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Gene expression profiling of mucopolidosis type IV fibroblasts reveals deregulation of genes with relevant functions in lysosome physiology

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Summary

Mucopolidosis type IV (MLIV, MIM 252650) is an autosomal recessive lysosomal storage disorder that causes mental and motor retardation as well as visual impairment. The lysosomal storage defect in MLIV is consistent with abnormalities of membrane traffic and organelle dynamics in the late endocytic pathway. MLIV is caused by mutations in the *MCOLN1* gene, which codes for mucolipin-1 (MLN1), a member of the large family of transient receptor potential (TRP) cation channels. Although a number of studies have been performed on mucolipin-1, the pathological mechanisms underlying MLIV are not fully understood. To identify genes that characterize pathogenic changes in mucopolidosis type IV, we compared the expression profiles of three MLIV and three normal skin fibroblasts cell lines using oligonucleotide microarrays. Genes that were differentially expressed in patients' cells were identified. 231 genes were up-regulated, and 116 down-regulated. Real-Time RT-PCR performed on selected genes in six independent MLIV fibroblasts cell lines was generally consistent with the microarray findings. This study allowed to evidence the modulation at the transcriptional level of a discrete number of genes relevant in biological processes which are altered in the disease such as endosome/lysosome trafficking, lysosome biogenesis, organelle acidification and lipid metabolism.

Introduction

Mucopolipidosis type IV (MLIV, MIM 252650) is an autosomal recessive lysosomal storage disease that causes severe neurologic abnormalities, achlorhydria, and visual impairment including corneal opacity, retinal degeneration, strabismus [1]. MLIV was first recognized in 1974 [2] and it predominantly affects people of Ashkenazi Jewish (AJ) origin, with an estimated frequency of 1/40000 and heterozygote frequency of 1/100 [3], [4]. The disease manifests itself in infancy, but is characterized by a slow progress, with most patients remaining in an apparent steady state for two to three decades.

Electron microscopy observations demonstrated lysosomal storage of both amphiphilic lipids in the shape of multi-concentric lamella together with granulated, water-soluble substances. Chemical analysis indicated that the storage materials, found in all the cells examined in MLIV patients, consisted mainly of phospholipids, gangliosides, neutral lipids, and mucopolysaccharides [5]. The disease was thus classified as a mucopolipidosis.

Interestingly, the storage bodies observed in MLIV fibroblasts are autofluorescent [6].

Studies with radioactive lipids (phosphatidylcholine or various gangliosides), or by fluorescent lipid analogues exogenously added to cultured fibroblasts indicated a significant retention of these compounds in lysosomes of MLIV patients during the pulse period when compared to normal control. However these substances were degraded during chase periods at a normal rate and pattern [7], [8] indicating that no defective hydrolytic activity was responsible for this heterogeneous storage. Analogue studies demonstrated a defective endocytosis process in MLIV cells [9], [10]. These observations imply that the lysosomal storage in MLIV might stem from a defect in the endocytic transport of membrane components, apparently as a result of a block in the transfer of these components down the endocytic route to the final lysosomal vacuole destination.

The loss of balance between transport of components into lysosomes and their catabolism offers an explanation for the heterogeneous nature of the stored molecules and the absence of organomegaly or skeletal deformations seen in many other lysosomal storage diseases. Nevertheless, it should be emphasized that changes in the endocytic trafficking of lipid analogues were observed in other lysosomal storage disorders [11].

MLIV is caused by mutations in *MCOLN1*, a gene mapping to 19p13.3 [12], [13], [14], [15] encoding mucolipin-1 (MLN1), a 580-amino acid protein with six predicted transmembrane domains. MLN1 presents a transient receptor potential (TRP) cation channel domain and an internal calcium and sodium channel pore region. The TRP domain spans transmembrane segments 3-6, with the putative pore-forming loop between the fifth and sixth segments. Two di-leucine motifs regulate trafficking of mucolipin-1 to lysosomes [16]. Overexpressed wild type mucolipin-1 colocalizes to late endocytic structures and induces an aberrant distribution of these compartments [17]. Two recent studies, suggest a novel role for MLN1 in regulating lysosomal function [18], [19], [20]. When intact in the lysosomes, mucolipin-1 provides a H⁺ leak pathway to prevent over-acidification of the lumen. When further acidification is needed to activate lipases, proteases or other lysosomal functions, MLN1 is then cleaved by a CatB dependent pathway that may be activated by very acidic lysosomal pH (pH_L). Continue insertion and cleavage of MLN1 results in cycles of decrease and increase in pH_L that is needed for the normal functioning of lysosomal lipases. MLIV associated mutations in mucolipin-1 disrupt regulation of pH_L and lead to aberrant activity of lysosomal lipases and the MLIV phenotype. MLN1 biological role seems to be highly conserved during evolution. A loss-of-function mutation in the *C. elegans* mucolipin-1 homologue, *cup-5*, was shown to result in decreased degradation of endocytosed protein and accumulation of large vesicles [21].

