

Analysis of Potential Biomarkers and Modifier Genes Affecting the Clinical Course of CLN3 Disease

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Mutations in the *CLN3* gene lead to juvenile neuronal ceroid lipofuscinosis, a pediatric neurodegenerative disorder characterized by visual loss, epilepsy and psychomotor deterioration. Although most CLN3 patients carry the same 1-kb deletion in the *CLN3* gene, their disease phenotype can be variable. The aims of this study were to (i) study the clinical phenotype in CLN3 patients with identical genotype, (ii) identify genes that are dysregulated in CLN3 disease regardless of the clinical course that could be useful as biomarkers, and (iii) find modifier genes that affect the progression rate of the disease. A total of 25 CLN3 patients homozygous for the 1-kb deletion were classified into groups with rapid, average or slow disease progression using an established clinical scoring system. Genome-wide expression profiling was performed in eight CLN3 patients with different disease progression and matched controls. The study showed high phenotype variability in CLN3 patients. Five genes were dysregulated in all CLN3 patients and present candidate biomarkers of the disease. Of those, *dual specificity phosphatase 2 (DUSP2)* was also validated in acutely *CLN3*-depleted cell models and in *CbCln3^{Aex7/8}* cerebellar precursor cells. A total of 13 genes were upregulated in patients with rapid disease progression and downregulated in patients with slow disease progression; one gene showed dysregulation in the opposite way. Among these potential modifier genes, *guanine nucleotide exchange factor 1 for small GTPases of the Ras family (RAPGEF1)* and *transcription factor Spi-B (SPIB)* were validated in an acutely *CLN3*-depleted cell model. These findings indicate that differential perturbations of distinct signaling pathways might alter disease progression and provide insight into the molecular alterations underlying neuronal dysfunction in CLN3 disease and neurodegeneration in general.

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INTRODUCTION

The neuronal ceroid lipofuscinoses (NCLs) are the most common inherited neurodegenerative disorders mainly affecting children. Typical symptoms comprise progressive visual loss, psychomotor deterioration and epilepsy. NCLs are incurable lysosomal storage diseases, and lysosomal accumulation of autofluorescent storage material, called ceroid lipofuscin, can be detected in almost all tissues (1). The most dramatically af-

ected organ is the brain, which undergoes massive neuronal cell atrophy and cell loss (2).

One of the most frequently occurring NCL forms in Northern European countries is caused by mutations in the *CLN3* gene (CLN3 disease, also called juvenile NCL or Batten disease). CLN3 disease starts at the age of 4–6 years with a progressive retinopathy leading to blindness. After several years, dementia, epilepsy and motor disturbances ensue.

The diagnostic hallmark of this NCL type are conspicuous vacuoles in the cytoplasm of lymphocytes (3).

CLN3 encodes a polytopic membrane protein of 438 amino acids (4). To date, >40 different mutations in *CLN3* have been described (summarized in a mutation database: <http://www.ucl.ac.uk/ncl/cln3.shtml>). CLN3 disease is recessive, and 85% of CLN3 patients carry a 1-kb deletion that causes the loss of exons 7 and 8 (5). Although the CLN3 protein is well conserved from yeast to humans, its function is a matter of debate. There are reports that CLN3 is involved in lysosomal acidification, arginine import, autophagy and apoptosis (6).

To understand the pathomechanisms underlying CLN3 disease, any natural

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Table 1. Distribution of CLN3 patients according to slow, average or rapid disease progression defined by the Index of Relative Severity.

Clinical course	Index of Relative Severity	Number of patients
Slow	>+0.5	5
Average	-0.5 to +0.5	9
Rapid	<-0.5	11

variability in the clinical course of CLN3 disease first has to be assessed. We applied a clinical scoring system to 25 CLN3 patients who were homozygous for the 1-kb deletion and classified them into patients with slow, average and rapid disease progression. To investigate whether the clinical variability can be correlated with changes in gene expression, we performed comparative genome-wide microarray analyses in lymphocytes of eight selected CLN3 patients with different rates of disease progression. We identified both genes that are dysregulated in CLN3 disease regardless of the clinical course that might be useful as biomarkers of the disease and genes for which expression correlated with clinical variability, possibly indicating differential expression of modifier genes. The general significance of selected genes of both sets was confirmed in a cell model system in which *CLN3* expression was depleted by small interfering RNA (siRNA).

MATERIALS AND METHODS

Distribution of CLN3 Patients

A total of 25 patients, 11 male and 14 female, participated in this study. Among the patients were three pairs of siblings. The mean age of the patients (corresponding to the mean time period of assessment) was 18.4 ± 6.2 years (mean \pm standard deviation [SD], range 12–34 years).

Clinical Scoring

The patients were analyzed according to a previously established clinical scoring system (7). Clinical scoring and calculation of an Index of Relative Severity

(Table 1) are described in the supplementary material.

Patients and Sample Isolation

Eight patients diagnosed with CLN3 disease and classified as having rapid ($n = 2$), average ($n = 4$) and slow disease progression ($n = 2$), and all homozygous for the 1-kb deletion in the *CLN3* gene, were recruited from the NCL specialty clinic at the University Medical Center Hamburg-Eppendorf (Table 2, patients 1–8). These eight patients did not receive anticonvulsive medication; therefore, they were particularly suitable for providing material to be used for microarray analysis. Two of the patients with average disease progression were siblings from one family, and one patient with average and one patient with rapid disease progression were siblings from another family. Seven age- and gender-matched controls were included in the microarray study.

