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Genomic and transcriptomic analyses distinguish classic Rett and Rett-like syndrome and reveals shared altered pathways

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ABSTRACT

Rett syndrome (RTT) is an X-linked neurodevelopmental disorder characterized by derangements in nervous system especially in cognition and behavior. The present study aims to understand the molecular underpinnings of two subtypes of RTT, classic RTT and Rett-like, and to elucidate common pathways giving rise to common RTT phenotype using genomic and transcriptomic approaches. Mutation screening on selected nuclear genes revealed only *MECP2* mutations in a subset of classic RTT patients. MLPA assays and mtDNA screenings were all negative. Genome-wide copy number analysis indicated a novel duplication on X chromosome. Transcriptional profiling revealed blood gene signatures that clearly distinguish classic RTT and RTT-like patients, as well as shared altered pathways in interleukin-4 and NF-κB signaling pathways in both subtypes of the syndrome. To our knowledge, this is the first report on investigating common regulatory mechanisms/signaling pathways that may be relevant to the pathobiology of the "common RTT" phenotype.
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1. Background

Rett syndrome (MIM 312750) is an X-linked neurodevelopmental disorder; first described by Rett, a Viennese pediatrician, who happened to notice peculiar behavior in two side by side sedating female patients [1]. Hagberg later on studied 35 patients and has essentially outlined the syndrome [2]. This is a disease almost exclusively encountered among autistic girls. Initially the development progresses normally for up to 18 months, then stops and succeeds a rapid deterioration of developmental milestones. By third year of life the neuro-degeneration progresses to severe dementia, acquired microcephaly, intermittent hyperventilation, severe autism, truncal ataxia and almost pathognomonic purposeful stereotypic hand movements as hand clapping or rubbing hands. The disease may remain stable for some years but slowly progresses with severe neurological abnormalities as spastic paraparesis, vasomotor disturbances and seizures. Various skeletal abnormalities and malformations are observed. The deformities related to hands and feet are studied in detail and revealed involvement of metacarpal and metatarsal bones. These malformations are seen both in typical and atypical RTT patients [3,4].

A systematic gene and related mutation screening studies linked *MECP2* as a cause of RTT [5]. The gene encodes a methyl-CpG-binding protein that represses transcription of some of the genes through interacting with histone deacetylase and corepressor SIN3A. Therefore,

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two global mechanisms of gene regulation, namely DNA methylation and histone decetylation underlie the *MECP2*'s action. Besides its role in brain, the expression of the gene shows unique developmental course in visceral organs [6]. It is well established that not all patients with the RTT have an *MECP2* abnormality [7,8]. Hoffbuhr et al. [9] indicated that only 63% with classic or typical RTT and 33% of atypical RTT patients had the *MECP2* abnormality. Another study showed that only 20% of patients carried the *MECP2* mutation [10]. Involvement of other genes for RTT has also been reported, such as *CDKL5*, *FOXG1*, and *NTNG1*. The mutations of *CDKL5* involved in early infantile epileptic encephalopathy may cause a severe atypical form of RTT. The frame-shift mutations of *CDKL5* were encountered in two girls with RTT [11]. The *FOXG1* mutations may also cause the atypical congenital variant of RTT [12]. This gene codes for a brain specific repressor of transcription and involves in the development of telencephalon. It also shares common mechanisms with *MECP2* during neuronal development. A break-point in the *NTNG1* gene was also implicated in RTT [13]. In a girl with characteristic features of RTT, a translocation with *t*(1;7) (p13.3;q31.3) was detected. In fact not only deletion or mutation but also duplication of *MECP2* may cause mental retardation and atypical RTT [14]. Besides these genes, involvement of other genes in RTT could be a possibility since there are still numerous RTT cases with no mutation in *MECP2, CDKL5*, *FOXG1*, or *NTNG1*. Several studies have been performed using different microarray-based and other techniques to evaluate alteration in gene expression in RTT [15–20]. Despite these efforts, the molecular basis and mechanisms involved in RTT-like patients and the common RTT phenotype among its subtypes remain unclear.

In this study, we sought to understand the molecular underpinnings of the two subtypes of the syndrome, classic RTT and RTT-like, and to elucidate the common pathways giving rise to common RTT phenotype using genomics and transcriptomic approaches. We performed genome-wide copy number variation (CNV) analysis using high density SNP mapping assays and identified a novel duplication in RTT-like patients. Moreover, we compared genome-wide gene expression profiles of whole blood samples from classic or typical RTT and RTT-like patients with the age–sex matching healthy controls. Recently, there is accumulating evidence indicating the similarities and common mechanisms between nerve and blood vessel wiring and function [21,22]. Such parallelism and resemblance were exploited for such genome-wide gene expression studies for the neurologic and neuropsychiatric diseases [23–26]. Using the same strategy, we identified blood gene signatures that clearly distinguish classic and atypical RTT patients from controls, as well as shared altered pathways in both subtypes of the syndrome. Thus, our results serve a window to deciphering underlying altered metabolic and signaling pathways that may be relevant to the disease pathogenesis.

2. Materials and methods

2.1. Patients

One hundred fifty patients were referred to KFSHRC clinics for the suspicion of having RTT disease during last 20 years, and 14 were re-characterized as classic RTT and two were as RTT-like according to Hagberg's criteria [27]. Among these, five patients were re-evaluated and followed-up in the clinics for at least few years. Two of these patients are from a consanguineous Saudi family (Fig. 1A) whereas the others are single cases. Two sisters showed four of the five obligatory manifestations, six of six inclusive criteria and three of 11 supportive manifestations according to criteria listed by Hagberg (Fig. 1B1–2 and B3–4) [27]. The early infantile onset precluded them from being classified as classic RTT syndrome. The detailed clinical characteristics of these two patients are given below. The other three patients who met all the postulated criteria were considered typical RTT or classic RTT. Moreover, they were being screened for *MECP2* mutations which were also found positive in these patients.

