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Transcriptomic Approach to Lesch-Nyhan Disease

Luce Dauphinot¹, Lionel Mockel², Julie Cahu², H. A. Jinnah³, Morgan Ledroit², Marie-Claude Potier¹, and Irène Ceballos-Picot^{2,4}

¹CRICM, UPMC Hôpital de la Pitié-Salpêtrière, Paris, France

²Department of Metabolic Biochemistry, Hôpital Necker-Enfants Malades, France

³Departments of Neurology, Human Genetics, and Pediatrics, Emory University; Atlanta, Georgia, USA

⁴School of Medicine, Paris Descartes University Sorbonne Paris Cité, Paris, France

Abstract

Lesch-Nyhan disease (LND) is an X-linked metabolic disease caused by various mutations in the gene *HPRT1* encoding an enzyme of purine metabolism, hypoxanthine guanine phosphoribosyltransferase (HPRT). In its most severe form, LND patients suffer from overproduction of uric acid along with neurological or behavioural difficulties including self-injurious behaviours. To gain more insight into pathogenesis, we compared the transcriptome from human LND fibroblasts to normal human fibroblasts using a microarray with 60,000 probes corresponding to the entire human genome. Using stringent criteria, we identified 25 transcripts whose expression was significantly different between LND and control cells. These genes were confirmed by quantitative RT-PCR to be dysregulated in LND cells. Moreover, bioinformatic analysis of microarray data using gene ontology (GO) highlighted clusters of genes displaying biological processes most significantly affected in LND cells. These affected genes belonged to specific processes such as cell cycle and cell-division processes, metabolic and nucleic acid processes, demonstrating the specific nature of the changes and providing new insights into LND pathogenesis.

Keywords

Lesch-Nyhan disease; transcriptome; microarray; gene ontology; hypoxanthine phosphoribosyltransferase; HPRT

Introduction

Mutations in the gene *HPRT1*, encoding the enzyme hypoxanthine guanine phosphoribosyltransferase (HPRT), are known to cause a rare X-linked metabolic disorder, Lesch-Nyhan disease (LND). Many different diseasecausing mutations have been reported

(www.lesch-nyhan.org). Spread through nearly the whole gene are missense mutations, nonsense mutations, splicing mutations, small and large coding and noncoding deletions or insertions, partial duplications, noncoding regulatory mutations, and more complex changes.^[1,2] Residual HPRT enzyme function is the most important factor for determining the variable severity of the clinical phenotype.^[3–7]

The HPRT enzyme plays a role in recycling the purine bases hypoxanthine and guanine back into the purine nucleotide pools (Figure 1). The pathogenesis of uric acid overproduction in HPRT deficiency is well understood. In the absence of HPRT, the purine bases are metabolized into uric acid because they cannot be recycled. Additionally, regulatory changes in purine synthesis result in increased synthesis of purines via the de novo pathway. These two processes cause uric acid overproduction. The excess uric acid tends to precipitate in specific body regions. Stones may form in any portion of the urogenital system where uric acid is concentrated resulting in nephrolithiasis or crystalluria. Uric acid may also precipitate in the synovial fluid of the joints, causing gout.^[7]

The pathogenesis of the neurological and neurobehavioral anomalies is less well understood. These problems do not appear to be the consequence of uric acid overproduction. Autopsy studies have not revealed overt morphological defects or signs of neurodegeneration.^[8,9] Instead, the absence of HPRT appears to influence the maturation of specific neural pathways in the brain, and specific dopamine pathways appear to be most vulnerable.^[10,11] Several studies have provided converging evidence for dysfunction of dopaminergic neurons and several hypotheses have been postulated to explain such dysfunction.^[12–15] Transcription factors, such as engrailed,^[10] have been shown to be dysregulated in HPRT-deficient neuronal cells, leading to impaired dopaminergic neurotransmission or early neurodevelopmental problems.^[10,16,17]

Thus a wide range of biological processes have been so far incriminated in LND pathogenesis. Transcript profiling using LND cells compared to normal cells could provide substantial insights into LND pathogenesis through the identification of abnormal pathways.

Materials and Methods

Cell Cultures and RNA Extraction

Fibroblasts from LND patients and controls were grown routinely at 37°C under 5% CO₂ and 95% air in RPMI medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 50 mg/mL streptomycin. Medium was renewed every 2–3 days until cells reached confluence. Cells were harvested at approximately 80% confluence by centrifugation to a pellet of an average of 10^[8] cells. Induced pluripotent stem (iPS) cells were obtained from LND patients' fibroblasts using the four-reprogramming Yamanaka factors (OCT4, SOX2, MYC, and KLF4)^[18] (i-Stem iPS workshop). Quality controls (morphology, karyotype, genotype, proliferation, epigenetic status of pluripotent cell-specific genes, differentiation capacities into cell types of the three germ layers in vitro) showed iPS cells were successfully obtained.