In addition, lymphocytes from three patients with CLN3 disease, 14, 13 and 14 years old (P14M, male; P13F, female; P14F, female), who received anticonvulsive medication and who were all homozygous for the 1-kb deletion in the *CLN3* gene, and age- and gender-matched controls (C14M, C13F and C14F) were used for RNA isolation for real-time polymerase chain reaction (RT-PCR) to validate *dual specificity phosphatase 2* (*DUSP2*) upregulation. Patient P14M was classified as having average disease progression,

patient P13F as slow disease progression, and patient P14F as rapid disease progression with Indexes of Relative Severity of 0.3 (P14M), 0.8 (P13F), and -1.0 (P14F). Patient P14M received valproate, patient P13F topiramate and patient P14F valproate and lamotrigine as anticonvulsive medications.

The patients' or parents' consent for participation in this study was obtained according to the Declaration of Helsinki (1991). The study was approved by the medical ethics committee of the Ärztekammer Hamburg. Lymphocytes were prepared from fresh patient blood samples by Ficoll-gradient centrifugation (Biocoll®; Biochrom, Berlin, Germany) according to the manufacturer's protocol and used for RNA isolation (RNeasy® Micro Kit; Qiagen, Hilden, Germany).

Microarray Analysis

Procedures for RNA extraction, cDNA synthesis, labeling and hybridization were carried out according to the manufacturer's protocol (Affymetrix, High Wycombe, UK), followed by microarray data analyses, as described in the supplementary material.

Cell Culture and RNA Preparation

HeLa cells were provided by K. von Figura (University of Göttingen, Göttingen, Germany) and were cultured in Dulbecco's minimal essential medium containing 10% fetal bovine serum and antibiotics. SH-SY5Y cells were provided

Table 2. Clinical course, age and gender distribution of the studied CLN3 patients who did not receive anticonvulsants.

Clinical course	Patient	Age (years)	Gender	Index of Relative Severity
Rapid	1	12	F	-1.4
	2 ^a	12	F	-0.8
Slow	3	29	M	1.2
	4	28	M	0.8
Average	5 ^a	18	F	-0.1
	6 ^b	10	M	0
	7 ^b	9	F	-0.3
	8	12	M	-0.3

^aSiblings family 1.

^bSiblings family 2.

by E. Kramer, Center for Molecular Neurobiology Hamburg (ZMNH) (Hamburg, Germany) and were cultured in Dulbecco's minimal essential medium containing 20% fetal bovine serum, antibiotics and 1 × GlutaMAX™. *CbCln3^{Δex7/8}* cerebellar precursor cells were cultured as previously described (8). RNA was extracted from the cells using an RNeasy® Mini Kit (Qiagen) according to the manufacturer's protocol.

RT-PCR

For RT-PCR, TaqMan® Gene Expression Assays (Applied Biosystems, Darmstadt, Germany) including predesigned probes and primer sets were used. For details, see the supplementary material.

Other Methods

cDNA synthesis and posttranscriptional gene silencing with siRNA for human *CLN3* in HeLa cells and SH-SY5Y cells was performed as previously described (9).

All supplementary materials are available online at www.molmed.org.

RESULTS

Clinical Course and Classification of *CLN3* Disease Progression

A total of 25 patients, 11 male and 14 female, participated in this study. The mean age of the patients (corresponding to the mean time period of assessment) was 18.4 ± 6.2 years (mean \pm SD; range 12–34 years). Scoring of vision, intellect, language and motor function was performed, both retrospectively and prospectively, at 6-month intervals starting at birth.

First clinical signs in these studied *CLN3* patients were observed at age 5.4 ± 1.4 years (mean \pm SD; range 1.5–7 years), which for the majority of patients ($n = 17$) was visual loss. However, in a few patients, other symptoms preceded the visual deterioration such as behavioral problems ($n = 5$), motor problems ($n = 1$), language problems ($n = 1$) and seizures ($n = 1$). Interestingly, the age of occurrence of these atypical first clinical