2.2. RTT-like clinical characteristics

Patient 1, a girl, is from consanguineous Saudi parents with no history of a neurological disease (Fig. 1A). She was first encountered at the age of 4 years for the evaluation of her seizure disorder (Fig. 1B-1 and B-2). Her younger sister also had the same condition (Fig. 1B-3 and B-4). She smiled at 4 months, sat at 18 months and could say syllables at 5 years of age. She had frequent vomiting, GE reflux during first year of life and repeated chest infections at 2 years of age. At third year of life, she started to show mental regression. On examination her head circumference was at 2nd percentile. She showed no eye-contact, she had hand-clapping, hand-wringing, puff blowing and bruxism. At 12 years of age her communication and social skills were at one month level. Her living skills were at 12 months and motor skills were at 20 months level. She showed increased muscle tonus in her lower extremities with brisk deep tendon reflexes and Babinski. At 16 years of age she underwent a very detailed metabolic workup including tandem MS in blood, urine GC/MS, biotinidase, blood amino acids and karyotyping; all of which were normal. The MR brain revealed mild prominence of ventricles. Her ECG was normal and her EEG on repeated occasions showed severely abnormal wave pattern with diffuse cortical neuronal irritability and predisposition to epileptic seizures with multifocal sensory generalized epileptic activities. All these findings indicated that according to the diagnostic criteria of Hagberg [27], she had 4/5 obligatory, 6/6 inclusion and 3/11 of the supportive manifestations. She did not have gait dyspraxia, breathing irregularities, neurogenic scoliosis, feet deformity, unprompted laughter, irregular breathing spells, and intensive eye communication. Considering the lack of an early infantile normal development before regression, the consensus was that she had a RTT-like syndrome.

Patient 2 is an 8-year-old sister of the previous patient at the time of initial encounter (Fig. 1B-3 and B-4). She was referred for the evaluation of her intractable complex partial seizures and secondary generalization. Her development was greatly delayed from early infancy on, for example she never smiled. On examination her head circumference was at 2nd percentile while it was at 25th percentile two years before. She showed no eye-contact, she had hand-clapping, hand-wringing, puff blowing and bruxism. Her overall developmental level was 1 year at the age of 8 years. She had increased tonus in her lower limbs with brisk deep tendon reflexes and Babinski. Her developmental delay was global and profound with no social or linguistic skills. Her EEG was markedly abnormal with diffuse background disorganization, paroxysmal activity predominantly involving the right hemisphere, mainly right centro-parietal and right occipital lobe paroxysmal discharges. All these findings indicated that according to Hagberg's criteria [27], she had 4/5 obligatory, 6/6 inclusion and 3/11 of the supportive manifestations. The consensus was that she had an RTT-like syndrome, similar to her sister, due to the lack of an early infantile normal development before regression.

2.3. Blood collection, nucleic acid isolation and PCR amplification

The collection of blood was as described before [28]. Five milliliters of whole blood from consented patients was collected into EDTA tubes for genomic DNA isolation. The DNA was isolated from the blood using PureGene DNA Purification Kit according to the manufacturer's instructions (Gentra Systems Inc., Minneapolis, MN, USA). RNA was extracted from the whole blood (2.5 ml) collected in PAXgene tubes (QIAGEN Inc., Valencia, CA, USA). Quality and quantities of the total RNA were determined by measuring the absorbance spectra on a UV/Vis spectrophotometer, the NanoDrop® ND-1000 Spectrophotometer (Nanodrop Inc., Wilmington, DE, USA), and further analyzed by RNA 6000 Nano Assay using 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The un-degraded, higher quality RNA was processed further for the real-time RT-PCR and microarray experiments. DNA