Overexpression of this protein caused the opposite phenotype, suggesting a role for CUP-

5 in the regulation of endocytosis. More recent studies demonstrated that CUP-5 functions in the biogenesis of lysosomes originating from hybrid organelles [22].

The aim of this study is to gain further insight into the MLIV phenotype through the use of the DNA microarray technology. The comparison of the gene expression profiles of cultured MLIV fibroblasts and healthy ones allowed us to identify a substantial number of differentially expressed genes, many of which have been shown to be relevant for the physiology and the biogenesis of the lysosomal compartment.

Materials and methods

Cell Lines

For expression profiling studies, GM02527 and GM02529 MLIV fibroblasts cell lines were obtained from the Coriell Cell Repositories (CRC), Camden, NJ, whereas for MLIV patient FM001 dermal fibroblasts have been obtained through external clinical collaborators. All patients gave their written informed consent. GM02527 is homozygous for an A->G transition at the 3' acceptor site of intron 3 (486-2 A->G mutation), resulting in skipping of exon 4; GM02529 is a compound heterozygote for a 6432-bp genomic deletion spanning a region from the 5' end of the gene to the first 12 bp of exon 7 and for 486-2 A->G splicing mutation; FM001 carries a 525insT frameshift allele which encodes a truncated protein of 148 aa. The control fibroblasts cell lines AB12, AB13 and AB14 have been derived from three different normal individuals.

Three additional fibroblast cell lines derived from MLIV patients (GM02526, GM02528, GM02629) were ordered from CRC and included in Real-Time PCR validation studies. The number of passages in tissue culture were comparable (ranging from 17th to 20th passage) among the fibroblast cell lines used in our study. Fibroblast cells were seeded in

175-cm² tissue culture flasks (Falcon), maintained in Eagle's minimum essential medium (MEM) (Euroclone Life Sciences), supplemented with 20% (v/v) fetal bovine serum (HyClone), 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine (Invitrogen Life Technologies) at 37°C in a 5% CO₂ humidified incubator. When the monolayer of adherent cells has reached confluence, they were trypsinized (0.25% trypsin; Sigma) and pelleted for the subsequent RNA extraction.

RNA extraction

Total RNA was isolated from confluent cells using Phase Lock Gel (Eppendorf) in conjunction with TRIzol (Invitrogen, Life Technologies) reagent according to manufacturers' protocols. The RNA was quantified using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc.) and checked for quality control with Agilent Bioanalyzer 2100 (Agilent Technologies). We routinely obtain a total RNA with a 260/280 absorbance ratio of approximately 1.9.

Target preparation and microarray hybridization

We pooled in a single tube equal amounts of the total RNA obtained from the three MLIV cell lines (MLIV pool). Similarly we created a pool starting from the RNAs of the fibroblasts of three normal individuals (AB pool). The cDNA target was generated using the First Strand cDNA Synthesis Kit (Amersham Biosciences), by direct incorporation of fluorescent nucleotide analogues during reverse transcription reaction. Briefly, for each labeling experiment 20 µg of total RNA were primed with anchored oligo(dT), incubated at 70°C and cooled for 5 min at 4°C. The RNA was reverse transcribed using the CyScript reverse transcriptase enzyme with dUTP nucleotide mix either with Cy3-dUTP or Cy5-dUTP (MLIV

pool was Cy5 labeled, whereas AB pool was Cy3 labeled) and incubated at 42°C for 3 hours. Following cDNA synthesis and labeling, RNA was degraded by RNase H (Roche Applied Science) treatment at 37°C for 20 minutes, alkaline hydrolysis with 2.5 M NaOH (Sigma) for 15 minutes at 37°C and neutralized with 2 M HEPES (Sigma). To minimize hybridization background and improve the sensitivity of detection of low abundance targets, unincorporated CyDye-nucleotides were removed from the reaction using the Qiaquick PCR purification kit (Qiagen). The cDNA was recovered in 60 µl of elution buffer. Labeling efficiency was assessed with the microarray module software of the NanoDrop ND-1000 spectrophotometer. For each hybridization, Cy3- and Cy5-labeled transcripts were mixed together using an equal amount of each label, assuming that the control and experiment targets were both labeled at about the same nucleotide/dye ratio. The mixed cDNAs were dried using a Microcon column (Millipore).

Human oligo arrays

The gene expression profile study was carried out using the Human 21K oligo arrays (Operon oligonucleotide set version 2.0) comprising 21,329 70-mer oligonucleotides designed from the UniGene build Hs 147 (February 2002). The oligonucleotides have been spotted in duplicate on MICROMAX glass slide SuperChip I (Perkin Elmer Life Sciences) at C.R.I.B.I. center of Padova University (<http://microcribi.cribi.unipd.it/>). Additional information on these microarrays can be found at the same URL.