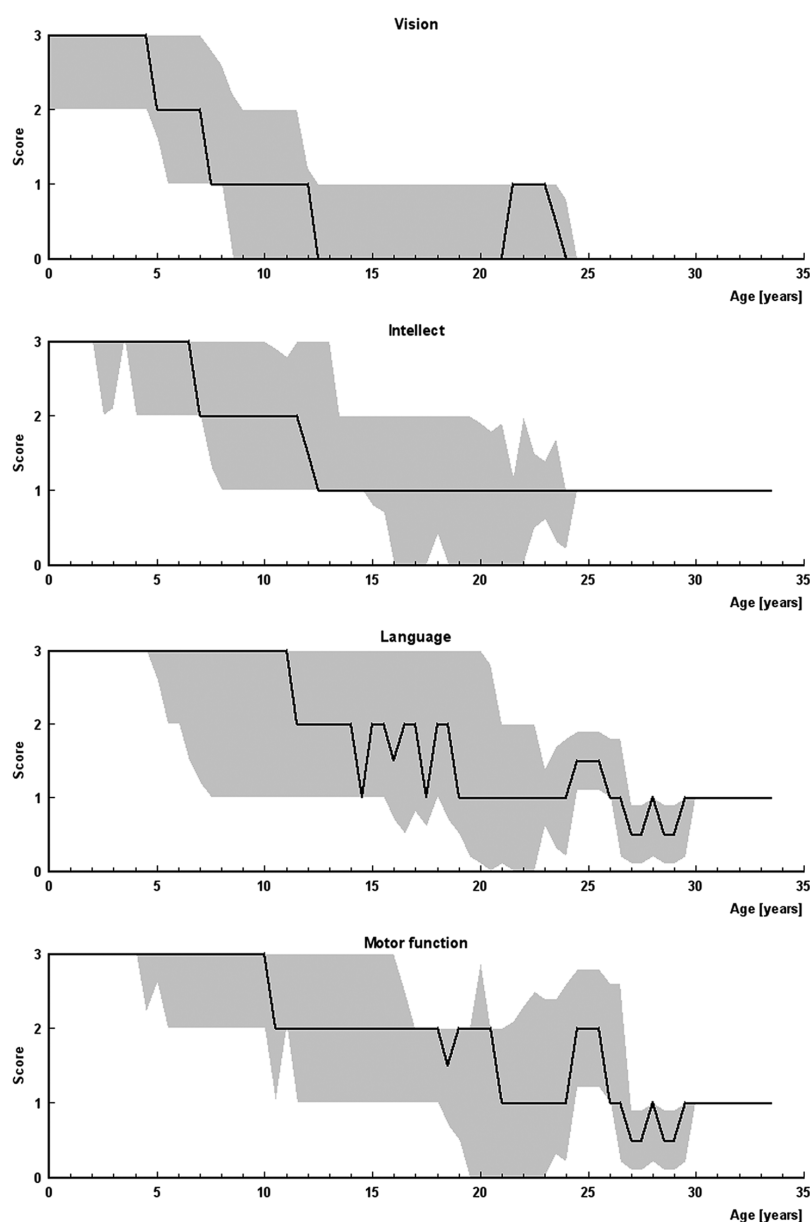


Figure 1. Clinical scoring of 25 *CLN3* patients homozygous for the 1-kb deletion in the *CLN3* gene. Scoring for each problem category was made as follows: normal function (3); problem slight, but readily recognized (2); problem severe (1); total loss of function (0). The range between the 10th and 90th percentile is represented by the shaded area. The black line represents the median.

signs was comparable to the age of onset of visual loss in the remaining 17 patients: 5.8 ± 1.1 years (mean \pm SD; range 3.5–7 years).

Figure 1 summarizes the clinical courses of all 25 patients over time for each problem category, indicating the median and the range between the 10th and 90th percentile. By calculating an

Index of Relative Severity (7), we classified 5, 9 and 11 *CLN3* patients into groups with rapid, average and slow disease progression, respectively (see Table 1), reflecting a high phenotype variability in *CLN3* patients despite an identical genetic defect.