Total RNAs were isolated from each cell sample using TRIzol following the manufacturer's recommendations (Invitrogen). RNA was quantified using a NanoDrop (ND-1000, ThermoFisher Scientific, Waltham, MA, USA) and checked for quality using the Agilent Bioanalyser.

cRNA Probe Preparation and Hybridization

Each RNA (100 ng) was amplified and labeled with Cy3 using the Low Input Quick Amp labeling kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. After purification and quantification on a Nanodrop, 1 μ g aliquots of each Cy3-cRNA were hybridized overnight on SurePrint G3 Human GE 8 $\times$ 60 K Microarray (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions.

Microarray Data Analysis

Microarray data were acquired on a Nimblegen MS200 scanner (Roche Applied Science) with a resolution of 2 μ m and analyzed with Mapix 5.0.0 software (Innopsys, Carbonne, France). For each sample, raw data consisted of the Median Feature Intensity—Median Background Feature (F-B) at 532 nm wavelength. These raw data were log²-transformed and quantilenormalized under the R freeware (www.r-project.org). The statistical analysis was performed on these normalized data under the R freeware. The dataset from LND cells versus controls was analyzed using an ANOVA test conducted on microarray data from LND fibroblasts ($n = 4$) and LND induced Pluripotent Stem cells (iPS) ($n = 3$) versus control fibroblasts ($n = 4$) and control iPS ($n = 3$) to find the specific genes linked to LND. Statistical significance was set to a p -value < 0.05 .

Gene ontology (GO) category enrichment analysis was performed to highlight biological processes using the web-based GOrilla application (<http://cbl-gorilla.cs.technion.ac.il/>).^[19]

Reverse Transcription and Real-Time Quantitative PCR

RT-qPCR was used to confirm the results obtained by expression arrays. cDNAs were synthesized using 1 μ g of total RNA with Super Script III Reverse Transcriptase (Life Technologies) in combination with oligo-dT and random hexamer priming according to the manufacturer's instructions. Thermal cycling was composed of 5 minutes at 25°C, 60 minutes of reverse transcription at 50°C, and 15 minutes at 70°C. For qPCR, cDNA, primers and SyBR green Master Mix (Life Technologies) were mixed according to manufacturer's instructions. The reaction was then cycled as follows: 10 minutes at 95°C and 40 cycles of 10 seconds at 95°C followed by 60 seconds at 60°C using the Applied Biosystems 7300 Real time PCR System. The genes *18S*, *PSMA2*, and *POLR2A* were used as normalization genes because, from microarray data, expression of these genes did not change in both LND fibroblasts and iPS cells. The fold change (FC) was calculated using the C_t method, where $FC = 2^{-C_t}$, and $C_t = [(Ct \text{ gene of interest} - \text{Mean of } Ct \text{ gene of reference})_{LND} - (Ct \text{ gene of interest} - \text{Mean of } Ct \text{ gene of reference})_{WT}]$. Significantly dysregulated genes were identified by a t -test.

Results

To assess gene expression differences between LND and control cells, RNAs were reverse-transcribed and labeled to perform whole genome transcriptomics. Among the initial 60,000 probes on the chip, 661 genes common to LND fibroblasts and iPS were found to be significantly differentially expressed by ANOVA ($p < 0.05$). iPS data lumped with the fibroblasts' data for statistical comparison of HPRT-deficient cells versus controls using ANOVA to find genes linked to the disease. Then we focused for qPCR validation on fibroblasts' data: as shown in Table 1, among the 661 significantly dysregulated genes, 25 genes were selected according to LND/control ratio in fibroblasts (<0.5 or >1.3): 18 were increased and 7 were decreased in LND when compared to control fibroblasts. Among the 11 genes selected for RT-qPCR validation 7 were confirmed to be dysregulated with a ratio similar to the microarray results (Table 1): *HPRT1*, myosin light chain kinase (*MYLK*), thymosin beta-15A (*TMBS15A*), actin alpha 2 smooth muscle aorta transcript variant 2 (*ACTA2*), and early growth response protein-1 (*EGR-1*) were confirmed to be reduced, whereas cathepsin (*CTSF*), nicotinamide N-methyltransferase (*NNMT*) were increased.

We also performed gene ontology (GO) category enrichment analysis to highlight clusters of genes displaying biological processes commonly affected in LND fibroblasts and iPS. An enrichment score was calculated to determine to what extent these identified processes were over-represented. The most significantly enriched processes are listed in Table 2.