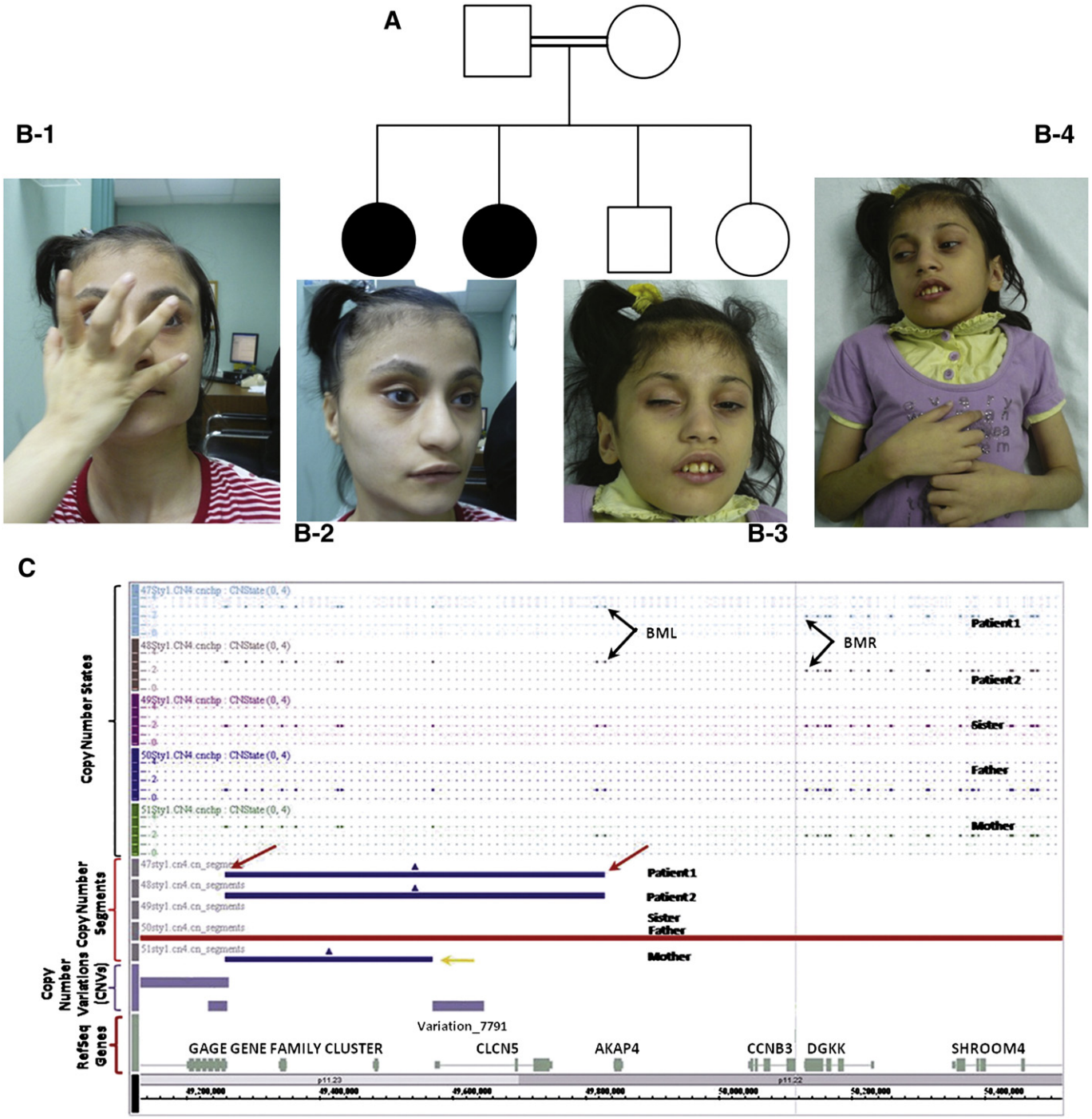

Fig. 1. (A) Family pedigree revealing two affected members (dark symbols) from a consanguineous Saudi family (B) RTT-like patients (affected girls) in the family. (C) Copy number and segregation analysis using GTC (Affymetrix). Copy number state (CNS) 2 refers to no change or wild type and 3 indicates single copy increase (duplication). Black arrows are pointing distal breakpoints. Left black arrow (BML) indicating duplication (CNS = 3) whereas right black arrow (BMR) is indicating no copy change (CNS = 2). Left and right red arrows are pointing the beginning and end of the CNV.

was amplified by PCR using intronic primers designed to amplify the coding exons of the *MECP2, CDKL5, NTNG1, FOXG1,* and *MBD2* genes. 5' and 3' UTR of *MECP2* were also included to the mutation screening study.

2.4. Mutation detection and analysis

Sequencing reactions after PCR amplifications were performed on ABI 3100 Automated DNA Sequence Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations. Briefly, purified PCR products was used for sequencing reactions using DNA Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and purified with Dye-Ex protocol before it was run on ABI 3100 Automated DNA Sequence Analyzer. Collected data from the sequencer was blasted to NCBI database, aligned with the publicly available reference sequences, and analyzed using Lasergene-SeqMan version 6.1 (DNA Star Inc., WI, USA) and ChromasPro 1.31 (Technelysium Pty Ltd, Australia).

2.5. mtDNA sequencing

DNA isolated from whole blood was used for PCR amplification of mtDNA. The entire coding region of the mitochondrial genome was amplified in 24 separate polymerase chain reactions (PCRs) using common cycling conditions as detailed elsewhere for the patients, certain family members, and controls [29]. Each successfully amplified fragment was directly sequenced using the BigDye Terminator V3.1 Cycle Sequencing kit (Applied Biosystems), and samples were run on the ABI Prism 3100 Sequencer (Applied Biosystems). Sequencing results were compared to the corrected Cambridge reference sequence [30]. All fragments were sequenced in both forward and reverse directions at least twice for confirmation of any detected variant. Sequence results were compared with the MITOMAP database [30].

2.6. Genome-wide SNP genotyping and copy number analysis

Genome-wide SNP genotyping of the patients were done using GeneChip® Human Mapping 500 K Array and Assay Kit from Affymetrix (Affymetrix Inc., Santa Clara, CA, USA) using manufacturer's protocols, manuals, and guidelines and by strictly following recommended protocols. Copy number analysis was done using Genotyping Console 3.0 (Affymetrix). We performed similar analysis on ethnically-matched 100 controls. CNV frequency between cases and controls was evaluated using Fisher's exact test. The estimated odds ratio (OR) of having a duplication in cases compared to controls as well as its confidence interval is calculated using the methodology designed to deal with the estimation of OR from a 2×2 table when one of the cells is zero [31].

2.7. X-chromosome inactivation

The assay was performed as described [32]. The experiments were repeated three times independently on the mother's DNA in the family (Fig. 1A).

2.8. Real-time RT-PCR and multiplex ligation-dependent probe amplification (MLPA)

After primer optimization, real-time RT-PCR experiments were performed on the cDNA using Quantitech SyBG Kit (QIAGEN), employing *GAPDH* as endogenous control gene. We randomly selected eight genes, *NRXN1, EPRS, DOCK8, NIPBL, SLC11A2, HNRNPL, PLA2G4B*, and *GABARAP*, and confirmed the differential expression in our patients compared to healthy controls (Supplementary Table 1). All reactions were conducted in triplicates and the data were analyzed using the delta delta C_T method [33]. The MLPA assays (SALSA MLPA kit P189 RETT-like and SALSA MLPA kit P015 *MECP2*, MRC-Holland, Amsterdam, Holland) were performed in patient samples for which mutations were not identified using standard sequencing analysis. The assay details and data analysis were done according to manufacturer's recommendations.