Oligo array hybridization

Slides were pre-hybridized at 42°C for 3 hours with a buffer composed by 5X SSC, 0.1% SDS, 100 ng/µl salmon sperm DNA (Invitrogen, Life Technologies), 5X Denhardt's

solution. Then the slides were washed with MilliQ water and dried by centrifugation. 50 μ l of hybridization mixture, composed by 250 ng poly(dA) (Amersham Biosciences), 50% deionized formamide and 25% Hybridization Buffer Version 2 (Amersham Biosciences), was added to dried labeled cDNA, heated to 92°C for 2 min and then centrifuged for 3 minutes. The hybridization of microarray slides was accomplished under a lifter slip (Implen GmbH) in a hybridization chamber (Genetix) at 42°C for at least 18 hours. Post hybridization washes were performed at room temperature for 5 min each in 1X SSC, 0.1% SDS; 0.1X SSC, 0.1% SDS; 0.2X SSC and transferred to 0.1X SSC for 2 min. Slides were dried by centrifugation.

Data acquisition and analysis

A confocal dual laser scanner (VersArray ChipReader, Bio-Rad Laboratories) was used to acquire the hybridization data at a 5 μ m of resolution. Laser strength and sensitivity detectors settings were selected in order to obtain a balanced signal ratio of Cy5 and Cy3 channels. Each pair of 16-bit TIFF files was processed using the GenePix Pro 4.1 software (Axon Instruments). These files were firstly examined for quality control: spots showing no signal or obvious defects were accordingly flagged by GenePix Pro and excluded from further analysis, as well as all control spots. All statistical and graphical analysis were carried out in the R computing environment (<http://www.R-project.org>) using the Linear models for microarray data package (Limma) [23] which is part of the Bioconductor project (<http://www.bioconductor.org/>) [24]. For each microarray, a local background subtraction was performed followed by global lowess normalization within each slide. The box plot analysis showed that between arrays normalization was necessary in order to obtain the best approximate equivalence among all the arrays in study [25]. Therefore each array was scaled so that the log transformed ratios had the same median absolute deviation

[25]. The two replicate spots present for every gene in the arrays were used to maximize the robustness of statistical analysis via the “lmFit” function within Limma [23]. This procedure uses a pooled correlation value to generate a more robust estimate of gene expression across replicate spots, compared to a straight average of replicate spots. To assess the significance of up- or down-regulation of the genes between MLIV and AB sample pools, an empirical Bayesian (EB) method (B-test) [23] followed by moderated t-statistics with adjusted p-values (FDR adjustment) [26], implemented in Limma, were applied. Genes were then ranked as being differentially expressed in decreasing order of the B-statistic (essentially the log-odds of differential expression) and p-value. A cut-off for differentially expressed genes was best determined empirically through volcano plot analysis which allows evaluating when selected genes are well separated from the data cloud [27]. We choose those genes with $B \geq 1$, $p\text{-value} \leq 0.01$ and $M \geq 1$ or $M \leq -1$ as “up-regulated” and “down-regulated” respectively. Chromosomal distribution of differentially expressed genes was tested using the ChrEx software (unpublished).

Gene Ontology

To classify genes according to standardized Gene-Ontology (GO) vocabulary for the categories of biological processes, molecular functions and cellular components, the entire dataset of differentially expressed genes (up- and/or down-regulated) was imported into gene ontology software tools such as EASE (<http://david.niaid.nih.gov/david/ease.htm>) [28] and Onto-Express (<http://vortex.cs.wayne.edu/projects.htm>) [29]. OE automatically translates the non redundant dataset using standardized GO terms for cellular component, biological process and molecular function providing statistical significance results for each category. GO groupings were determined by comparing the number of genes expected for each GO category based on their representation on the array. Significant ($p\text{-value} < 0.05$)

differences from the expected were calculated by binomial test with a FDR-corrected significance for the dataset analyzed.

Real-Time RT-PCR

We used quantitative Real-Time RT-PCR to validate the results obtained from microarray experiments, focusing our attention on a limited number of selected genes. One microgram of total RNA from fibroblast cell lines was dissolved in 20 μ l of final volume reaction mixture. Briefly the RNA was incubated with 50 μ M Oligo(dT)₂₀ and 10 mM dNTP mix (Invitrogen, Life Technologies) at 65°C for 5 min and at 4°C for 3 min. A reaction mixture was added containing: 10X RT buffer, 25 mM MgCl₂, 0.1 M DTT, 200 U SuperScript III RT, 40 U RNaseOUT (Invitrogen, Life Technologies). An identical reaction without reverse transcriptase was performed to verify the absence of genomic DNA (no-RT control). The mixture was incubated at 50°C for 50 min, followed by 5 min at 85°C for to inactivate the reverse transcriptase. 2 units of RNase H was added to each tube and incubated for 20 min at 37°C to remove the RNA template from the cDNA:RNA hybrids. The final reaction's volume was set to 100 μ l. Real-Time RT-PCR was performed using the ICycler iQ detection system (Bio-Rad Laboratories). The PCR cycling protocol was optimized to maximize the reaction efficiency and ensure that only the target product was contributing to the SYBR Green fluorescence signal. For each quantification, a standard curve was created using suitably appropriate quantity of cDNA obtaining amplification efficiencies values close to 2 for all primer combinations.