Figure 2 compares the disease progression of two patients (1 and 2) with rapid

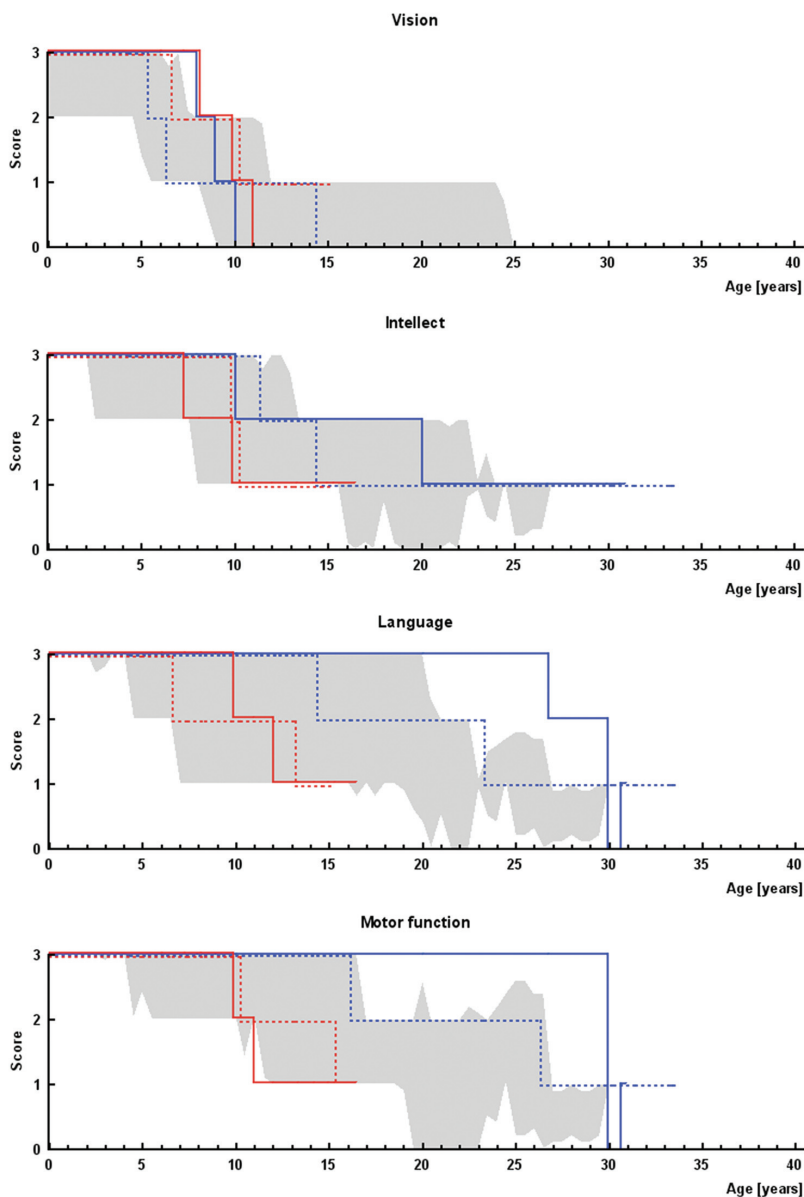


Figure 2. Comparison of clinical scoring of CLN3 patients with rapid ($n = 2$) versus slow disease progression ($n = 2$). Clinical scoring is shown in red for patients with rapid disease progression (patient 1, solid line; patient 2, broken line) and in blue for patients with slow disease progression (patient 3, solid line; patient 4, broken line). The shaded area represents the range between the 10th and 90th percentile calculated from clinical scoring of 25 patients who are homozygous for the 1-kb deletion in the *CLN3* gene (including the two patients with rapid and slow disease progression).

versus two patients (3 and 4) with slow disease progression (see Table 2). None of the four patients received anticonvulsants. Patients 1 and 2 had scorings for motor function, language and intellect that decreased rapidly over time, following the 10th percentile for intellect and

language and being briefly beyond the 10th percentile for motor function. For patient 1 (solid red line), regression of visual function was similar to the average disease course until age 10 years and led to complete blindness 1 year later. In contrast, in patient 2, regression of visual

function appeared to be slow, with scoring mostly following the 90th percentile.

Patients 3 and 4 had scorings for motor function, language and intellect mainly on or above the 90th percentile. The regression of visual function was similar to (patient 3, solid blue line), or started even earlier (patient 4, broken blue line) than, the two patients with rapid progression. Patient 3 was diagnosed with sinus bradycardia and received a pacemaker implant at age 30 years. Six months after this procedure, language and motor function each improved to score 1, which remains to date. Even though scoring of epilepsy has not been used to classify these patients, it should be noted that this patient has not had any detectable seizure to date and has a normal electroencephalogram reading.

For patient 4, scoring for intellect, language and motor function mainly remained on or above the 90th percentile until age 14.5 years and then deteriorated slowly. Like patient 3, this patient was diagnosed with sinus bradycardia and received a pacemaker implant at age 26 years. Since then, neither motor nor intellect or language deteriorated further, remaining constant to date at age 34 years.

Dysregulated Genes in CLN3 Patients

In addition to the CLN3 patients 1–4, four patients with average progression of the disease (patients 5–8) were selected whose clinical course was not influenced by anticonvulsants (see Table 2). To gain insight into molecular processes possibly related to the different disease courses in CLN3 patients, we performed RNA microarray analyses in freshly isolated lymphocytes. These cells are easily accessible, and since no culturing is required, they are not affected by subsequent handling. The entire set of the original microarray data obtained is accessible at the Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=bbqprmqisgkmgvq&acc=GSE22225>).

A total of 89 genes were dysregulated in patients with average disease progression compared with healthy controls

Table 3. List of potential modifier genes with opposite dysregulation in patients with rapid versus slow disease progression.

Gene symbol	Gene name	Fold change	
		Rapid	Slow
<i>KLF6</i>	<i>Kruppel-like factor 6</i>	+2.0	-1.9
<i>MARCKS</i>	<i>Myristoylated alanine-rich protein kinase C substrate</i>	+2.0	-1.7
<i>LOC283663</i>	<i>Hypothetical protein LOC283663</i>	+1.9	-1.7
<i>BLK</i>	<i>B lymphoid tyrosine kinase</i>	+1.9	-2.0
<i>FCGR2B/FCGR2C</i>	<i>Fc fragment of IgG, low affinity IIb, receptor (CD32)/ Fc fragment of IgG, low affinity IIc, receptor for (CD32)</i>	+1.9	-2.1
<i>SPIB</i>	<i>Spi-B transcription factor (Spi-1/PU.1 related)</i>	+1.8	-6.2
<i>MS4A1</i>	<i>Membrane-spanning 4-domains, subfamily A, member 1</i>	+1.8	-2.0
<i>RAPGEF1</i>	<i>Rap guanine nucleotide exchange factor (GEF) 1</i>	+1.8	-1.9
<i>ZNF718</i>	<i>Zinc finger protein 718</i>	+2.1	-26.9
<i>BACE2</i>	<i>β-Site APP-cleaving enzyme 2</i>	+2.1	-2.8
<i>LOC283663</i>	<i>Hypothetical protein LOC283663</i>	+2.0	-1.9
<i>GPR109B</i>	<i>G protein-coupled receptor 109B</i>	+1.9	-3.9
<i>CLU1</i>	<i>Chronic lymphocytic leukemia upregulated 1</i>	+1.7	-4.4
<i>FLJ10357</i>	<i>Protein SOLO</i>	-1.7	+2.0

(Supplementary Figure 1). Using gene ontology tools to categorize the dysregulated genes (10), the encoded proteins were predicted to play a wide variety of cellular roles (Supplementary Table 1). In patients with rapid and slow progression, 643 and 839 genes were dysregulated, respectively (Supplementary Figure 1, Supplementary Tables 2A and 3A). Of these dysregulated genes, 89 and 121 genes, respectively, encode proteins involved in functions of the nervous system, lysosomes and proteolysis, protein transport and autophagy, apoptosis and visual perception (Supplementary Tables 2B and 3B).