2.9. Affymetrix GeneChip genome-wide gene expression experiments

Briefly, the high-quality total RNA samples were converted to cDNA and then labeled with biotin during the synthesis of cRNA. The latter was fractionated and then hybridized to the gene chips. The experimental procedures and quality control procedures at each step (before hybridization as well as post-hybridization) were strictly followed according to manufacturer's instructions. Washing, staining, and scanning were performed using Affymetrix's Fluidics Station 450 and GCS 3000 G7, respectively, according to the manufacturer's instructions and guidelines. The Affymetrix GeneChip/GCOS software (Affymetrix) was used to calculate the raw expression value of each gene from the scanned image. Then the total RNA quality was assessed by the values of the 3'–5' ratios for actin and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The dChip [34] outlier detection algorithm also used to identify outlier arrays. All samples/chips passed the above-mentioned quality controls.

2.10. Microarray analysis

Following previously described protocols, the transcriptional profiles of twelve samples from RTT-like ($n=2$) with no *MECP2* mutation (denoted as "*MECP2*−"), classic RTT ($n=3$) with MECP2 mutation (denoted as "*MECP2*+") and age and sex-matched normal controls ($n=7$) isolated from whole blood were probed using Affymetrix's GeneChip® Human Genome U133 Plus 2.0 Arrays representing over 47,000 transcripts and variants using more than 54,000 probe sets. The open source R/Bioconductor packages [35] were used for processing and analysis of microarray data. The data were normalized by the GC Robust Multi-array Average (GC-RMA) algorithm [36,37]. One-way analysis of variance (ANOVA) was performed to identify genes varying significantly across RTT groups and normal controls with adjusting the probability (p) values for multiple comparisons by false discovery rate (FDR) according to Benjamini–Hochberg procedure [38]. Significantly modulated genes were defined as those with absolute fold change (FC)>1.5 and the false discovery rate (FDR) of less than 5%. The hierarchical clustering using Pearson's correlation with average linkage clustering was performed by Multi Experiment Viewer (MeV4.0) [39,40]. Functional annotation and biological term enrichment analysis were performed using DAVID Bioinformatics Resources [41], Expression Analysis Systematic Explorer (EASE) [42] and Ingenuity Pathways Analysis (IPA) 6.3 (Ingenuity Systems, Mountain View, CA). A right-tailed Fisher's exact test was used to calculate a p-value determining the probability that the biological function (or pathway) assigned to that data set is explained by chance alone. Statistical analyses were performed with the MATLAB software packages (Mathworks, Natick, MA, USA), and PARTEK Genomics Suite (Partek Inc., St. Lois, MO, USA).

3. Results

3.1. Mutation analysis

We screened *MECP2*, *NTNG1, CDKL5* and *MBD2* for any putative pathogenic changes in our patients. A subset of classic RTT patients were found to be positive only for the *MECP2* mutation and three of these *MECP2* positive patients were chosen for further studies for gene expression profiling; however we could not identify any mutation in RTT-like patients. Moreover, we sequenced *FOXG1* to identify any putative mutations, as *FOXG1* has been reported to be involved in an early onset variant of RTT. However, we could not find any mutation either.

3.2. Copy number analysis using MLPA, oligo aCGH and SNP based mapping assay and X-inactivation experiment

We first employed commercially available MLPA assays to identify any gross changes in *MECP2, CDKL5*, *NTNG1*, and *ARX* regions in all patients. There were no detectable gross changes in these genes. Next, high density SNP based Affymetrix Mapping Assays were utilized for familial segregation analysis in the RTT-like family. We identified chromosomal imbalances in the X-chromosome (duplication) comprising genes, *VCX3B, STS, VCX, PNPLA4, GAGE1, PAGE1, PAGE4, BRWD3, NSBP1 (HMGN5), SH3BGR,* in both patients as well as the mother, and *CLCN5* (possibly *AKAP4* and *CCNB3*) that is specific to the patients only (Table 1, Fig. 1C). We also screened autosomal chromosomes related pathogenic CNVs and did not find any likely candidate for further analysis. We evaluated the region on X-chromosome comprising *VCX* genes and *PNPLA4* due to its involvement in developmental delay, speech developmental and autistic behaviour, ichthyosis and mental

Table 1
List of CNVs identified at X chromosome.

CN region	Gene	Start position	End position	Genomic size	Cytoband start	Known CNV	CN state[a]	Loss/gain	Family members
1	VCX3B	6492092	6601161	109069	p22.31	YES	3	Gain	All
2	STS,VCX,PNPLA4	7261432	7991300	729868	p22.31	YES	3	Gain	Mother
3	VCX,PNPLA4	7554925	7991300	436375	p22.31	YES	3	Gain	Patients1 and 2
4	GAGE1,PAGE1, PAGE4	49257848	49569993	312145	p11.23	NO	3	Gain	Mother
5	CLCN5, AKAP4, CCNB3	49257848	49829311	571463	p11.23p11.22	NO	3	Gain	Patients1 and 2
6	BRWD3?, NSBP1, SH3BGR	80011604	80452110	440506	q21.1	YES	3	Gain	Mother and Patient1
7	No Gene	80011604	80251750	240146	q21.1	YES	3	Gain	Patient2

[a] Copy number (CN) state of 2 refers to no change or wild type and 3 indicates single copy increase (duplication).

retardation in other studies [43–45]. Since the mother was asymptomatic, we performed an X-inactivation assay on the mother's DNA using previously established methods [43]. A non-random skewness was not detected in the mother (data not shown). To exclude the possibility of being a benign CNV, we screened 100 ethnically matched healthy controls. Besides the evidence found in the Database of Genomic Variants [46], we found two control samples that had such a duplication for the regions comprising *VCX* genes and *PNLP4* genes. However, the breakpoint and segregation analysis indicated that the distal region of the duplication comprising *CLCN5* (and possibly *AKAP4* and *CCNB3*) unique to both affected ones, not shared among the parents and the healthy sister (Fig. 1C), and could not be found in unrelated ethnically matched 100 nondiseased controls as well as Database of Genomic Variants [46] (Fisher's exact test, p-value<0.001) and hence likely to be novel. The estimated odds ratio of this duplication in the diseased individuals relative to the nondiseased is OR$=1005$ [95% CI $16.4–6.1\times10^4$] (p-value<0.001).