Reactions were performed in a 25 μ l reaction volume, containing a variable concentration (from 200 to 400 nM) of specific primers, 12.5 μ l iQ SYBR Green Supermix (Bio-Rad Laboratories), and 2 μ l of reverse transcription reaction solution. The amplification profile used was: denaturation program (95°C for 3 min), 35 cycles of two steps amplification

(95°C for 15 sec and 60°C for 30 sec), and melting curve (60–90°C with a heating rate of 0.5°C/10 sec). Each reaction was performed in triplicate. Gene specific primers were designed with the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), using published sequence data from the NCBI Reference Sequence (RefSeq) database (<http://www.ncbi.nlm.nih.gov/RefSeq>), and Oligo Analyzer software (<http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/>) to avoid potential hairpin formation and self dimerization. Primer sequences are listed in Supplementary Table S1. Gel electrophoresis and the dissociation curve were used to assess the specificity of the amplicon (data not shown). To evaluate differences in gene expression we chose a relative quantification method based on the standard curve approach as described by Pfaffl, M.W. [30]. Levels of expression obtained by this method were normalized with that of an endogenous control transcript glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) for MLIV pool versus AB pool comparison and ribosomal protein, large, P0 (*RPLP0*) for GM02527, GM02529, FM001, GM02526, GM02528, GM02629 versus AB pool comparison) that does not appear to be differentially expressed under the experimental conditions assayed in our work.

Results and Discussion

In this study we exploited DNA microarray technology to compare the gene expression profiles of cells from individuals affected by mucopolisaccharidosis type IV and healthy controls. The analysis was carried out on skin fibroblasts cell lines derived from three different MLIV patients and three normal individuals. The study of gene expression profiles in a genetic disease is often impaired by the lack of suitable biological material from the patients as

source of RNA. In the case of MLIV, *MCOLN1*, the gene mutated in the disease, is ubiquitously expressed and, although psychomotor retardation is the most relevant phenotypic consequence of MLIV, tissues other than brain are affected in the disease, such as skeletal muscles, cornea, retina, pituitary gland. A relevant number of researches (described in the introduction) have been performed on fibroblasts of affected individuals demonstrating both metabolic and structural alterations with respect to normal controls. MLIV fibroblasts thus represent a suitable cell type for expression profiling experiments that may ultimately provide a global picture of the molecular changes occurring in patients' cells. Pooling of RNA samples was performed to reduce the genetic and biological variability that may occur within individuals. The pooling strategy for microarray analysis presents both advantages and disadvantages as described in a discrete number of publications [31], [32], [33]. Since our main goal is to generate the first global picture of the transcriptional changes occurring in MLIV rather than comparing the expression profiles of individual patients, we believe that pooling of biological samples is a suitable strategy for our study.

The experiments were carried out using glass slides spotted with the Operon Human Genome Array-Ready Oligo Set (Version 2.0) comprising 21,329 70-mer oligonucleotides representing the same number of genes. Oligo array hybridization, washes and scanning were performed as described in the Methods section. Analysis of four technical replicates was performed using the Limma package. The microarray data were prepared according to minimum information about a microarray experiment (MIAME) recommendations [34] and deposited in the public GEO database (<http://www.ncbi.nlm.nih.gov/geo/>). The GEO accession number for Micro-CRIBI Human Oligo Array platform is GPL2136 and for expression dataset is GSE5444.

From now on, the terms “up-regulated” or “overexpressed” will indicate genes that are expressed more than two-fold ($B \geq 1$ and $p\text{-value} \leq 0.01$) in MLIV fibroblasts than in the

pool of normal control cells. Vice versa, genes expressed more than two-fold in normal control cells (using the same statistical criteria indicated above) are defined “down-regulated”. This analysis led to the identification of 231 up-regulated and 116 down-regulated transcripts. To better visualize the results, a volcano plot of the B against M values was generated (Figure 1). No chromosomal-skewed distribution was observed for differentially expressed genes (data not shown).

Validation of microarray data

We used Real-Time RT-PCR to test the consistency and reproducibility of the DNA microarray data. The study has been carried out on a set of selected differentially expressed genes: *LAPTM5*, *LAPTM4A*, *CD63*, *CTSK*, *MANBA*, *ATP6V0E* and *VPS39* encode proteins belonging to the lysosomal compartment and are up-regulated in MLIV fibroblasts; *LDLR* and *SREBF2* are involved in lipid metabolism and are also up-regulated in MLIV; *FADS2*, one of the genes more up-regulated identified in our study and *POSTN*, down-regulated in MLIV. The Real-Time PCR data have been generated from the same MLIV and AB (normal controls) RNA pools used for microarray expression profiling studies. The data from triplicate experiments were quantified, and the expression ratios of genes between the MLIV pool and the AB pool were calculated and compared with the array data (Figure 2). There is a general agreement between the Real-Time PCR and DNA microarray datasets even if, as previously reported by other investigators, the differential expression values obtained using the two techniques are not identical. The same genes were also studied by Real-Time PCR on the RNA from skin fibroblasts of six different MLIV patient's (the three included in gene expression profiling studies plus three additional ones). The majority of the genes we tested behave similarly (Figure 2) suggesting a limited variability of gene expression among the different MLIV samples present in our study.

