in Nagase et al. [10]). These nuclei are involved in ADHD and obsessive–compulsive behaviour [11]. The deletion of this gene could thus explain not only the behavioural problems seen in our patients, but also the more severely affected   behaviour associated to Prader–Willi syndrome patients with a type I deletion that overlaps the deletion that we have found in the six patients. No knockout mouse is currently available. **NIPA1** is the "Nonimprinted gene in PWS/AS chromosome region 1"; it contains 5 exons and spans 41.1 kb. This gene is highly expressed in neuronal tissue and encodes a putative membrane   transporter, most likely involved in intracellular magnesium transport. Gain-of-function mutations are described in patients with spastic paraplegia type 6 [12, 13]. No knockout mouse is currently available. The **NIPA2** gene contains 7 exons and spans 29 kb; it encodes a membrane transport protein that plays a role in renal magnesium metabolism [14]. There is no further information about the function of this gene or

the encoded protein [15]. No knockout mouse is currently available. Finally, the **CYFIP1** gene, ''Cytoplasmic FMRP interacting protein 1'', contains 1253 amino acids and is a member of a highly conserved protein family. CYFIP1 interacts with FMRP (fragile X mental retardation protein) and GTPase RAC1, a small GTP-binding protein that is involved in the development and maintenance of neuronal structures. CYFIP1 is present in synaptosomal extracts, indicating co-localisation with FMRP and RAC1 in dendritic fine structures [15, 16]. Related to CYFIP1 gene, a model knock-out mice, has been studied by Nishimura et al. [17]. Authors identified a potential molecular link between FMR1-FM and dup(15q), the cytoplasmic FMR1 interacting protein 1 (CYFIP1), which was up-regulated in dup(15q) patients. Authors also confirmed this link in vitro by showing common regulation of two other dysregulated genes, JAKMIP1 and GPR155, downstream of FMR1 or CYFIP1 and confirmed the reduction of the Jakmip1 protein in Fmr1 knock-out mice, demonstrating in vivo relevance.

Based on the roles that NIPA1 and CYFIP1 have in cell processes, it can be hypothesised that they are important in neurodevelopment, and thus their haploinsufficiency might play a role in the motor and speech delays seen in all six of our patients. None of the four genes involved in this microdeletion are known to undergo imprinting.

A clear statement on the pathogenic role of this deletion in our patients as well in the patients studied by Dombos et al., [9] is

not possible at this time due to the fact that the latter Authors describe in their study that in some cases the deletion is transmitted to the children from *unaffected* parents. However, it can be hypothesized that other modififier loci may be required together with this deletion to express the abnormal phenotypes observed in all these patients. Thus, further studies are required to establish the existence of a novel microdeletion syndrome possibly linked to this CNV of 15q11.2.

Three de novo CNVs each borne by a different patient containing only 1 gene

Each of three (FBXO11, SOX5 and EHBP1) genes were present in three de novo CNVs detected in 3 patients respectively. We investigated further on these three genes because they comply with most stringent selection criteria (described above). Detailed information on their function is reported below.

SOX5 (**Transcription factor SOX-5)**

Member Of:	Sox
Entrez Gene Name:	SRY (sex determining region Y)-box 5
Synonym(s):	A730017D01RIK, AI528773, LOC312831, L-SOX5, MGC124352, MGC35153, Sox5
Source Id:	--
Protein Family, Domain:	DNA binding, HMG box, protein heterodimerization, transcription factor, transcription regulator
Subcellular Location:	Nucleus
Canonical Pathway:	Wnt/β-catenin Signaling
microRNA:	MIR125B1, MIR125B2, MIR15A (includes EG:406948), MIR224 (includes EG:407009), MIR34A (includes EG:407040)

Top Findings from Ingenuity Knowledge Base ([show all 166 categorized literature Findings](#))

regulates:	COL2A1, BMP6, ACAN, RUNX2, HSPG2 (includes EG:3339), FGFR3, Cbp/p300
regulated by:	NFkB, HRAS, EDN1, ABL1, BCR, IL3, retinoic acid, SOX9, SCXA, Wnt, RUNX1, RUNX2, BRD2, RAC1, CDC42
binds:	SOX6, SOX5 (includes EG:6660), SMAD7, SMAD1, SMAD5, Cbp/p300, MIR224 (includes EG:407009), MIR125B1, MIR15A (includes EG:406948), MIR125B2, MIR34A (includes EG:407040), LIPE, FGF3
role in cell:	commitment, apoptosis, development
disease:	rheumatoid arthritis, Parkinson's disease, acquired immunodeficiency syndrome, ischemic stroke, coronary artery disease, non-insulin-dependent diabetes mellitus, insulin-dependent diabetes mellitus, hypertension, Crohn's disease, bipolar disorder, schizophrenia

Descriptions from External Databases

Entrez Gene Summary:	This gene encodes a member of the SOX (SRY-related HMG-box) family of transcription factors involved in the **regulation of embryonic development and in the determination of the cell fate**. The encoded protein may act as a transcriptional regulator after forming a protein complex with other proteins. The encoded protein may play a role in chondrogenesis. A pseudogene of this gene is located on chromosome 8. Multiple transcript variants encoding distinct isoforms have been identified for this gene. [provided by RefSeq]

GO Annotations

Molecular Function:	transcription factor activity; protein binding; promoter binding; protein heterodimerization activity
Biological Process:	in utero embryonic development; transcription from RNA polymerase II promoter; **central nervous system neuron differentiation**; cell fate commitment; regulation of transcription; negative regulation of transcription, DNA-dependent; positive regulation of transcription from RNA polymerase II promoter; oligodendrocyte differentiation; cartilage development; regulation of timing of neuron differentiation
Cellular Component:	nucleus