3.3. Genome-wide gene expression changes associated with RTT subtypes

We analyzed whole-genome mRNA expression profiling of 12 samples from classic RTT and RTT-like patients, and age–sex-matched healthy controls isolated from whole blood probed using Affymetrix's GeneChip® Human Genome U133 Plus 2.0 Arrays which includes over 47,000 transcripts and variants using more than 54,000 probe sets. This technology is well established and reliable method to assess the global gene expression profiling [47]. To find differentially expressed genes (DEG) across three subjects groups, we performed one-way analysis of variance (ANOVA). An unsupervised principle components analysis (PCA), which contained about 90% of the variance in the data matrix, clearly distinguished individuals as classic RTT, RTT-like and normal controls (Fig. 2A), hence supporting the conclusion that gene expression profiles robustly reflected the clinical diagnosis. Comparing *RTT*-like group with the normal controls, we found statistically significant differential expression of 1910 genes; of which 398 significantly down-regulated, 1512 significantly up-regulated, whose expression varied at least 1.5-fold and were statistically significant at a false discovery rate of <5% between patients and normal controls. Additionally, comparison of classic RTT with the normal controls revealed significant modulation of 2369 genes (1467 down-regulated, 902 up-regulated). Comparison of gene lists with the Venn diagram approach revealed a significant overlap between classic RTT and RTT-like patients compared to normal controls (Supplementary Table 2, Fig. 2B). The significance of overlaps is calculated using hypergeometric distributional assumption [48]. Unsupervised two-dimensional hierarchical clustering of commonly dysregulated genes in both RTT-like and classic RTT compared to normal controls was performed using Pearson's correlation with average linkage clustering (Fig. 2C). The hierarchical clustering revealed clear pattern of genes deregulation defining two main transcriptome clusters, one was composed of RTT (that is also subclustered as RTT-like or classic RTT) patients, and another composed of normal controls (Fig. 2C).

3.4. Functional pathway and gene network analysis of dysregulated genes in RTT subtypes

The gene ontology (GO) and functional analysis of differentially expressed genes (up/down-regulated) in RTT-like vs. Control, classic RTT vs. Control, and DEG common to both comparison (up- or down-regulated concordantly in both subtypes, whose heatmap shown in Fig. 2C) were performed using the Ingenuity knowledge base and using DAVID Bioinformatics Resources [41]. The biological functions assigned to the data set are ranked according to the significance of that biological function to the dataset. The enriched functional categories of significantly dysregulated genes for RTT-like patients showed great similarity to the classic RTT patients (Fig. 3A). Indeed, the DEG common to both subtypes were enriched with functional categories including cellular development, immune cell trafficking, nervous system development and function, cell death, cellular movement, and cellular growth and proliferation. The shared significantly altered canonical pathways included glucocorticoid receptor signaling, IL-4 signaling and NF-κB pathways. However, oxidative phosphorylation, mitochondrial dysfunction, p53 signaling, docosahexaenoic acid (DHA) signaling seem to be altered uniquely in RTT-like patients (Fig. 3B). The diseases and disorders significantly associated with the dysregulated genes include neurological disease, genetic disorder, skeletal and muscular disorders, inflammatory response and connective tissue disorders (all p-values<0.01, Supplementary Table 3). To elucidate how significantly deregulated genes shared by both subtypes of RTT are interacting with genes in various pathways, the DEG common to both subtypes were mapped to the gene networks using the Ingenuity knowledge base. The network analysis revealed potential critical regulatory role of IL1, IL1R1, TGf-β, Interferon-alpha and beta, and NF-κB in pathophysiology of both classic RTT and RTT-like syndromes (Figs. 3C and D). It appears that several altered pathways, processes and genes described in the present study are also implicated in various central nervous system disorders [49,50].

3.5. mtDNA sequencing

Since functional pathway and gene network analysis revealed perturbed mitochondria related pathways; we screened complete mtDNA genome for putative mutations in our patients. However, we could not identify any pathogenic changes in the genome. Therefore, we exclude the possibility of involvement of mitochondrial mutations in our patients.

3.6. Comparison of RTT differentially expressed genes with multi-disorder autism gene set

Recently, Wall et al. [51] created a phylogeny that grouped autism together with 13 related disorders, including mental retardation, ataxia, Rett, Fragile X, microcephaly and seizure disorder, and called them as “autism sibling disorders”. Using OMIM and GeneCards, the authors identified 66 genes that have been linked to autism as well as at least one other autism sibling disorder, and called multi-disorder