These results further support the effectiveness of the RNA pooling strategy for microarray experiments on MLIV fibroblasts.

Analysis of the differentially expressed genes

To facilitate the analysis of the differentially expressed genes identified, we used the publicly available software Expression Analysis Systematic Explorer (EASE) (<http://david.niaid.nih.gov/david/ease.htm>) [28] which allows to automate the process of creation of descriptive annotation tables. Furthermore, to translate the data into a more meaningful biological context and to characterize more thoroughly sets of functionally related genes, the differentially expressed datasets were subsequently organized into Gene Ontology (GO) groupings using the Onto-Express (OE) software (<http://vortex.cs.wayne.edu/projects.htm>) [29]. The results of the Onto-Express analysis are presented in Tables 1-4. Tables 1 and 2 report the Gene Ontology classification (cellular component category) for up- and down-regulated gene datasets, respectively. The biological process GO categories for up-regulated and down-regulated genes are shown in Table 3 and 4, respectively. The output of the EASE and Onto-Express software allowed us to manually curate a list of differentially expressed genes (either up- or down-regulated in MLIV fibroblasts) that are involved in important biological and molecular processes that are altered in mucopolipidosis type IV (Table 5). The relevance of these findings is discussed below for every Gene Ontology category present in Table 5.

Lysosomal genes

In our study we have identified a number of differentially expressed genes that are related to the physiology of these organelles.

The only down-regulated gene in this category is scavenger receptor class B, member 2 (*SCARB2*), encoding a type III glycoprotein located primarily in limiting membranes of lysosomes and endosomes that may participate in membrane transportation and the reorganization of endosomal/lysosomal compartment.

Twelve genes belonging to the lysosomal compartment category are up-regulated. Interestingly, a number of them is involved in the pathogenesis of autosomal recessive lysosomal storage disorders. Mannosidase, beta A, lysosomal (*MANBA*) encodes a protein involved in the pathway for N-linked glycoprotein oligosaccharide catabolism. Mutations in this gene are associated with beta-mannosidosis. Iduronidase, alpha-L- (*IDUA*) encodes an enzyme that hydrolyzes the terminal alpha-L-iduronic acid residues of two glycosaminoglycans, dermatan sulfate and heparan sulfate. *IDUA* mutations lead to mucopolysaccharidosis Type I (MPS I). Another enzyme encoding gene is the Hexosaminidase B (*HEXB*); mutations in this gene lead to an accumulation of GM2 ganglioside in neurons and neurodegenerative disorders termed Sandhoff disease. *CLN2* and *CLN3*, encode proteins that are important in lysosomal function: mutations in these, as well as other neuronal ceroid-lipofuscinosis (*CLN*) genes, cause neurodegenerative diseases commonly known as neuronal ceroid-lipofuscinoses (NCLs). Cathepsin K (*CTSK*) gene encodes a member of the papain cysteine proteinase family, and is a key component of the lysosomal proteolytic system. Mutations in the gene are responsible for an osteochondrodysplasia named pycnodysostosis. An additional cathepsin gene, *CTSF*, is up-regulated in MLIV fibroblasts.

Interestingly, gene expression profiling studies on a murine model of another lysosomal storage disorder (mucopolysaccharidosis type VII) also show an up-regulation of a number of lysosomal enzymes [35].

Other lysosomal genes that are differentially expressed are not directly involved in the pathogenesis of metabolic storage disorders. Among them lysosomal-associated protein

transmembrane 4A (*LAPTM4A*) and 5 (*LAPTM5*) genes, encoding multispanning transmembrane proteins that reside in the late endosome/lysosome. While *LAPTM4A* has a role in the transport of small molecules across membranes of these organelles [36] the biological role of *LAPTM5* is still unknown [37]. *CD63* (also known as *LAMP-3*) encodes for a membrane protein common to lysosomes, melanosomes, and platelet dense bodies [38].

Endosome to lysosome transport

The two up-regulated genes, EH-domain containing 1 (*EHD1*) and EH-domain containing 3 (*EHD3*), take part to endocytic process, in particular the EH domain has been shown to be an important motif in proteins involved in protein-protein interactions and in intracellular sorting. *EHD1* interacts with *EHD3* in regulating the movement of recycling endocytic vesicles along microtubule-dependent tubular tracks [39]. The protein encoded by *EHD1* is thought to play a role in the endocytosis of IGF1 receptor (*IGF1R*) [40]. Interestingly, *IGF1R* gene is 2.39 fold more expressed in MLIV fibroblasts.