FBXO11 **F-box only protein 11**

ntrez Gene Name:	F-box protein 11
Synonym(s):	C80048, FBX11, FLJ12673, JF, MGC156416, MGC44383, PRMT9, UBR6, UG063H01, VIT1
Source Id:	--
Protein Family, Domain:	enzyme, F-box domain, histone-arginine N-methyltransferase, protein binding, zinc finger domain
Subcellular Location:	Unknown
Canonical Pathway:	--
microRNA:	MIR124-1 (includes EG:406907), MIR124-2 (includes EG:406908), MIR124-3 (includes EG:406909), MIR129-1 (includes EG:406917), MIR129-2 (includes EG:406918), MIR184 (includes EG:406960), MIR199A1, MIR199A2, MIR204 (includes EG:406987), MIR24-1 (includes EG:407012), MIR294 (includes EG:100049712), MIR298 (includes EG:723832), MIR325 (includes EG:100313970), MIR339 (includes EG:100314146), MIR34A (includes EG:407040), MIR34C (includes EG:407042)

Top Findings from Ingenuity Knowledge Base (show all 38 categorized literature Findings)

regulates:	--
regulated by:	--
binds:	SKP1, CUL1, COPS5, COPS6, RBX1 (includes EG:9978), TXN, EFHD1, HSPE1, GXYLT1, MIF, GDI1, PSMA6, TP53, TKT, MIR339 (includes EG:100314146)
role in cell:	--
disease:	--

Descriptions from External Databases

Entrez Gene Summary:	This gene encodes a member of the F-box protein family which is characterized by an approximately 40 amino acid motif, the F-box. The F-box proteins constitute one of the four subunits of ubiquitin protein ligase complex called SCFs (SKP1-cullin-F-box), which function in phosphorylation-dependent ubiquitination. The F-box proteins are divided into 3 classes: Fbws containing WD-40 domains, Fbls containing leucine-rich repeats, and Fbxs containing either different protein-protein interaction modules or no recognizable motifs. The protein encoded by this gene belongs to the Fbxs class. It can function as an arginine methyltransferase that symmetrically dimethylates arginine residues, and it acts as an adaptor protein to mediate the neddylation of p53, which leads to the suppression of p53 function. This gene is known to be down-regulated in melanocytes from patients with vitiligo, a skin disorder that results in depigmentation. Polymorphisms in this gene are associated with chronic otitis media with effusion and recurrent otitis media (COME/ROM), a hearing loss disorder, and the knockout of the homologous mouse gene results in the deaf mouse mutant Jeff (Jf), a single gene model of otitis media. Alternatively spliced transcript variants encoding distinct isoforms have been identified for this gene. [provided by RefSeq]

GO Annotations	
Molecular Function:	ubiquitin-protein ligase activity; protein binding; zinc ion binding; protein-arginine N-methyltransferase activity; metal ion binding
Biological Process:	protein modification process; ubiquitin-dependent protein catabolic process; protein ubiquitination
Cellular Component:	ubiquitin ligase complex; nucleus; cytoplasm

EHBP1 (**EH domain-binding protein 1**)

Entrez Gene Name:	F-box protein 11
Synonym(s):	C80048, FBX11, FLJ12673, JF, MGC156416, MGC44383, PRMT9, UBR6, UG063H01, VIT1
Source Id:	--
Protein Family, Domain:	enzyme, F-box domain, histone-arginine N-methyltransferase, protein binding, zinc finger domain
Subcellular Location:	Unknown
Canonical Pathway:	--
microRNA:	MIR124-1 (includes EG:406907), MIR124-2 (includes EG:406908), MIR124-3 (includes EG:406909), MIR129-1 (includes EG:406917), MIR129-2 (includes EG:406918), MIR184 (includes EG:406960), MIR199A1, MIR199A2, MIR204 (includes EG:406987), MIR24-1 (includes EG:407012), MIR294 (includes EG:100049712), MIR298 (includes EG:723832), MIR325 (includes EG:100313970), MIR339 (includes EG:100314146), MIR34A (includes EG:407040), MIR34C (includes EG:407042)

Top Findings from Ingenuity Knowledge Base (show all 38 categorized literature Findings)	
regulates:	--
regulated by:	--
binds:	SKP1, CUL1, COPS5, COPS6, RBX1 (includes EG:9978), TXN, EFHD1, HSPE1, GXYLT1, MIF, GDI1, PSMA6, TP53, TKT, MIR339 (includes EG:100314146)
role in cell:	--
disease:	--

Descriptions from External Databases	
Entrez Gene Summary:	This gene encodes a member of the F-box protein family which is characterized by an approximately 40 amino acid motif, the F-box. The F-box proteins constitute one of the four subunits of ubiquitin protein ligase complex called SCFs (SKP1-cullin-F-box), which function in phosphorylation-dependent ubiquitination. The F-box proteins are divided into 3 classes: Fbws containing WD-40 domains, Fbls containing leucine-rich

repeats, and Fbxs containing either different protein-protein interaction modules or no recognizable motifs. The protein encoded by this gene belongs to the Fbxs class. It can function as an arginine methyltransferase that symmetrically dimethylates arginine residues, and it acts as an adaptor protein to mediate the neddylation of p53, which leads to the suppression of p53 function. This gene is known to be down-regulated in melanocytes from patients with vitiligo, a skin disorder that results in depigmentation. Polymorphisms in this gene are associated with chronic otitis media with effusion and recurrent otitis media (COME/ROM), a hearing loss disorder, and the knockout of the homologous mouse gene results in the deaf mouse mutant Jeff (Jf), a single gene model of otitis media. Alternatively spliced transcript variants encoding distinct isoforms have been identified for this gene. [provided by RefSeq]

GO Annotations	
Molecular Function:	ubiquitin-protein ligase activity; protein binding; zinc ion binding; protein-arginine N-methyltransferase activity; metal ion binding
Biological Process:	protein modification process; ubiquitin-dependent protein catabolic process; protein ubiquitination
Cellular Component:	ubiquitin ligase complex; nucleus; cytoplasm

Though based on our selection strategy all three genes have passed several selection steps suggesting they would be good candidates for MR, the most compelling support based on the available information in the literature and databases (summarized above) is for SOX65.