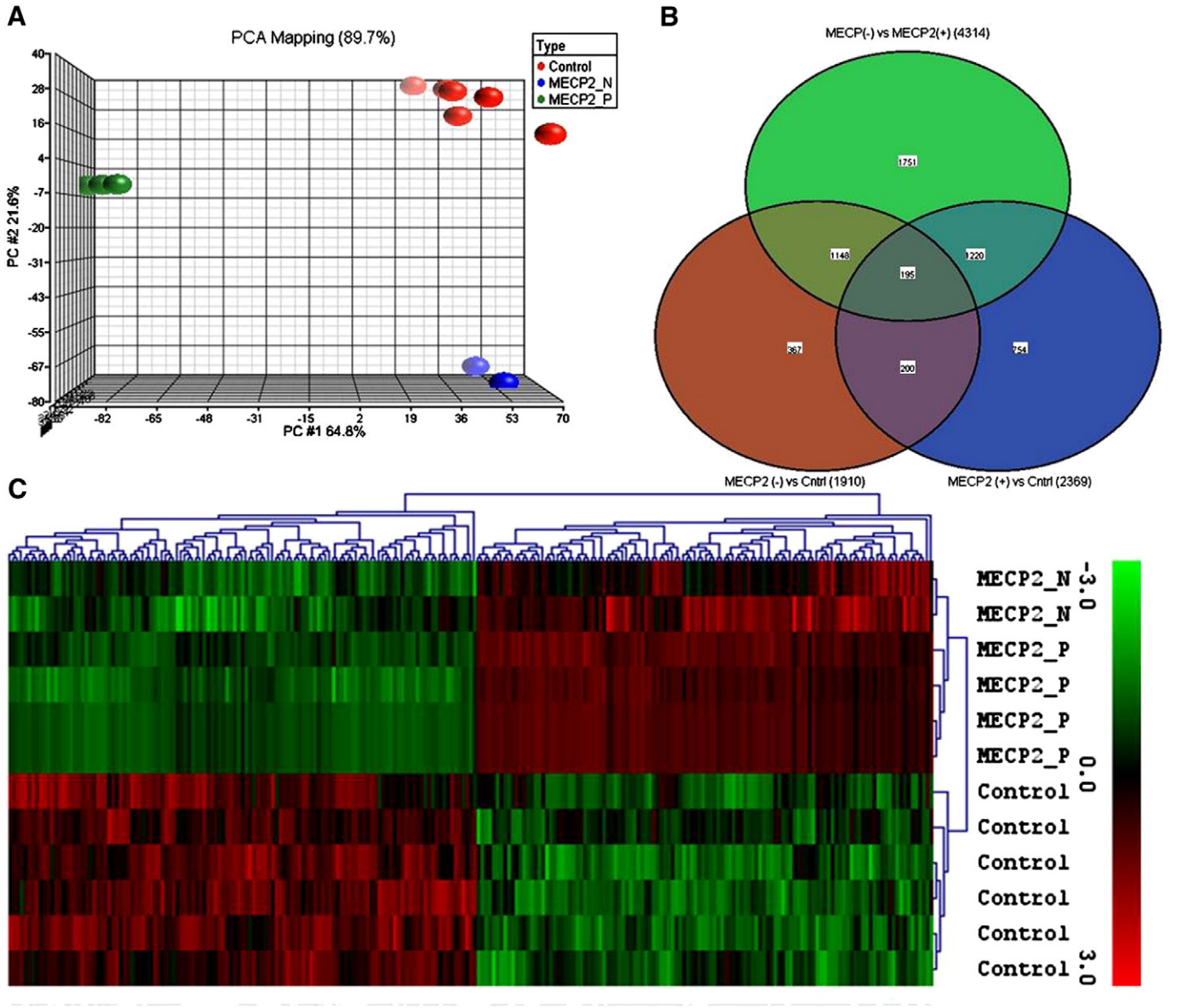

Fig. 2. (A) The three dominant PCA components that contained about 90% of the variance in the data matrix clearly distinguished individuals as RTT-like (denoted as MECP2_N or *MECP2*−), classic RTT (denoted as MECP2_P or *MECP2*+), and normal controls. (B) Venn diagram representing the common and subtype-specific genes that resulted from disease-induced significantly differentially regulated genes (FDR<5% and FC >1.5) from two RTT subtypes. (C) Unsupervised two-dimensional hierarchical clustering of commonly dysregulated genes in both subtypes compared to normal controls was performed using Pearson's correlation with average linkage clustering. The hierarchical clustering revealed clear pattern of genes deregulation defining two main transcriptome clusters, one was composed RTT patients (that is also subclustered as RTT-like (*MECP2*_N) or classic RTT (*MECP2*_P)), and another composed of normal controls.

autism gene set (MDAG) [51]. To evaluate our data, we compared the differentially regulated genes in classic RTT and RTT-like patients with the MDAG gene set. The MDAG gene set showed significant number of genes in common with our analysis results. Indeed, we found that 61% and 33% of MDAG genes were significantly dysregulated in classic RTT and RTT-like, respectively (Supplementary Table 4).

4. Discussion

The present study sought to identify common regulatory mechanisms and signaling pathways that may cause the "common RTT" phenotype" in classic RTT and RTT-like patients using genomic and transcriptomic approaches. We investigated DNA copy number alterations using 500 K SNP mapping array technology and identified a novel CNV likely to be associated with the RTT-like syndrome. The transcriptomic analysis using whole blood samples identified disease subtype-specific genes as well as genes commonly dysregulated in both subtypes; hence our results provide initial evidence indicating that whole blood gene expression may harbor valuable information for identifying etiological subsets of RTT and exploring its pathophysiology.

Copy number variations (CNV) are DNA segments with gains or losses in copy number longer than 1 kb compared to a reference genome. Increasing evidence has shown important causal role of CNVs in human disease [52,53]. We performed genome-wide copy number variation (CNV) analysis using high density SNP mapping assays, and identified several known CNVs as well as a novel duplication. The breakpoint and segregation analysis indicated that the distal region of the duplication comprising *CLCN5* present only in patients and not found in parents, healthy sibling, and unrelated ethically matched 100 nondiseased controls (p-value $= 1.9 \times 10^{-4}$). This gene is responsible for chloride transport and its mutations have been associated with DENT' s disease, an X-linked renal tubular disorder [54].