In patients with rapid or slow disease progression, 105 genes were dysregulated that were not changed in patients with average disease progression. Of

these, 13 genes were upregulated in patients with rapid disease progression and downregulated in patients with slow disease progression. One gene was identified that was upregulated in patients with slow disease progression and downregulated in patients with rapid disease progression (Table 3).

A total of five genes were significantly dysregulated in all CLN3 patients (Table 4). Of these, *regulator of G protein signaling 1 (RGS1)*, *DUSP2* and *poly(ADP-ribose) polymerase family member 15 (PARP15)* were significantly upregulated in all three CLN3 patient groups. Two genes, *DNA-directed RNA polymerase II polypeptide J-related (POLR2J2)* and *CDC42 small effector 2 (CDC42SE2)*, were significantly downregulated in all three CLN3 patient groups.

Table 4. List of genes that were significantly dysregulated in all CLN3 patients.

Gene symbol	Gene name	Fold change		
		Rapid	Average	Slow
<i>POLR2J2</i>	<i>DNA-directed RNA polymerase II polypeptide J-related</i>	-2.2	-1.7	-2.6
<i>CDC42SE2</i>	<i>CDC42 small effector 2</i>	-3.2	-1.8	-2.5
<i>RGS1</i>	<i>Regulator of G protein signaling 1</i>	+1.9	+2.5	+1.8
<i>DUSP2</i>	<i>Dual specificity phosphatase 2</i>	+2.3	+2.4	+2.4
<i>PARP15</i>	<i>Poly(ADP-ribose) polymerase family, member 15</i>	+1.5	+1.7	+1.8

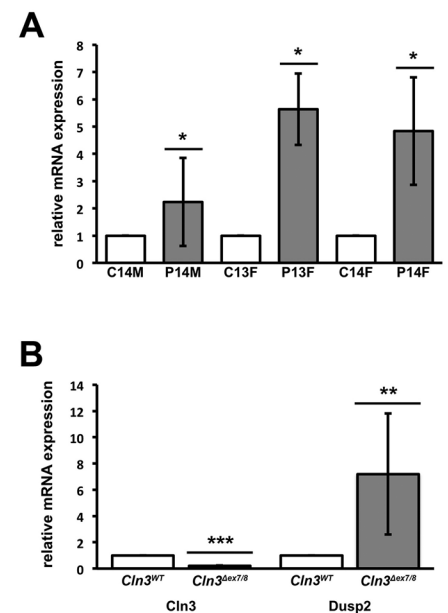


Figure 3. Relative mRNA expression of *DUSP2*. (A) The relative mRNA expression of *DUSP2* in lymphocytes derived from CLN3 patients receiving anticonvulsive medication compared with age- and gender-matched controls was measured by RT-PCR and normalized to *RPS18* expression. The patients were 14, 13 and 14 years old (P14M, male; P13F, female; P14F, female) and were all homozygous for the 1-kb deletion in the *CLN3* gene. They were classified as having average (P14M), slow (P13F) and rapid disease progression (P14F). (B) The relative mRNA expression of *Dusp2* in CbCln3^{Δex7/8} cerebellar precursor cells compared with wild-type cells was measured by RT-PCR and normalized to *Actb* expression. Data are means \pm SD of three independent RT-PCR experiments. * $P < 0.05$ compared with controls; ** $P < 0.01$ compared with controls; *** $P < 0.001$ compared with controls.

To verify the significance of the biomarker candidates, the relative mRNA expression of *DUSP2* was determined in lymphocytes derived from three CLN3 patients under anticonvulsive treatment with average, slow and rapid disease progression (P14M, P13F and P14F, respectively) and age- and gender-matched controls (Figure 3A). The mRNA expression level of *DUSP2* was upregulated 2.2- to 5.6-fold in extracts from all three