One microRNA (hsa-miR-150) overlaps a CNV_gain on chromosome 19 borne by one RM patient

miRNAs are small non-coding RNA molecules that regulate the translation of target mRNAs. Binding of miRNAs to target mRNAs results in translational arrest and, in some instances, transcript degradation. At least 1000 miRNAs have been identified in the human genome, and most of them have their genes in the

non-coding regions. Of all human whole genome mRNAs, approximately one-third are predicted to be miRNA targets by bioinformatics analysis. These non coding RNAs can simultaneously control post-transcriptionally the expression levels of hundreds of genes

miRNAs have key roles in diverse physiological processes, including nervous system development and function and the evidences that they are involved in disease etiology are steadily increasing.

Abnormal variation of the expression levels of a microRNA may deregulates the synthesis of the many proteins encoded by its mRNA targets . Thus, because a microRNA embedded in a CNV is expected to lead to increased repression (CNV gain) or upregulation (CNV loss) of its mRNA targets, we have screened CNVs detected in MR patients to look for overlapping microRNAs. Interestingly, using an in silico approach we have found that patient # 03177 bears a de novo CNV_gain on chromosome 19q13.33 overlapping the hsa-miR-150 microRNA. The duplicated genomic region also overlaps 40 coding genes listed below: FUT1, FGF21, BCAT2, HSD17B14, PLEKHA4, PPP1R15A, TULP2, NUCB1, DHDH, BAX, FTL, GYS1, RUVBL2, LHB, CGB, SNAR-G2, CGB2, CGB1, SNAR-G1, CGB5, CGB8, CGB7, NTF4, KCNA7, SNRNP70, LIN7B, C19orf73, PPFIA3, HRC, TRPM4, SLC6A16, CD37, TEAD2, DKKL1, CCDC155, PTH2, SLC17A7, PIH1D1, ALDH16A1, FLT3LG, RPL13.

We have also queried several microRNA databases (Targetscan, miRanda, PicTar) to identify which mRNA targets are regulated by hsa-miR-150. Only mRNA identified as targets in all three databases were considered. By this procedure we selected 15 mRNAs predicted to be regulated by this microRNA, (listed below): CACNA1G, CALCR, COL4A4, EGR2, FOXO4, FZD4, GABRG2, GLDC, GLP1R, NOTCH3, PAFAH1B1, PAK1, PPARGC1A, RAD23B, SLC2A4.

An IPA analysis was then performed to uncover possible associations between the (i) coding genes overlapping this CNV or (ii) the mRNAs/protein targets for the hsa-miR-150 from one side and the biofunctions related to nervous system development and function from the other side. Many statistically significant neurofunctions were identified. In Table 2 we list a selection of the statistically significant IPA ontologies associated to the genes regulated by the has-miR-150

Correlation of genotype (genes in CNV) to the clinical phenotype in patient # 03177

Patient # 03177 has a complex phenotype that can be summarized in the following list of clinical signs:

Mental retardation (mild), Chiari malformations type I and syringomyelia, white-matter abnormalities, (hypodensity), cerebral edema, partial epilepsy, paroxysmal manifestations, episodes of crisis, psychosis, familial headache, delayed psychomotor

development, hypotonia, dorsal-lumbar scoliosis, hearing
disorder, sleep disorder.

It is interesting to note that several genes regulated by has-miR-150 have been individually implicated in previous studies in clinical phenotypes that overlaps those present in this patient [i.e., PAFAH1B1 and PPARGC1A in schizophrenia (psychotic manifestations are present in our patient), GABRG2 CACNA1G in epilepsy, FZD4 in deafness (mouse), NOTCH3 an CALCR in migraine, PAFAH1B1 FGF2R (the receptor for FGF2) in Chiari syndrome type I (see Box below for details)].

Looaking at the many functions (see Table 2) played the genes regulated by has-miR-150 in the morphogenesis of neurons, nervous tissue and CNS as well as in bone and connective tissue morphogenesis it is tempting to speculate that dosage variation in this single miRNA at one or more critical developmental stages would be largely responsible for the spectrum of phenotypic manifestations displayed by patient # 03177 especially mental retardation, epilepsy, Chiari syndrome, migraine. We also need to emphasize that certain genes (e.g., EGR2) among those regulated by the miRNA appear to act pleiotropically on multiple cellular pathways, in different cell types and tissues as well as in concert with the other genes regulated by the same miRNA.

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What are the Chiari malformations? a sketch on phenotypic and biological aspects

_____

Chiari malformations (CMs) are structural defects in the cerebellum. If there is an occipital bone hypoplasia with foramen magnum obstruction at the lower rear of the skull , the cerebellum and brainstem can be pushed downward to form the so-called cerebellar tonsils through the foramen magnum. The resulting pressure on the cerebellum can block the flow of cerebrospinal fluid (the liquid that surrounds and protects the brain and spinal cord) thus giving rise to syringomyelia. This condition can cause a range of symptoms including dizziness, muscle weakness, numbness, vision problems, headache, and problems with balance and coordination. There are three primary types of CM. The most common is Type I, which may not cause symptoms and is often found by accident during an examination for another condition.

As far the embryological cause of this condition is concerned it has been postulated that the etiology of CM1 involves underdevelopment of the occipital bone, perhaps due to abnormal development of the occipital somite originating from the paraxial mesoderm, resulting in overcrowding in the posterior fossa. In other words, current findings suggest that the bony components of the posterior fossa are not fully developed in these patients, supporting the concept that CM1 is a disorder of the paraxial mesoderm

It is hypothesized that genetic factors play a role in the etiology of abnormalities of these hindbrain structures.