Interestingly, these affected genes belonged to specific processes and particularly to cell-cycle and cell-division processes (p -value: 2.2×10^{-25} to 2.0×10^{-12}), metabolic processes (p -value: 1.5×10^{-13} to 1.3×10^{-12}) and nucleic acid processes (p -value: 8.9×10^{-12} to 2.2×10^{-11}) demonstrating the specific nature of the changes in LND. A Venn diagram detailing shared and distinct genes between mitotic cell cycle (p -value: 2.2×10^{-25}), cell cycle (p -value: 4.9×10^{-25}), and cell cycle processes (p -value: 1.3×10^{-23}), is shown in Figure 2A.

Among the 58 dysregulated genes common to these processes, we selected several for RT-qPCR validations in fibroblasts: cyclin-dependent kinase 1 (*CDK1*), cell-division cycle associated 8 (*CDCA8*), cyclin b2 (*CCBN2*), centromere protein a (*CENPA*), and centromere protein f (*CENPF*). These genes were among the 661 differentially expressed genes common to HPRTdeficient fibroblasts and iPS cells identified by ANOVA. As shown in Figure 2B, *CDK1* and *CDCA8* were statistically confirmed to be downregulated by RT-qPCR in LND fibroblasts as compared to controls.

Discussion

Complete deficiency of the enzyme HPRT causes LND, a neurobehavioral disorder characterized by hyperuricemia, cognitive impairment, motor dysfunction, and compulsive self-mutilation.^[20,21] Although treatment with the xanthine oxydase inhibitor allopurinol effectively prevents hyperuricemia and renal damage in LND and thereby markedly extends the lifespan of patients, neither allopurinol nor any other treatment produces lasting improvement in neurological manifestations.^[7] We have used microarraybased methods of global gene expression together with qPCR to identify dysregulation of genes and aberrant cellular processes in human HPRTdeficient fibroblasts. Analysis of the microarray

expression data by gene ontology (GO) enrichment reveals that HPRT deficiency is accompanied by perturbations in specific processes known to regulate cell cycle/cell division, nucleic acid processes, and metabolic processes. Interestingly, prior studies have shown that HPRT-deficient neuroblastoma subclones grow more slowly in culture than the parent cell from which they were derived.^[22,23] Moreover, dysregulation of cell-cycle/cell-division genes also could play an important role in brain development: Cdk1, we found significantly downregulated in LND cells, has been linked to cell death of postmitotic neurons in brain development^[24] and the Cdk1 complex plays a prime role in regulating N-myc phosphorylation and turnover in neural precursors.^[25] CDCA8, also found downregulated in LND cells, is a component of a chromosomal passenger complex required for stability of the bipolar mitotic spindle.^[26] Consequently, any subtle defects in this regulation may have consequences in neurogenesis and/or neuronal migration that could affect specific neural pathways. Recent studies also have established roles of nucleotides in controlling several aspects of neurogenesis, neural differentiation,^[27–29] and migration in the developing mammalian brain through interactions with two classes of purinergic P2 receptors.^[30,31] Because LND is a classical inborn error of purine metabolism, and because we and other groups have demonstrated aberrant neurogenesis and neural pathway development in LND [10,11,16,21,22,32], we have based much of our recent work on human iPS cells obtained from LND patients' fibroblasts for studying the development of dopaminergic pathways.

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Figure 1.

Schematic overview of the purine metabolism. 1: PRPP synthetase; 2: Adenylosuccinase; 3a: ATIC: AICAR transformylase; 3b: ATIC: IMP cyclohydrolase; 4: adenylosuccinate synthetase; 5: AMP desaminase; 6: adenosine desaminase; 7: 5' nucleotidase; 8: purine nucleoside phosphorylase; 9: hypoxanthine guanine phosphoribosyltransferase (HPRT), the deficient enzyme in LND; 10: adenine phosphoribosyltransferase; 11: adenosine kinase; 12: xanthine oxydase; 13: deoxyguanosine kinase. In grey: salvage pathways.

Figure 2.

GOrrilla cell cycle processes. (A) Venn diagram of cell cycle/cell division biological processes showing genes commonly dysregulated among these processes and those of interest: *CDK1*: cyclindependent kinase 1; *CDCA8*: cell division cycle associated 8; *CCNB2*: cyclin b2; *CENPA*: centromere protein a; *CENPF*: centromere protein f. (B) RT-qPCR validation of the genes of interest in fibroblasts. The y-axis of the graphic represents the gene expression fold changes of the genes indicated on the x-axis. (*t*-test: * *p*-value < 0.05; ** *p*-value < 0.01).