The *VPS39* gene (also named *hVam6p*) is overexpressed 2.06 times in MLIV fibroblasts vs. controls. *VPS39* exhibits homology to the *Saccharomyces cerevisiae* vacuolar protein sorting gene product Vam6p/Vps39p. Overexpression of *VPS39* results in massive clustering and fusion of lysosomes and late endosomes into large (2-3 micron) juxtannuclear structures [41] that resemble to the aberrant lysosomes in MLIV cells. The differential expression of this gene observed in MLIV cells is particularly intriguing, considering that studies carried out on *C. elegans* strongly suggest a defect in lysosome biogenesis in cells of worms with mutations in the *cup-5* homolog of the *MCOLN1* gene [22].

Proton transport

Recently, it has been reported that the lysosomes of MLIV cells are more acidic than those of wild type cells and that MLN1 regulates lysosomal pH (pH_L) [19]. The authors demonstrated that MLN1 can function as a H^+ channel, and the increased lysosomal acidification in patients' cells is likely caused by the loss of MLN1-mediated H^+ leak. These results are in contrast with data previously presented by Bach *et al* who reported elevated lysosomal pH in mucopolipidosis type IV cells [42]. pH_L controls most lysosomal hydrolytic activities and, above all, it's essential for the normal function of lysosomes [43]. Interestingly, in our study we found overexpression of five genes encoding for subunits of vacuolar ATPase (V-ATPase) in MLIV fibroblasts (Table 5). V-ATPase is a multi-subunit enzyme complex and a member of a family of ATP-driven proton pumps responsible for the acidification of intracellular compartments such as endosomes, lysosomes, Golgi-derived vesicles, and clathrin-coated vesicles [44]. V-ATPase dependent organelle acidification is necessary for such intracellular processes as protein sorting, zymogen activation, receptor-mediated endocytosis, and synaptic vesicle proton gradient generation. Our data suggest that the overexpression of vacuolar ATPase subunits may also contribute to the acidification described in lysosomes of MLIV patients.

Cholesterol and lipid metabolism

MLIV is characterized by accumulation of cholesterol and/or glycosphingilipids in the endocytic compartments [7], a phenomenon whose molecular basis is not well understood. It has been hypothesized that the impaired endosomal/lysosomal fusion limits access of lipids to lysosomal lipases leading to the accumulation of unprocessed lipids [45]. Recent findings indicate that mutation in *MCOLN1* disrupt regulation of pH_L [19] which in turn may be responsible for the aberrant activity of lysosomal lipases. This may explain the

lysosomal accumulation of lipids in MLIV cells. It is thus interesting that cholesterol and, more generally, lipid metabolism emerged as one of the most significant GO process category in our gene expression analysis, with a range of regulation from -3 to 7.5 fold (Table 5).

The low density lipoprotein receptor (*LDLR*), 7.57-fold more abundant in the MLIV pool, encodes a cell surface protein involved in receptor-mediated endocytosis of specific ligands. Low density lipoprotein (LDL) is normally bound at the cell membrane and taken into the cell ending up in lysosomes where the protein is degraded and the cholesterol is released. It has been demonstrated that non physiological overexpression of low-density lipoprotein receptors leads to the accumulation of large amounts of LDL which eventually lead to massive intracellular lipid deposition and the formation of crystal structures [46], [47]. Interestingly, Jansen *et al.* performed a study that suggest that MLIV fibroblasts have a defect that affects the low-density lipoprotein-receptor mediated endocytosis pathway [10].

SREBF1 and *SREBF2* genes are both up-regulated in MLIV fibroblasts. Sterol-regulatory element binding proteins (SREBPs) play a key role in transcriptional regulation of cholesterol metabolism in response to cholesterol levels in the cell [48]. Binding of SREBPs to the *LDLR* promoter increases the expression of LDL receptor on the cell surface and increases the internalization of LDL from plasma, increasing cellular cholesterol levels and lowering LDL cholesterol in the plasma. The up-regulation of *LDLR* and SREBP genes observed in MLIV fibroblasts is consistent with the model proposed for sphingolipid storage diseases in which excess of sphingolipids in the endocytic compartments serve as a "molecular trap" for cholesterol, leading to a reduction in cholesterol at the endoplasmic reticulum, induction of sterol regulatory element-binding protein-1 cleavage, and up-regulation of LDL receptors [49].

This is the first study based on gene expression profiling on mucopolipidosis type IV.

In this analysis, carried out on fibroblast cell lines from three MLIV patients, allowed us to detect the modulation at the transcriptional level of a discrete number of genes belonging to biological processes which are altered in the disease such as endosome/lysosome trafficking, lysosome biogenesis, organelle acidification and lipid metabolism. To support the validity of our data, the differential expression of selected genes has been mostly confirmed in biological samples from three other MLIV patients.