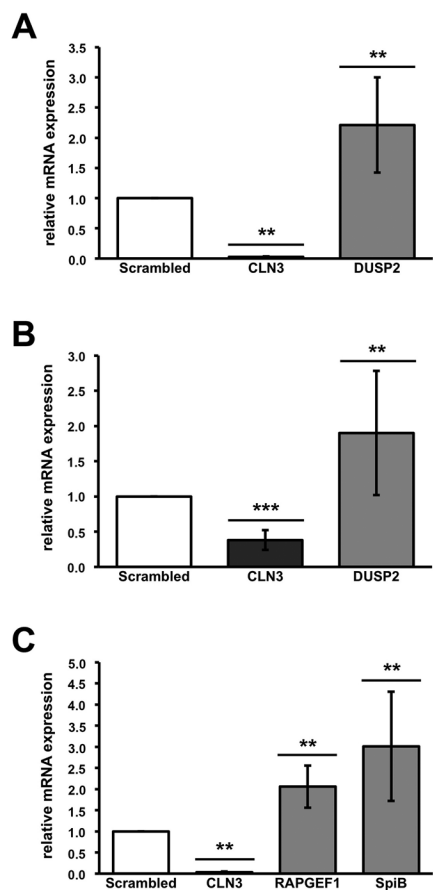


Figure 4. Relative mRNA expression of candidate genes in siRNA-mediated acutely *CLN3*-depleted cells. HeLa cells or SH-SY5Y cells were transfected with *CLN3* siRNA for 72 h in total. The relative mRNA expressions of candidate genes were measured by RT-PCR and normalized to *ACTB* expression. mRNA levels of scrambled siRNA-treated cells (white bar) were set as 1 and used as control. (A) Relative mRNA expression of *CLN3*, and *DUSP2* in *CLN3* siRNA-treated HeLa cells. (B) Relative mRNA expression of *CLN3*, and *DUSP2* in *CLN3* siRNA-treated SH-SY5Y cells. (C) Relative mRNA expression of *CLN3*, *RAPGEF1* and *SPIB* in *CLN3* siRNA-treated HeLa cells. Data are mean \pm SD of three (B) or five (A, C) independent transfection experiments. ** $P < 0.01$ compared with controls; *** $P < 0.001$ compared with controls.

patients cells, indicating that *DUSP2* upregulation is not influenced by anticonvulsive medication.

In addition, we tested *DUSP2* mRNA expression in *CbCln3^{Δex7/8}* cerebellar pre-

cursor cells derived from *Cln3^{Δex7/8}* knock-in mice that represent precise genetic replicas of the mutation that causes disease in most CLN3 patients: the 1-kb deletion of exons 7 and 8 (8). *DUSP2* mRNA expression was upregulated 3.5- to 12.3-fold in *CbCln3^{Δex7/8}* cerebellar precursor cells compared with controls (Figure 3B). These data show that upregulation of *DUSP2* mRNA expression is not restricted to CLN3 patient lymphocytes but is also detected in murine neuronal cells that harbor the exact same genetic defect like the CLN3 patients studied here.

Expression of Candidate Genes in Acutely CLN3-Depleted Cells

To further prove the general significance of our results, we explored whether acute loss of CLN3 in two different cell models affects expression of the same genes that are altered in lymphocytes from patients. mRNA expression of several candidate genes was analyzed in HeLa and SH-SY5Y neuronal cell lines treated with *CLN3* siRNA. Quantitative RT-PCR revealed a downregulation of *CLN3* mRNA expression of at least 97% in HeLa cells and 62% in SH-SY5Y cells under the conditions used (Figures 4A, B) (11). The relative mRNA expression of *DUSP2*, for which expression was 2.4-fold upregulated in all CLN3 patients (see Table 4), was increased in both cell types ranging from 1.4- to 2.8-fold in *CLN3*-depleted HeLa cells and 1.4- to 2.9-fold in SH-SY5Y cells compared with controls (see Figures 4A, B). The *CDC42SE2* mRNA level, which was decreased (1.8- to 3.2-fold) in CLN3 patient cells, showed a slight reduction ranging from 0.6- to 0.9-fold in *CLN3*-depleted HeLa cells compared with controls (Supplementary Figure 2). The alterations in the amounts of mRNA of three other genes (*POLR2J2*, *RGS1* and *PARP15*) were similar in *CLN3*-depleted HeLa and control cells.

Next, mRNA expression of 5 of the 14 genes that appear to modify the progression rate of CLN3 disease were analyzed in HeLa cells after acute siRNA-mediated *CLN3* depletion (Figure 4C). Four of

the genes tested were upregulated in CLN3 patients with rapid disease progression and downregulated in patients with slow disease progression (*RAPGEF1* [guanine nucleotide exchange factor 1 for small GTPases of the Ras family], *SPIB* [transcription factor Spi-B], *MARCKS* [myristoylated alanine-rich protein kinase C substrate], *BACE2* [β -site APP [β -amyloid precursor protein] cleaving enzyme 2]), and one was dysregulated in the opposite way (*FLJ10357*). Quantitative RT-PCR of *RAPGEF1* revealed a significant increase of mRNA expression ranging from 1.7- to 2.1-fold in *CLN3*-depleted cells compared with controls. *SPIB* mRNA level was also significantly increased (range 1.7- to 3.6-fold). mRNA levels of *MARCKS*, *BACE2* and *FLJ10357* were not significantly altered compared with controls.

DISCUSSION

High Phenotype Variability in CLN3 Patients with Identical Genotype

This study shows that the natural variability in CLN3 disease caused by the same 1-kb deletion in the *CLN3* gene is wide and independent of genotype. We performed a detailed analysis of the natural history of 25 CLN3 patients homozygous for the 1-kb deletion over a mean period of about 18 years, using an established scoring system and an Index of Relative Disease Severity (7). Of these patients, 36%, 44% and 20% exhibited an average, rapid and slow progression of the disease, respectively.