It is noteworthy that certain genes regulated by the miRNA and several statistically significant IPA terms associated to these genes are involved in the morphogenesis of bone and cerebellum namely (taken from Table 2):

- purkinje cell degeneration (CACNA1G)

- ossification of bone (CALCR, EGR2)

- developmental process of bone (CALCR, GR2, GLP1R)

- resorption of bone (CALCR, GLP1R)

- development of rhombomere 3 (EGR2)

- development of rhombomere 5 (EGR2)

- development of rhombomere 6 (EGR2)

- degeneration of cerebellum (FZD4)

Conclusions

In summary, the results of our study show that MLPA is a valid and efficient (large number of samples can be processed simultaneously) method for the evaluation of cryptic genomic imbalances detected by array−CGH in mental retardation and other disorders. Although the molecular bases of mental retardation still remain largely unknown our study has generated a set of novel candidates CNVs and genes etiologically linked to MR. Confirmation of the potential pathogenic role of such CNVs and genes shall await the description of other MR patients bearing the same genomic imbalances and phenotypes. Such *reverse phenotyping* approach shall be increasingly effective in

the near future thanks to the increased use of high throughput technologies such as whole-genome aCGH and next-generation sequencing. As a result of special interest from this thesis there is the likely identification of a novel microduplication syndrome in a MR patient with a complex phenotype involving a microRNA overlapping a CNV of chromosome 19.

Acknowledgements

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Figures

Figure1: MLPA technique

1. Denaturation and Hybridization

PCR primer sequence X

PCR primer sequence Y

Stuffer sequence

Hybridization sequence (left)   Hybridization sequence (right)

2. Ligation

3. PCR with universal primers X and Y
exponential amplification of ligated probes only
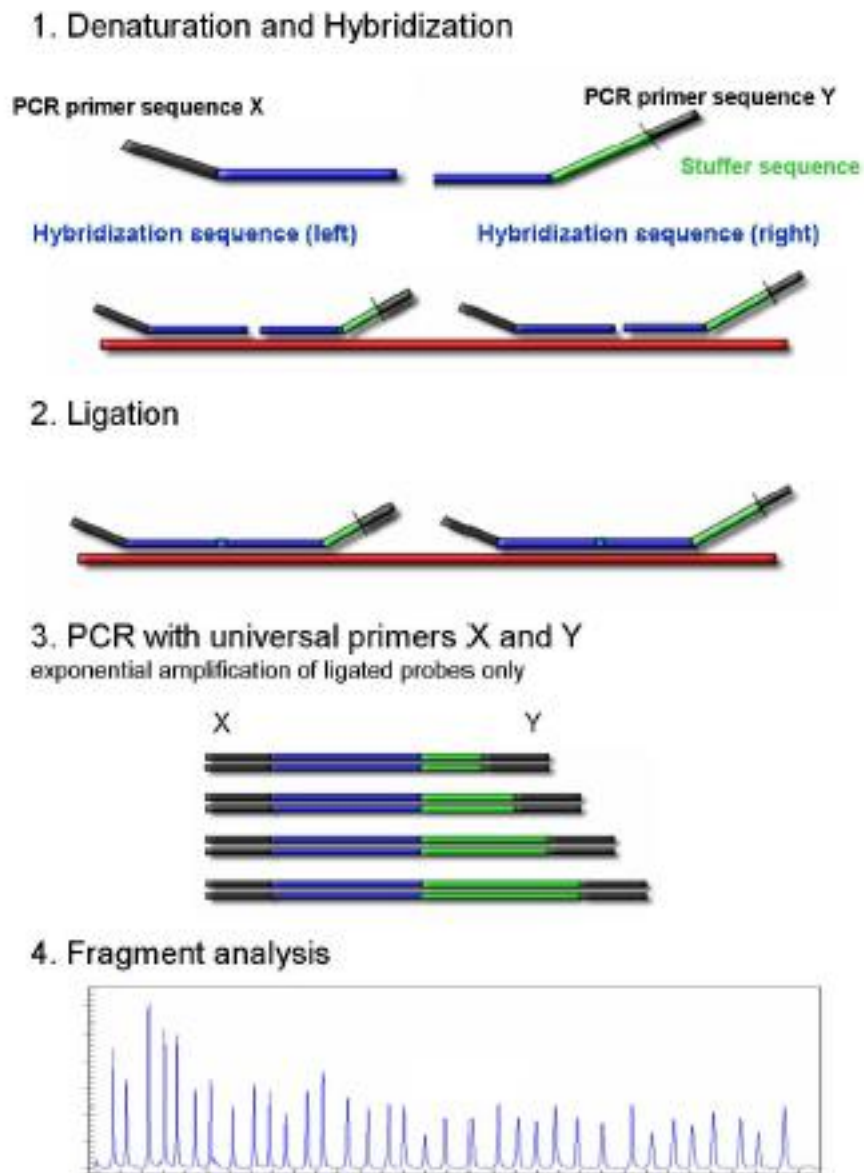
X   Y