The use of whole blood as a surrogate tissue to study potential changes in brain gene expression that potentially underlie neurological

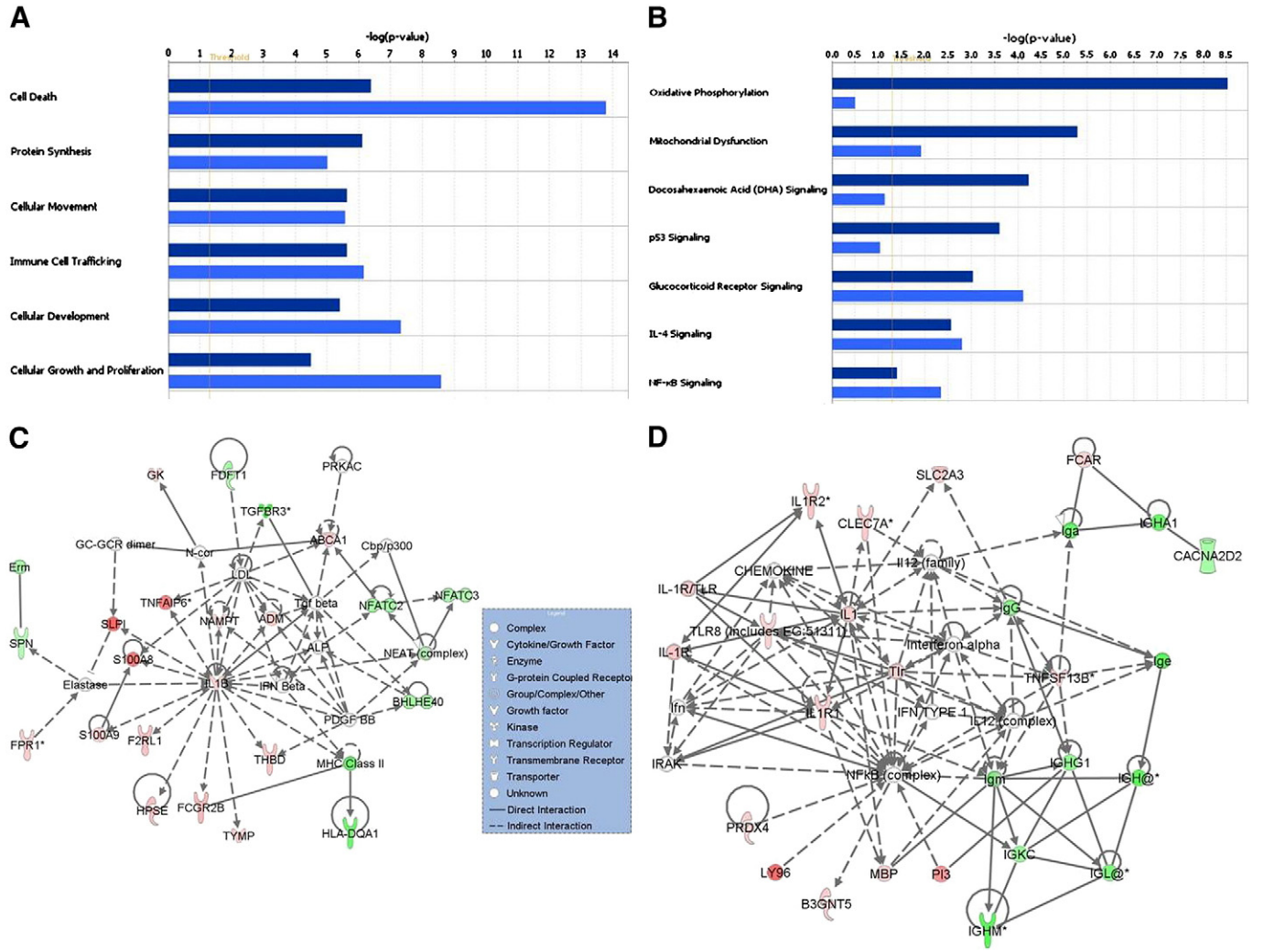

Fig. 3. (A) Functional and (B) canonical pathway analysis of DEG (up- or down-regulated) in classic RTT and RTT-like patients. *X*-axis indicates the significance (−log *p*-value) of the functional/pathway association that is dependent on the number of genes in a class as well as biologic relevance. Dark bars represent RTT-like, and light bars represent classic RTT. The threshold line represents a *p*-value of 0.05. (C) Gene network analysis of DEG commonly dysregulated in both RTT subtypes. Top two scoring gene interaction networks (with highest relevance scores) were shown. Nodes represent genes, with their shape representing the functional class of the gene product, and edges indicate biological relationship between the nodes (*see legend*). Green (red) indicates down- (up-) regulated, in RTT compared to controls. The color intensity is correlated with fold change. DEG, differentially expressed genes.

disorders has been exemplified in a number of studies. Indeed, gene expression profiles of different brain regions have been shown to have significant similarity to whole blood [25]. The previous studies have found that cells derived from peripheral blood could be used to assess neurological disease-associated gene signatures [23,26,55,56]. In our study, the gene expression profiling clearly distinguished individuals as classic RTT, RTT-like and normal controls (Fig. 2A), hence supporting the conclusion that gene expression profiles robustly reflected the clinical diagnosis, and exploited the usefulness of using whole blood, an easily accessible tissue, in identifying etiological subtypes of RTT.

The functional pathway and network analysis revealed that primary immunodeficiency signaling, interleukin-4 (IL-4) and NF-κB signaling pathways were significantly altered in both RTT-like and classic RTT patients (Fig. 3). NF-κB's involvement in the nervous system was comprehensively detailed in a previous report [57]. As a pathway and a complex system with various members, NF-κB plays critical role in nervous system development and function, particularly in synaptic transmission, plasticity, cognition and behavior [57]. Indeed, recent work has identified NF-kappaB signaling defect as a cause of autosomal-recessive mental retardation [58]. In this study, the functional analysis indicated that notable number of genes commonly dysregulated in both subtypes of RTT syndrome were significantly associated with immune, inflammatory response and nervous system development and function (p-value<0.001), that is consistent with the previous studies of neurological disorders [23,55,59]. Indeed, many genes that were once thought to encode proteins relevant only to the immune system-including cytokines, chemokines, major histocompatibility complex (MHC) are now known to have major functions in the central nervous system (CNS) at all stages of development [60,61]. Numerous cytokines with clearly defined functions in the immune system, including IL-1β and IL-4, are expressed in the developing CNS and show a key role in the regulation of cognitive function [62]. Most recently, the immune malfunction is suggested as a potentially contributory or even causative factor in the etiology of neurodevelopmental, cognitive, and psychiatric diseases [63].