In conclusion, we believe that the data presented in this study will provide useful information for a better understanding of the molecular mechanisms leading to mucopolipidosis type IV.

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Figure legends

Fig. 1. Volcano plot of statistical significance versus gene expression ratio

Values were plotted for the B and M for each transcript expressed on the array. The horizontal line, in bold, indicates $B = 0$ and the bold vertical lines the 2-fold threshold. Black symbols represent changes below the threshold, red and green symbols represent significant changes above the threshold.

Fig. 2. Validation of microarray data by Real-Time RT-PCR

Real-Time RT-PCR for selected differentially expressed genes has been carried-out on total RNA of “MLIV pool”, individual MLIV cell lines and “AB pool” (normal controls). The relative expression is represented as the mean \pm SEM, in 2log scale between “MLIV pool” vs “AB pool” together with the microarray data obtained comparing the same biological samples. The figure also shows the relative expression of the selected genes between individual MLIV cell lines (GM02527, GM02529, FM001, GM02526, GM02528 and GM02629) vs the “AB pool”. For all genes tested, with the exception of those marked with an asterisk, the results obtained were statistically significant ($p < 0.05$) based on Student's t test.

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Table 1.

Gene Ontology classification for up-regulated gene dataset.

Cellular component	Genes	p-value
Integral to membrane	48	5.25E-03
Plasma membrane	14	3.05E-02
Extracellular matrix	12	1.45E-04
Endoplasmic reticulum	12	1.95E-02
Lysosome	9	1.58E-04
Proton-transporting two-sector ATPase complex	5	1.28E-03
Golgi apparatus	4	2.00E-02

Gene Ontology classification performed by Onto-Express software.
We reported only significant cellular components categories (p-value < 0.05)
and with a number of genes greater than 3.

Table 2.

Gene Ontology classification for down-regulated gene dataset.

Cellular component	Genes	p-value
Cytoplasm	15	1.09E-02
Extracellular matrix	6	7.72E-03

Gene Ontology classification performed by Onto-Express software. We reported only significant cellular components (p-value < 0.05) and with a number of genes greater than 3.

Table 3.

Gene Ontology classification for up-regulated gene dataset.

Biological process	Genes	p-value
Signal transduction	24	3.43E-02
Cell adhesion	14	4.24E-03
Protein amino acid phosphorylation	11	4.49E-02
Lipid metabolism	9	3.73E-03
Ion transport	7	3.91E-02
ATP synthesis coupled proton transport	5	1.14E-03
Phosphate transport	5	3.85E-03
Phospholipid biosynthesis	4	1.29E-03
Cholesterol metabolism	4	1.67E-03
Endocytosis	4	1.08E-02
Blood coagulation	3	1.90E-02

Gene Ontology classification performed by Onto-Express software. We reported only significant biological processes (p-value < 0.05) and with a number of genes greater than 3.

Table 4.

Gene Ontology classification for down-regulated gene dataset.

Biological Process	Genes	p-value
Cell adhesion	11	8.691E-04
Protein folding	6	1.277E-03
Integrin-mediated signaling pathway	5	2.106E-05
Immune response	5	1.508E-02
Induction of apoptosis	4	2.068E-03
Cell motility	4	4.658E-03
Protein biosynthesis	4	1.929E-02
rRNA processing	3	2.992E-03
Generation of precursor metabolites and energy	3	3.304E-03
Transmembrane receptor protein tyrosine kinase signaling pathway	3	4.085E-03
Nuclear mRNA splicing, via spliceosome	3	1.079E-02
Intracellular protein transport	3	1.852E-02
Inflammatory response	3	2.545E-02

Gene Ontology classification performed by Onto-Express software. We reported only significant biological processes (p -value < 0.05) and with a number of genes greater than 3.