4. Fragment analysis
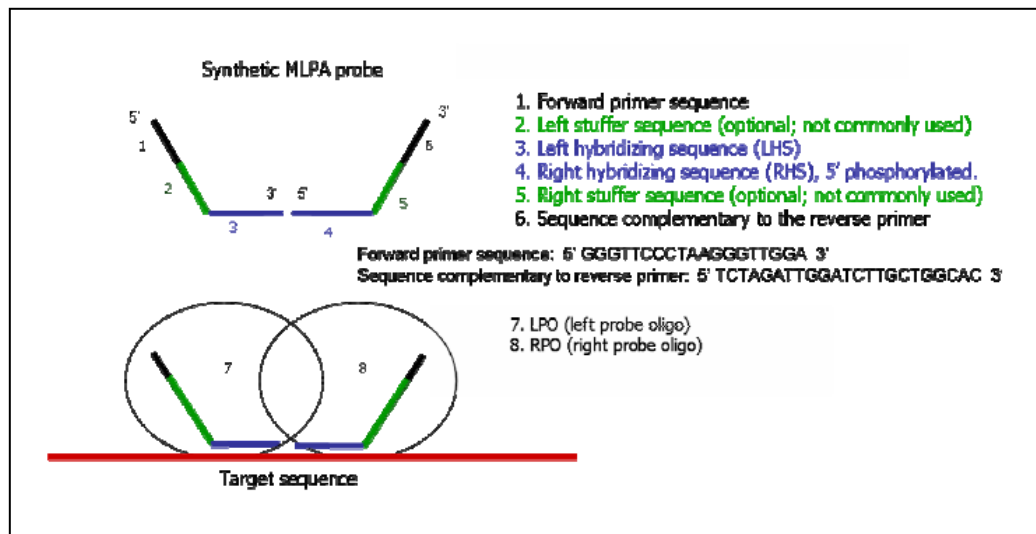
Figure 2: MLPA probe terminology

Figure 3: Chromosomal positions of Copy Number Variants identified by aCGH and MLPA in 120 mentally retarded patients
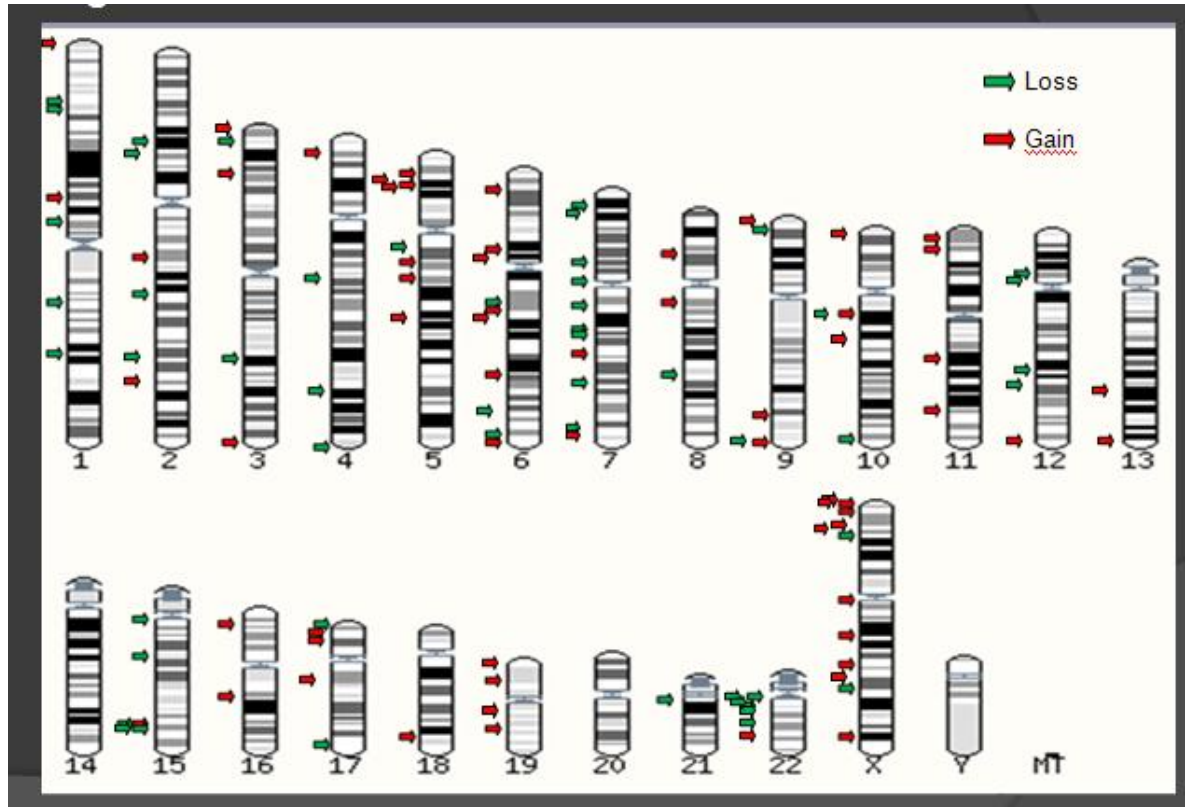
Figure 4: De novo CNVs identified in this study

N° paziente	sesso	cromosoma	citobanda	Kb del/dup	gain	n geni	geni
01395	F	chrX	(p22.12)	212,26	gain	4	MAP7D2 EIF1AX SCARNA9L RPS6KA3
03177	F	chr19	(q13.33)	915,265	gain	41	FUT1 FGF21 BCAT2 HSD17B14 PLEKHA4 PPP1R15A TULP2 NUCB1 DHDH BAX FTL GYS1 RUVBL2 LHB CGB SNAR-G2 CGB2 CGB1 SNAR-G1 CGB5 CGB8 CGB7 NTF4 KCNA7 SNRNP70 LIN7B C19orf73 PPFIA3 HRC TRPM4 SLC6A16 CD37 TEAD2 DKKL1 CCDC155 PTH2 SLC17A7 PIH1D1 ALDH16A1 FLT3LG RPL13
03401	F	chr7	(q22.1)	1310,94	gain	39	C7orf59 C7orf43 GAL3ST4 GPC2 STAG3 GATS PVRIG SPDYE3 PMS2L1 PILRB PILRA ZCWPW1 MEPCE C7orf47 LOC402573 TSC22D4 C7orf51 AGFG2 LRCH4 FBXO24 PCOLCE MOSPD3 TFR2 ACTL6B GNB2 GIGYF1 POP7 EPO ZAN EPHB4 SLC12A9 TRIP6 SRRT UFSP1 ACHE MUC17 TRIM56 SERPINE1 AP1S1
01512	M	chr3	(q29)	1676,481	gain	12	TNK2 SDHALP1 TFRC ZDHHC19 OSTalpha PCYT1A TCTEX1D2 TM4SF19 UBXN7 RNF168 C3orf43 WDR53 FBXO45 LRRC33 C3orf34 PIGX PAK2 SENP5 NCBP2 LOC152217 PIGZ MFI2 DLG1 BDH1
03861	F	chr5	(q21.1)	1798,318	gain	6	SLCO4C1 SLCO6A1 PAM GIN1 HISPPD1 C5orf30
02944	F	chr2	(p16.3)	21,022	loss	1	FBXO11
03309	M	chr17	(q25.3)	41,334	loss	2	WDR45L RAB40B
03696	M	chr2	(p15)	117,359	loss	1	EHBP1
02919	M	chr6	(p21.32)	128,446	loss	6	LYPLA2P1 KIFC1 PHF1 CUTA SYNGAP1 ZBTB9
02985	F	chr22	(q11.21)	139,387	loss	5	DGCR6 PRODH DGCR5 DGCR9 DGCR10
03633	M	chr1	(p34.2)	195,344	loss	6	LEPRE1 C1orf50 CCDC23 ERMAP ZNF691 SLC2A1
03056	F	chr5	(q32)	204,831	loss	4	JAKMIP2 SPINK1 SCGB3A2 C5orf46
02416	M	chr12	(p12.1)	218,98	loss	1	SOX5
02824	M	chr16	(p11.2)	578,662	loss	28	SLC7A5P1 SPN QPRT C16orf54 MAZ PRRT2 C16orf53 MVP CDIPT LOC440356 SEZ6L2 ASPHD1 KCTD13 TMEM219 TAOK2 HIRIP3 INO80E DOC2A C16orf92 FAM57B ALDOA PPP4C TBX6 YPEL3 GDPD3 MAPK3 LOC100271831 CORO1A
03009	F	chrX	(p22.31)	1029,209	loss	4	HDHD1A STS VCX PNPLA4
03001	F	chr2	(p25.2)	1110,158	loss	3	SOX11 LOC150822 LOC400940