Bioinformatics and gene ontological analyses revealed that the differentially expressed genes shared by both subtypes involved in calcium homeostasis and cholesterol metabolism (*NFAT, CACNA2D2, ERM* and *FDFT1)*. The initial step in neuronal connections is outgrowth of axons which require calcineurine/NFAT signaling [64]. The Ca2+/calcineurine-NFAT mediated signaling pathways regulate diverse biological functions by either stimulating or inhibiting them [65]. Calcineurin/NFAT signaling is required for neuregulin-regulated Schwann cell differentiation [66]. Schwann cells develop from multipotent neural crest

cells and wraps axons by producing myelin sheath. A normal myelination is an absolute requirement for normal neurotransmission. Neuregulins initiate an increase in cytosolic Ca2+ which in turn activates calcineurin and in downstream the NFATC3 and NFATC4. Hence, the deficient NFAT complex activity is primarily responsible for the abnormal neuronal development in both subtypes of RTT and the decrease of axonodendritic connections and disruption of synaptic proliferation [67].

The calcium channel voltage dependent alpha2/subunit 2 (*CACNA2D2*) encodes the alpha-2/delta subunit of a protein in the voltage-dependent calcium channel. The decreased mRNA in both RTT subgroups should be highly relevant to the phenotype. Indeed, the mouse mutant ducky that represent a model for absence epilepsy characterized by spike-wave seizures and cerebellar ataxia have mutations in Cacna2d2, which results in abnormalities in their Purkinje cell dendritic trees [68]. Ermin (*ERM*, also known as ERMN) is a protein homologous to Ezrin, Radixin and Moesin. It is expressed in fetal and adult brains and particularly in oligodendrocytes. During embryogenesis, its expression parallels the period when oligodendrocytes are actively myelinating. ERMN is an inducer of various cellular processes among which is the reorganization of the cytoskeleton arrangement therefore causing multiple changes in cell morphology in neural and other cells [69]. It may be hypothesized that in this manner it participates in the neuropathology observed in the brain of RTT patients.

Another gene that we found significantly altered in both subtypes of RTT is Farnesyldiphosphate farnesyl transferase (*FDFT1*) that encodes the first specific enzyme in cholesterol biosynthesis. Cholesterol is required for the development of a normal brain since it modifies the hedgehog signaling proteins in development [70]. It is noteworthy that deficiency of 7-α-dhyro-cholesterol reductase, the terminal enzyme in cholesterol biosynthesis, is the genetic cause of Smith–Lemli–Opitz (SLO) syndrome, which is an autosomal recessive malformation and mental retardation syndrome [71]. The study of Sikora et al. [71] indicated that most children with SLO syndrome have some variant of autism and suggested a link between cholesterol metabolism and autism. Also maternal apo E genotype influences the efficiency of cholesterol transport to the fetus thus modulating embryonic development and malformations [72]. These observations may also be relevant to the usual mild malformations observed among RTT girls.

The network analysis indicated potentially important role of genes involved in nervous system development and function, neuronal growth, bone changes, mitochondrial function, G protein signaling and morphogenesis (*PRDX4, IL1R1, TYMP, FPR1* and *B3GNT5).* Peroxiredoxin 4 (*PRDX4*) encodes for an antioxidant enzyme. Antioxidants govern intracellular reduction-oxidation (redox) status, which plays a critical role in activation of NF-κB transcription factor [73]. As detailed previously, NF-κB plays critical role in nervous system development and function, synaptic transmission, cognition and behavior [57]. The protein encoded by Interleukin 1 Receptor Type 1 (*IL1R1*) is a cytokine receptor that belongs to the interleukin 1 receptor family. It is an important mediator involved in many cytokine induced immune and inflammatory responses. *IL1R1* is a hypothalamic receptor which activates pathways that suppress bone formation [74]. The overrepresentation of this gene activity in both types of RTT might be linked to the well-established metacarpal and metatarsal malformations in the RTT patients.

The dysregulated genes shared by both subtypes (Supplementary Table 2) appears to play a major role in defining the RTT phenotype, regardless of *MECP2* mutation findings. The involvement of genes related to immune and nervous systems, coupled with dysregulation of the cytosolic calcium balance, cholesterol metabolism, and regulation of neuronal functions appear to be of significance to the pathobiology of RTT. Our gene network analysis revealed potential critical regulatory role of IL1, IL1R1, TGf-β, Interferon-α and β, and NF-κB in pathobiology of both typical or classic RTT and RTT-like syndromes; some of which have been implicated in various neurological disorders (Supplementary Tables 3 and 4) [49–51,59]. To validate our results, we compared the dysregulated genes in our RTT patients with the multi-disorder autism gene (MDAG) set which includes genes that have been linked to autism as well as at least one other "autism sibling disorder" compiled from 13 autism related disorders [51] and found significant number of genes in common (Supplementary Table 4). Moreover, we confirmed the high expression of eight randomly selected genes by using realtime RT-PCR from the blood of RTT patients, adding to the validity of the present observations. The genes identified in common with the MDAG set could be potentially useful as indicators of genes or metabolic/signaling pathways that may contribute to the autistic phenotype.

In conclusion, to our knowledge, this is the first study reporting a possible involvement of a novel CNV inclusive of *CLCN5* (and possibly *AKAP4* and *CCNB3)* in RTT-like syndrome; however, the link between this CNV and its pathogenic affect on RTT-like phenotype needs to be further studied. Moreover, this study reports the first time, the common regulatory mechanisms and signaling pathways that may cause the "common RTT" phenotype in RTT-like and classic RTT patients that may be relevant to the pathobiology of the RTT common clinical phenotype. Furthermore, our results provide evidence that whole blood gene expression is likely to be useful for identifying etiological subsets of RTT and exploring its pathophysiology.

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