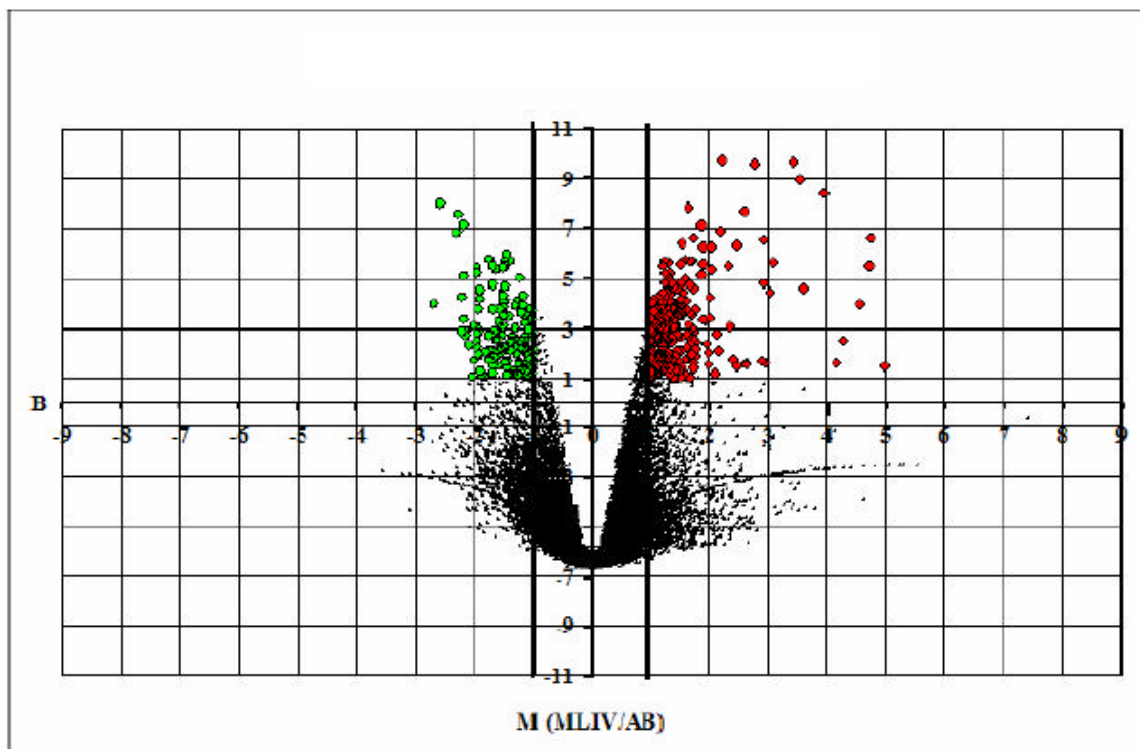
Table 5.

List of differentially expressed genes related to biological and molecular processes altered in MLIV.

Category	Molecular Function	Gene Symbol	Entrez Gene ID	Fold Change
Lysosome				
	carrier activity	<i>CD63</i>	967	2.84
	serine-type endopeptidase activity; tripeptidyl-peptidase I activity	<i>CLN2</i>	1200	2.28
	chaperone activity	<i>CLN3</i>	1201	3.39
	cysteine-type endopeptidase activity	<i>CTSF</i>	8722	2.60
	cathepsin K activity	<i>CTSK</i>	1513	6.82
	beta-N-acetylhexosaminidase activity	<i>HEXB</i>	3074	2.60
	L-iduronidase activity	<i>IDUA</i>	3425	3.66
	nucleobase transporter activity	<i>LAPTM5</i>	7805	7.57
	nucleobase transporter activity	<i>LAPTM4A</i>	9741	2.99
	beta-mannosidase activity	<i>MANBA</i>	4126	2.31
	receptor activity	<i>SCARB2</i>	950	-2.04
	hydrolase activity;sphingomyelin phosphodiesterase activity	<i>SMPD1</i>	6609	2.08
	electron transporter activity	<i>QSCN6</i>	5768	3.29
Endocytosis				
	ATP binding;DNA binding;calcium ion binding	<i>EHD1</i>	10938	2.57
	ATP binding;DNA binding;calcium ion binding	<i>EHD3</i>	30845	3.34
	GTP binding	<i>RAB32</i>	10981	2.46
	protein transporter activity;small GTPase regulatory/interacting protein activity	<i>VPS39</i>	23339	2.06
Proton Transport				
	hydrogen ion transporter activity; hydrolase activity	<i>ATP6V0D1</i>	9114	2.39
	hydrogen-transporting ATPase activity\, rotational mechanism	<i>ATP6V0E</i>	8992	2.50
	ATP binding;hydrogen ion transporter activity;hydrolase activity	<i>ATP6AP1</i>	537	2.00
	hydrogen ion transporter activity;hydrolase activity;receptor activity	<i>ATP6AP2</i>	10159	2.00

	hydrogen-transporting ATP synthase activity\, rotational mechanism	<i>ATP6V0C</i>	527	2.31
Cholesterol and Lipid metabolism				
	isopentenyl-diphosphate delta-isomerase activity;magnesium ion binding	<i>IDI1</i>	3422	2.61
	dimethylallyltranstransferase activity; geranyltranstransferase activity	<i>FDPS</i>	2224	2.61
	farnesyl-diphosphate farnesyltransferase activity;magnesium ion binding;	<i>FDFT1</i>	2222	2.86
	oxidoreductase activity	<i>DHCR24</i>	1718	2.87
	RNA polymerase II transcription factor activity;protein binding	<i>SREBF2</i>	6721	3.60
	RNA polymerase II transcription factor activity;protein binding;	<i>SREBF1</i>	6720	3.33
	lipid transporter activity;low-density lipoprotein receptor activity	<i>LDLR</i>	3949	7.57
	steroid hormone receptor activity; transcription factor activity	<i>PPARD</i>	5467	2.38
	cell adhesion receptor activity	<i>ITGA4</i>	3676	-3.34
	acetyl-CoA C-acetyltransferase activity	<i>ACAT1</i>	38	-2.37

In the category column is reported the name of a GO grouping, either cell component or biological process.



ACCEPTED