16 patients (5 gain – 11 loss)

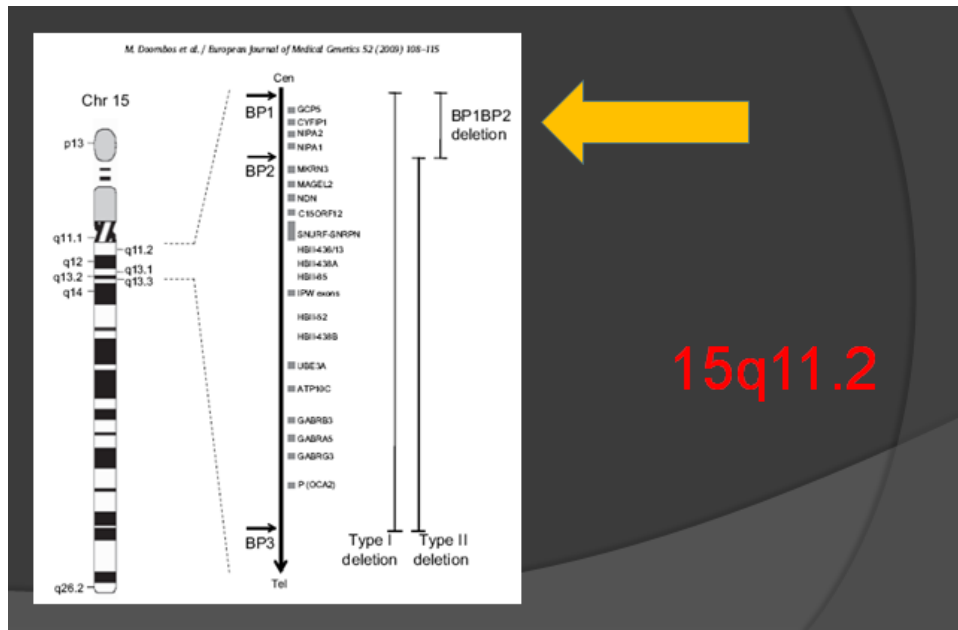Figure 5: CNV in 15q11.2 region shared by six unrelated MR patients