Previous studies also described a variable phenotype, but for these patients, genetic diagnosis was not possible, since the *CLN3* gene had not been identified at that time (11,12). In contrast, a study by Adams *et al.* (13) analyzing only the behavioral phenotype of a heterogeneous cohort of CLN3 patients did not find differences between these CLN3 patients. Loss of vision was previously described to occur within a narrow age range in juvenile NCL patients (7). We confirm this for our patients, including those whose clinical progression in other categories

(intellect, language and motor function) varied widely.

Interestingly, both patients with slow disease progression (patients 3 and 4) received pacemaker implants because of sinus bradycardia at age 30 and 26 years, respectively. Scoring of these two patients remained stable (patient 4) or even improved (patient 3) after treatment of the cardiac problem by a pacemaker implant. Other groups also reported on few CLN3 patients with cardiac pathology (14,15). By electrocardiography, significant rhythm abnormalities, the most frequent being sinus bradycardia and sinus arrhythmia, were found in 16 of our 25 CLN3 patients. Ten of the 25 CLN3 patients examined had abnormal results in echocardiographic examinations, with left ventricular hypertrophy being the most common finding. The mean age of CLN3 patients with pathologic cardiac findings was 17.7 ± 7.3 years (mean \pm SD, range 11–34 years) (A Schulz, unpublished results). These results might point to additional system involvement in NCL. Whether cardiac involvement in CLN3 patients is a common phenomenon and how it might influence the course of the disease needs to be studied in more detail. However, these observations suggest that regular cardiologic examinations are warranted in CLN3 patients.

The observation of a wide clinical variability in CLN3 patients with the same mutation (apart from the conformity of the visual loss) is intriguing. To exclude any effect of anticonvulsants on the general progression of the disease (16), we selected eight patients who did not receive anticonvulsants over the entire assessment period. Scoring of these patients classified them into four individuals with average disease progression and two each with slow and rapid disease progression.

The present and previous studies suggest that genetic variations in addition to the defect in the *CLN3* gene are responsible for the rapid or slow progression rate in CLN3 patients. To identify genetic variations responsible for this clinical variability, we performed genome-wide mi-

croarray analyses in lymphocytes derived from patients 1 to 8 with strikingly different disease progression. Lymphocytes are ideal as the source of the required RNA because (i) they contain large vacuoles with lysosomal storage material characteristic for CLN3 disease, indicating a specific disease phenotype effect in this tissue (17,18), and (ii) the rapid isolation of lymphocytes within a time frame of 5 min after taking the blood sample prevents or reduces any secondary effects on gene expression patterns that might be induced by cell culture conditions and repeated passaging of cells such as fibroblasts from a skin biopsy were used.

Identification of a Potential New Biomarker for CLN3 Disease

Five genes were found to be dysregulated in all three patient groups. The expression of *POLR2J2* and *CDC42SE2* were decreased, whereas *RGS1*, *DUSP2* and *PARP15* were increased, in all CLN3 patients compared with controls. Of these, *DUSP2* was studied in more detail because of its (i) high dysregulation and (ii) potential relevance for brain function. *DUSP2* dephosphorylates both phosphotyrosine and phosphoserine/threonine residues simultaneously within the mitogen-activated protein kinases ERK (extracellular signal regulated kinase), p38 and JNK (c-jun N-terminal kinase) and thereby acts as an antagonist of associated signaling cascades (19). It has been reported that overexpression of *DUSP2* increases susceptibility to p53-mediated cell death induced by oxidative stress (20). In mice, seizures can induce *DUSP2* expression in microglial cells and dying neurons of the hippocampus (21). Although *DUSP2* was found to be upregulated in cells of CLN3 patients not receiving anticonvulsive medication, a biomarker should also be dysregulated in patients under medication. Therefore, *DUSP2* mRNA expression was also tested in CLN3 patients receiving anticonvulsive medication, where it was again found to be significantly upregulated regardless of which group of dis-

ease progression these patients belonged. Moreover, *DUSP2* mRNA expression was slightly higher in patients under anticonvulsive medication compared with untreated patients, suggesting that seizures do not significantly influence *DUSP2* upregulation in CLN3 patient lymphocytes.

Of note, expression of *DUSP2* already serves as a biomarker of therapeutic responsiveness, since it is associated with poor outcome in ovarian cancer patients (22). *DUSP2* upregulation in *CbCln3^{Δex7/8}* cerebellar precursor cells harboring the same genetic defect like most CLN3 patients shows that both neuronal and non-neuronal regulation of *DUSP2* is affected in CLN3 disease. This result is also supported by the result that acute and short-term induced *CLN3* depletion leads to *DUSP2* upregulation, not only in HeLa cells, but also in SH-SY5Y neuronal cells.

DUSP2 expression was increased in lymphocytes of all CLN3 patient groups without and with medication as well as in murine neuronal cells harboring the patients genetic *CLN3* defect and in both acutely *CLN3*-depleted nonneuronal and neuronal cells. This *DUSP2* expression increase makes *DUSP2* suitable as a potential biomarker of CLN3 disease.