Table 1

Differentially expressed genes in LND fibroblasts

Gene symbol	Gene ID	Gene name	Ratio LND/ Cont. microarray	p- value	Ratio LND/ Cont. RT-qPCR	p-value
OCRL	NM 000276	oculocerebrorenal syndrome of Lowe transcript variant a	0.24	0.035	—	—
HPRT1	NM 000194	hypoxanthine phosphoribosyltransferase 1	0.26	0.005	0.19	0.0385
MYLK	NM 053025	myosin light chain kinase transcript variant 1	0.42	0.012	0.42	0.0005
TMSB15A	NM 021992	thymosin beta 15a	0.43	0.018	0.15	0.0447
ACTA2	NM 001613	actin alpha 2 smooth muscle aorta transcript variant 2	0.44	0.027	0.34	0.0095
IGF2BP1	NM 006546	insulin-like growth factor 2 binding protein 1 transcript variant 1	0.49	0.031	—	—
EGR1	NM 001964	early growth response 1	0.49	0.001	0.42	0.0004
CTSF	NM 003793	cathepsin F	1.30	0.024	2.28	0.0004
DSEL	NM 032160	dermatan sulfate epimerase-like	1.35	0.005	—	—
CALY	NM 015722	calcyon neuron-specific vesicular protein	1.47	0.007	—	—
JDP2	NM 130469	jun dimerization protein 2 transcript variant 1	1.51	0.047	—	—
SPATA20	NM 022827	spermatogenesis associated 20	1.53	0.041	—	—
MXI1	NM 130439	MAX interactor 1 transcript variant 2	1.54	0.030	—	—
LOC100130825	NM 001720026	PREDICTED: hypothetical protein LOC100130825	1.56	0.006	—	—
C6orf132	NM 001164446	chromosome 6 open reading frame 132	1.58	0.025	—	—
APLP2	NM 001642	amyloid beta (A4)precursor-like protein 2 transcript variant 1	1.61	0.029	—	—
NNMT	NM 006169	nicotinamide N-methyltransferase	1.64	0.023	3.20	0.0023
ZNF28	NM 006969	zinc finger protein 28 transcript variant 1	1.67	0.039	—	—
RUNDC2A	NM 032167	RUN domain containing 2A	1.70	0.039	1.51	0.1480
RECK	NM 021111	reversion-inducing-cysteine-rich protein with kazal motifs	1.70	0.044	1.59	0.1396
DHRS3	NM 004753	dehydrogenase/reductase (SDR family) member 3	1.80	0.044	1.89	0.2310
KIAA1161	NM 020702	KIAA1161	2.00	0.039	—	—
IGFBP3	NM 001013398	insulin-like growth factor binding protein 3 transcript variant 1	2.07	0.019	2.33	0.0968
OSCAR	NM 206818	osteoclast associated immunoglobulin-like receptor transcript variant 1	2.13	0.015	—	—
CCDC18	NM 206886	coiled-coil domain containing 18	2.37	0.034	—	—

Among 661 differentially expressed genes common to LND fibroblasts and iPS (ANOVA; p -value <0.05), 25 genes were selected according to LND/control ratio in fibroblasts (<0.5 or Cell cycle/cell division processes Mitotic cell cycle >1.3). Among the 11 genes selected for RT-qPCR validation 7 were confirmed to be dysregulated with a ratio similar to the microarray results. (— : not tested).

Table 2
GORilla enrichment of specific biological processes in LND cells

Biological processes	Description	<i>p</i> -value	Enrichment score
Cell cycle/cell division processes	Mitotic cell cycle	2.24×10^{-25}	5.16
	Cell cycle	4.92×10^{-25}	4.02
	Cell cycle process	1.27×10^{-23}	3.17
	Cell cycle phase	9.42×10^{-21}	3.75
	M phase	4.59×10^{-20}	8.87
	Mitotic prometaphase	6.42×10^{-17}	8.46
	Cell division	1.98×10^{-12}	3.60
Metabolic processes	Cellular macromolecule metabolic process	1.53×10^{-13}	1.45
	Cellular metabolic process	4.67×10^{-13}	1.34
	Organelle organization	4.72×10^{-13}	1.96
	Nucleobase-containing compound metabolic process	1.27×10^{-12}	1.56
Nucleic acid processes	RNA splicing	8.86×10^{-12}	4.68
	Nucleic acid metabolic process	1.14×10^{-11}	1.58
	Response to DNA damage stimulus	1.29×10^{-11}	2.76
	RNA splicing, via transesterification reactions	2.07×10^{-11}	4.52
	DNA metabolic process	2.23×10^{-11}	2.58