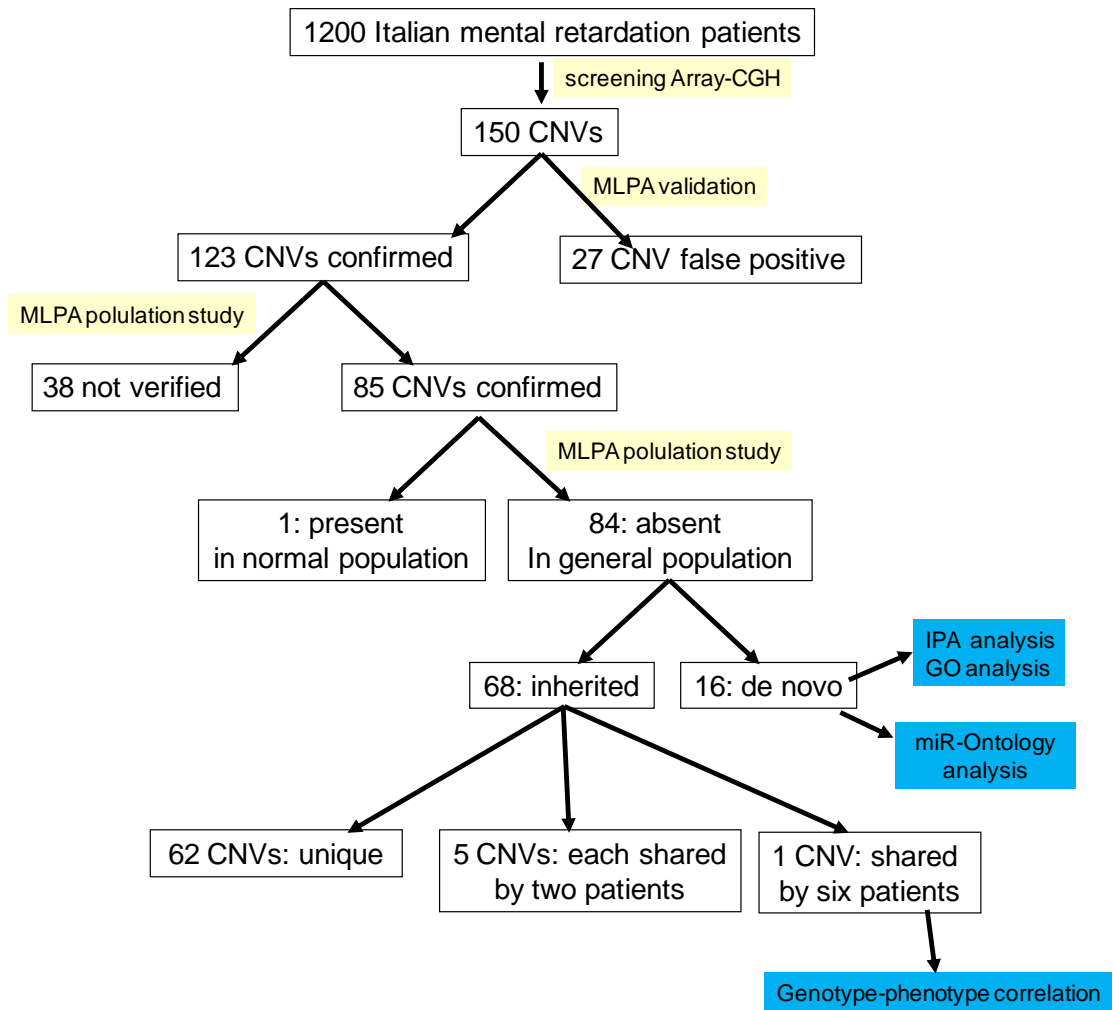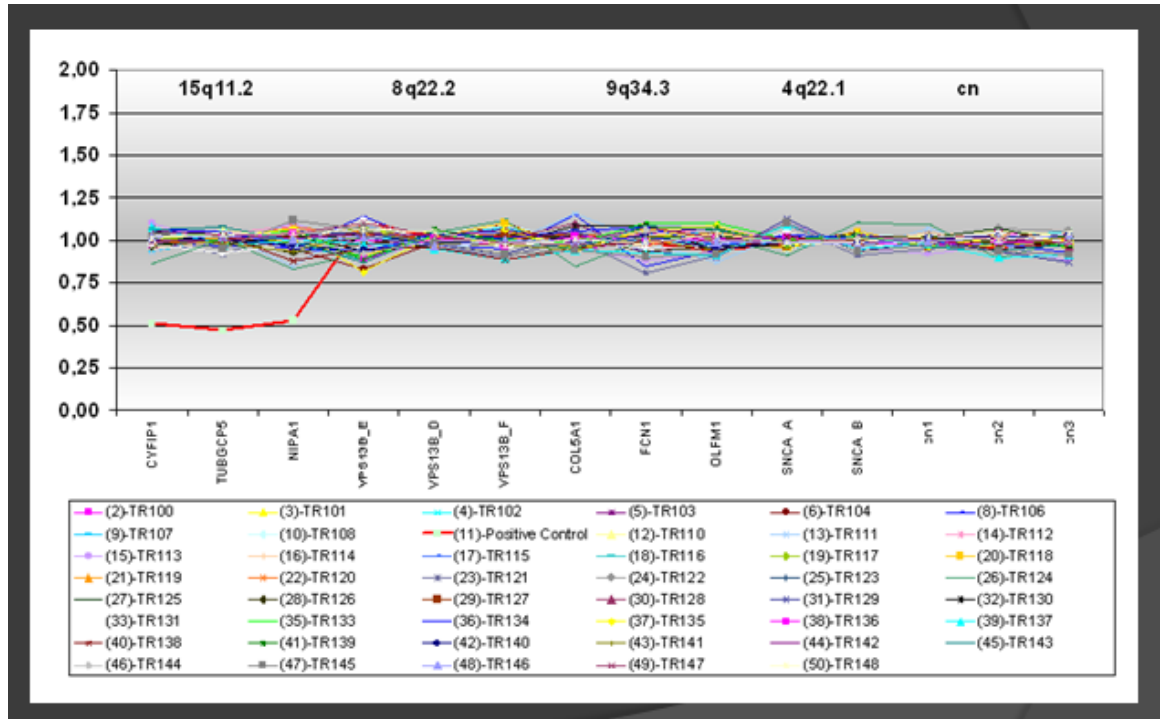Figure 6: Algorithm used to select the best candidates genes

Figure 7: A typical MLPA Analysis performed in the general population and carried out on 4/123 CNVs identified by our study (see methods for explanations).

Tables

Table 1: Overview of the clinical features present in five out

patients sharing a deletion on chromosome 15q11.2

	03103	03476	03598	03290	02224
Motor Delay	+	+		+	+
Speech Delay	+	+	+	+	+
Dysmorphisms		+	-	+	+
behavioural deficit		+		+	
Learning disabilities	+		+	+	
Astigmatism	+	+	+	+	+

Table 2: significant IPA ontologies associated to the genes regulated by the has-miR-150

Function Annotation	B-H p-value	Molecules	# Molecules
absence seizure	4,86E-02	GABRG2	1
purkinje cell degeneration	4,24E-02	CACNA1G	1
neurological disorder	1,27E-02	CACNA1G, COL4A4 (includes EG:1286), EGR2, FZD4, GABRG2, GLDC, GLP1R, PAFAH1B1, PAK1, PPARGC1A, SLC2A4	11
ossification of bone	1,27E-02	CALCR, EGR2	2
developmental process of bone	1,45E-02	CALCR, EGR2, GLP1R	3
resorption of bone	1,66E-02	CALCR, GLP1R	2
differentiation of connective tissue cells	1,83E-02	CALCR, PPARGC1A, SLC2A4	3
mass of connective tissue	2,17E-02	CALCR, SLC2A4	2
Alport's syndrome	2,10E-02	COL4A4 (includes EG:1286)	1
strength of neuromuscular junctions	1,45E-02	COL4A4 (includes EG:1286)	1
apoptosis of Schwann cells	2,87E-02	EGR2	1
Charcot-Marie-Tooth disease	4,16E-02	EGR2	1
congenital hypomyelination	1,60E-02	EGR2	1
Dejerine-Sottas disease	1,67E-02	EGR2	1
development of motor nucleus of trigeminal nerve	1,27E-02	EGR2	1
development of peripheral nervous system	4,86E-02	EGR2	1
development of rhombomere 3	1,67E-02	EGR2	1
development of rhombomere 5	1,45E-02	EGR2	1
development of rhombomere 6	1,27E-02	EGR2	1
development of trigeminal motor neurons	1,27E-02	EGR2	1
differentiation of Schwann cells	3,11E-02	EGR2	1
formation of rhombomere 3	1,27E-02	EGR2	1
formation of rhombomere 5	1,27E-02	EGR2	1
fusion of cranial nerve ganglion	1,45E-02	EGR2	1
fusion of cranial nerve ganglion	1,45E-02	EGR2	1
guidance of motor axons	3,35E-02	EGR2	1
misrouting of axons	1,45E-02	EGR2	1
myelination of neurons	2,38E-02	EGR2	1