The mRNA expression of another member of the dual-specificity phosphatases group, *DUSP7*, was increased 1.7-fold in embryonic, primary neuron cultures of *Cln3*-deficient mice in comparison with control neuronal cells (23). These data support our findings and suggest members of the DUSP family as likely candidates linked to CLN3 deficiency. Detailed analysis of the specificity of *DUSP2* expression in CLN3 disease and its regulatory function may contribute to our understanding of the molecular mechanisms of inflammatory responses and cell survival pathways in CLN3 disease. In addition, the identification of biomarkers for CLN3 disease will support the identification of therapeutic targets for experimental therapies and will provide valuable tools to monitor the effect of such new treatment strategies.

Previously, we found that *ACP2* encoding the lysosomal acid phosphatase was

significantly upregulated at the mRNA and protein level in *CLN3*-deficient mouse brain and patient fibroblasts (9). The basal mRNA expression of *ACP2* in lymphocytes, however, appears to be too low to allow detection of alterations between *CLN3* patients and controls.

Identification of Potential Modifier Genes for *CLN3* Disease

We also identified genes that were dysregulated in opposite directions in *CLN3* patients with rapid and slow progression of the disease and that may therefore represent disease-modifier genes. Thirteen genes were upregulated in patients with rapid disease progression and downregulated in patients with slow disease progression. One gene only was dysregulated in the opposite direction, suggesting that the course of the disease is determined more by genes that aggravate rather than by genes that reduce deterioration. Expression of two of the genes upregulated in patients with rapid disease progression and downregulated in patients with slow disease progression was evaluated in *CLN3* siRNA-treated HeLa cells: *RAPGEF1*, which is relevant for brain function, and *SPIB*, which is a lymphocyte-specific gene.

RAPGEF1 protein plays a role in multiple signal transduction pathways that regulate growth, differentiation and neuronal migration processes during brain development (23,25,26). *RapGef1*-deficient mouse embryos exhibit a cortical neuron migration defect resulting in a failure to split the preplate into marginal zone and subplate and a failure to form a cortical plate (27). Quantitative RT-PCR of *RAPGEF1* in *CLN3*-depleted cells confirmed the significant increase in mRNA expression in *CLN3* patients with rapid progression.

Expression analysis of *SPIB*, which is involved in the regulation of development and differentiation of T- and B-lymphocytes (28,29), was of interest for two reasons. First, changes in the expression of lymphocyte-specific genes such as *SPIB* are not surprising because pathological storage products are found both

in T- and B-lymphocytes of *CLN3* patients (30,31). However, it remains to be examined whether the differentiation of B-lymphocytes to plasma cells and T-cell-dependent immune responses are differentially impaired in *CLN3* patients with rapid and slow progression of disease. Second, in *CLN3*-depleted cells, *SPIB* showed the highest and most significantly increased mRNA level of the genes tested.

Together, these findings suggest that *CLN3* depletion causes increases in the expression of genes that were also found to be dysregulated in patients with rapid progression but decreased in patients with slow progression. Additional dysregulated genes listed in Table 4 have yet to be evaluated to demonstrate that *CLN3* depletion in HeLa cells truly represents a useful cell model to follow molecular alterations in *CLN3* patients with rapid rather than slow progression of the disease. However, of note, the expression of *FCGR2B*, which is increased in *CLN3* patients with rapid disease progression and decreased in patients with slow disease progression, is also elevated in brain tissues of mouse models of the more severe infantile *CLN1* disease and variant late infantile *CLN5* disease, suggesting that some of these genes may be general markers for NCL (32).

Several comparative gene expression analyses have been performed in whole brain, cerebellum and eyes of presymptomatic *Cln3*-deficient mice with targeted deletion of exons 1–6 or in embryonic cultured neuronal cells of these mice (33–35). More than 800 genes were found to be altered. The physiological relevance of these genes in the pathogenesis of neurodegeneration or for the function of *CLN3* has not yet been proven, nor can they explain differences in the progression rates of human *CLN3* disease.

In contrast to studies performed in NCL mouse models, which are not limited by the number of samples, our small sample number is due to the rareness of the *CLN3* disease and especially that we included patients who were without any medication. Because

of the progressive course of a disease with multiple neurologic symptoms including epilepsy, few patients who are unmedicated exist at any one time. Therefore, we used this to guide our approach for statistical analysis. To compensate for the small sample size, a sophisticated preanalytical selection of control samples was performed to reduce the number of degrees of freedom by gender and age and in turn the biological background noise. Considering the rareness of the disease, the challenge of finding unmedicated patients and matching these with defined age and gender in controls, this study represents a significant step toward the identification of potential biomarker and modifier genes in juvenile *CLN3* disease.

To conclude, our study showed a high phenotype variability in *CLN3* patients with an identical genotype. Among five genes identified by microarray analysis as dysregulated in all *CLN3* patients regardless of the progression of the disease, *DUSP2* is a potential and much-needed biomarker of the disease. Furthermore, 13 genes were found that appear to modify the progression of the disease. Among these potential modifier genes, *RAPGEF1* and *SPIB* were validated in an acutely *CLN3*-depleted cell model. These findings indicate that differential perturbations of distinct signaling pathways might alter the progression. Future longitudinal studies are required to evaluate the regulation of these identified biomarker or modifier genes during the different stages of the disease. Finally, this project provides valuable insights into the molecular alterations that underlie neuronal dysfunction in juvenile *CLN3* disease, and the genes identified in the work reported here should become a focus for future studies.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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