myelination of peripheral nerve	1,45E-02	EGR2	1
proliferation of Schwann cells	3,47E-02	EGR2	1
quantity of motor neurons	4,90E-02	EGR2	1
differentiation of neuroglia	1,67E-02	EGR2, NOTCH3	2
development of brain	1,45E-02	EGR2, NOTCH3, PAFAH1B1	3
deafness of mice	2,99E-02	FZD4	1
degeneration of cerebellum	2,38E-02	FZD4	1
development of ear	3,11E-02	FZD4	1
formation of body axis	2,71E-02	FZD4	1
binding of hippocampus	1,45E-02	GABRG2	1
childhood absence epilepsy	1,67E-02	GABRG2	1
childhood absence epilepsy	1,67E-02	GABRG2	1
fear response by mice	3,35E-02	GABRG2	1
generalized epilepsy with febrile seizures plus	1,67E-02	GABRG2	1
generalized tonic-clonic seizure	4,90E-02	GABRG2	1
hyperpolarization	4,55E-02	GABRG2	1
idiopathic generalized epilepsy	2,20E-02	GABRG2	1
myoclonic seizure	3,60E-02	GABRG2	1
panic-like anxiety	3,35E-02	GABRG2	1
status epilepticus	3,71E-02	GABRG2	1
Parkinson's disease	4,86E-02	GABRG2, GLP1R, PAK1	3
glycine encephalopathy	1,60E-02	GLDC	1
fragility of bone	1,45E-02	GLP1R	1
osteopenia of tibia	1,60E-02	GLP1R	1
vasodilation of artery	4,90E-02	GLP1R	1
learning	2,37E-02	GLP1R, PAFAH1B1	2
locomotion	4,86E-02	GLP1R, PAFAH1B1	2
disease of connective tissue	1,45E-02	GLP1R, PPARGC1A	2
movement of mice	2,91E-02	GLP1R, PPARGC1A	2
cadasil	1,45E-02	NOTCH3	1
commitment of neurons	4,67E-02	NOTCH3	1
differentiation of neuronal progenitor cells	3,75E-02	NOTCH3	1
neurogenesis	2,91E-02	NOTCH3, PAFAH1B1, PAK1	3
cell division of neuronal progenitor cells	1,45E-02	PAFAH1B1	1
disorganization of cortical subplate	1,27E-02	PAFAH1B1	1
ectopia of neurons	1,67E-02	PAFAH1B1	1
elongation of axons	4,67E-02	PAFAH1B1	1
excitation of collateral synapses	1,27E-02	PAFAH1B1	1

hydrocephalus of mice	4,24E-02	PAFAH1B1	1
hypertrophy of dentate granule cells	1,27E-02	PAFAH1B1	1
length of pyramidal neurons	1,27E-02	PAFAH1B1	1
lissencephaly	1,97E-02	PAFAH1B1	1
migration of neural precursor cells	2,20E-02	PAFAH1B1	1
morphogenesis of brain	2,20E-02	PAFAH1B1	1
morphology of dentate granule cells	1,45E-02	PAFAH1B1	1
positioning of cortical neurons	1,45E-02	PAFAH1B1	1
scattering of neurons	1,83E-02	PAFAH1B1	1
stratification of cerebral cortex	1,60E-02	PAFAH1B1	1
stratification of hippocampus	1,45E-02	PAFAH1B1	1
stratification of hippocampus	1,45E-02	PAFAH1B1	1
thickness of ventricular zone	1,45E-02	PAFAH1B1	1
transmigration of neurons	1,27E-02	PAFAH1B1	1
transmission of synapse	3,23E-02	PAFAH1B1	1
cell death of neurons	2,77E-02	PAFAH1B1, PAK1, PPARGC1A	3
disease of neurons	1,67E-02	PAFAH1B1, PPARGC1A	2
development of dendritic spines	2,99E-02	PAK1	1
development of dendritic spines	2,99E-02	PAK1	1
development of neuromuscular junctions	4,30E-02	PAK1	1
growth of dendrites	4,30E-02	PAK1	1
initiation of formation of dendrites	1,27E-02	PAK1	1
cell death of cerebral cortex cells	3,11E-02	PAK1, PPARGC1A	2
cell death of striatal neurons	1,27E-02	PAK1, PPARGC1A	2
apoptosis of CA1 neuron	1,60E-02	PPARGC1A	1
atrophy of neurons	3,23E-02	PPARGC1A	1
cell death of dopaminergic neurons	4,90E-02	PPARGC1A	1
cell death of neuronal progenitor cells	1,83E-02	PPARGC1A	1
damage of brain	4,86E-02	PPARGC1A	1
damage of hippocampal cells	2,20E-02	PPARGC1A	1
damage of pyramidal neurons	1,83E-02	PPARGC1A	1
degeneration of striatal neurons	2,20E-02	PPARGC1A	1
hyperactive behavior of mice	3,75E-02	PPARGC1A	1
hyperactivity of mice	2,55E-02	PPARGC1A	1
neurodegeneration of striatal	1,83E-02	PPARGC1A	1

neurons			
neurological disorder of central nervous system	**3,47E-02**	**PPARGC1A**	**1**
neurological disorder of striatum	**1,97E-02**	**PPARGC1A**	**1**
organismal abnormalities of connective tissue	**2,20E-02**	**PPARGC1A**	**1**
volume of neurons	**1,60E-02**	**PPARGC1A**	**1**

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