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Expression signature of the Leigh syndrome French-Canadian type

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ABSTRACT

As a result of a founder effect, a Leigh syndrome variant called Leigh syndrome, French-Canadian type (LSFC, MIM / 220,111) is more frequent in Saguenay–Lac-Saint-Jean (SLSJ), a geographically isolated region on northeastern Quebec, Canada. LSFC is a rare autosomal recessive mitochondrial neurodegenerative disorder due to damage in mitochondrial energy production. LSFC is caused by pathogenic variants in the nuclear gene leucine-rich pentatricopeptide repeat-containing (*LRPPRC*). Despite progress understanding the molecular mode of action of *LRPPRC* gene, there is no treatment for this disease.

The present study aims to identify the biological pathways altered in the LSFC disorder through microarray-based transcriptomic profile analysis of twelve LSFC cell lines compared to twelve healthy ones, followed by gene ontology (GO) and pathway analyses.

A set of 84 significantly differentially expressed genes were obtained ($p \geq 0.05$; Fold change (Flc) ≥ 1.5). 45 genes were more expressed (53.57%) in LSFC cell lines compared to controls and 39 (46.43%) had lower expression levels. Gene ontology analysis highlighted altered expression of genes involved in the mitochondrial respiratory chain and energy production, glucose and lipids metabolism, oncogenesis, inflammation and immune response, cell growth and apoptosis, transcription, and signal transduction. Considering the metabolic nature of LSFC disease, genes included in the mitochondrial respiratory chain and energy production cluster stood out as the most important ones to be involved in LSFC mitochondrial disorder. In addition, the protein-protein interaction network indicated a strong interaction between the genes included in this cluster. The mitochondrial gene *NDUFA4L2* (NADH dehydrogenase [ubiquinone] 1 alpha subcomplex, 4-like 2), with higher expression in LSFC cells, represents a target for functional studies to explain the role of this gene in LSFC disease.

This work provides, for the first time, the LSFC gene expression profile in fibroblasts isolated from affected individuals. This represents a valuable resource to understand the pathogenic basis and consequences of *LRPPRC* dysfunction.

1. Introduction

The principal function of the mitochondria is to carry out the oxidative energy metabolism [1,2]. It produces adénosine-5′-triphosphate (ATP), by oxidative phosphorylation (OXPHOS), that is used by most mammalian cells for growth, survival and regular function [3]. The

Abbreviations: LSFC, Leigh syndrome, French-Canadian type; SLSJ, Saguenay–Lac-Saint-Jean; *LRPPRC*, leucine-rich pentatricopeptide repeat-containing; GO, gene ontology; Flc, fold change; *NDUFA4L2*, NADH dehydrogenase [ubiquinone] 1 alpha subcomplex, 4-like 2; ATP, adénosine-5'-triphosphate; OXPHOS, oxidative phosphorylation; COX, cytochrome c-oxidase; DMEM, Dubelcco's Modified Essential Medium; RMA, robust multi-array analysis; PPI, protein-protein interaction; *ND6*, NADH dehydrogenase, subunit 6; *PFKFB4*, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4; *HES1*, hairy and enhancer of split 1; *RPL13A*, ribosomal protein L13a; qRT-PCR, Real-time PCR; SRA, steroid receptor RNA activator; SLIRP, stem-loop interacting protein; *HIF-1*, hypoxia inducible factor-1; ETC, electron transport chain; ROS, reactive oxygen species; NAFLD, non-alcoholic fatty liver disease; COPD, chronic obstructive pulmonary disease.

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OXPHOS system is located in the inner mitochondrial membrane and comprises five enzyme complexes (complexes I-V) [4]. More than 150 distinct genetic mitochondrial syndromes have been defined [5]. Leigh syndrome, a metabolic disease affecting 1/40,000 newborn infants worldwide [6], is one of these disorders. It is characterized by a psychomotor regression, hypotonia, ataxia, lactic acidosis and by an estimated mean life expectancy of 3 to 5 years and can be caused by more than 30 genes [2]. A variant known as Leigh Syndrome French-Canadian type (LSFC, MIM / 220,111) was described in the founder population of Saguenay–Lac-Saint-Jean region (SLSJ) of Quebec, Canada where the largest cohort of LSFC patients was identified (56 patients in 2011) [7]. In SLSJ, around 1/2000 births are affected by LSFC and the carrier rate is 1/23 [8,9]. LSFC is an autosomal recessive form of neurodegenerative congenital lactic acidosis that presents with developmental delay, hypotonia, ataxia, failure to thrive, and mild dysmorphic facial features [8,10,11]. It is biochemically characterized by tissue-specific defect in the respiratory chain complex IV (cytochrome *c*-oxidase, COX). In LSFC individuals, liver and brain are severely affected while fibroblasts and skeletal muscle are 50% affected, and kidney and heart have almost normal activities [10–12]. LSFC individuals presented also severe and often deadly neurological and/or acidotic crisis [13]. The responsible gene, *LRPPRC,* encoding for a pentatricopeptide repeat (PPR) family protein, was identified in 2003 [9]. Most SLSJ patients are homozygous for the founder missense mutation p.Ala354Val in exon 9 of this gene. Subsequently, significant advances in understanding the molecular mechanisms of LSFC were succeeded. A low steady state levels of the mutated LRPPRC protein was observed in all LSFC patient tissues [9] resulting in a defect in the translation of most mitochondrial messengers particularly those of the complex IV [14,15]. Other evidences show implication of *LRPPRC* in various other diseases ranging from viral to tumour infections [16,17].

All these recent findings and data illustrate the complexity of the LRPPRC function and the need to identify the downstream dysregulated pathways in LSFC patients and other caused diseases. This is why we conducted the present study on the gene expression profile of LSFC patients cell lines by microarray technology. It revealed several affected pathways and induction of cellular mechanism compensation and raised the possibility of designing novel therapeutic strategies for LSFC patients.

2. Patient and methods

2.1. Patients

Twelve unrelated French-Canadian LSFC patients were included in this study. Their samples were available in the LSFC Consortium Biobank (Université du Québec à Chicoutimi, Saguenay, QC, Canada) and clinical information was extracted from their medical reports (Table 1). Twelve healthy individuals were also recruited in this study and were paired to the LSFC patients according to their age (±3 years) and sex to perform comparative gene expression microarray analysis. The inclusion criteria for the control individuals included no health problems or being affected by diseases that did not involve nervous system degeneration or the mitochondrial respiratory chain. The ethic committee of the *Centre intégré universitaire de santé et de services sociaux du SLSJ* located in Saguenay, Quebec, Canada approved the study and all individuals (or their parents for affected children) gave informed consent.

2.2. Mutation screening

Genomic DNA was extracted from peripheral blood lymphocytes using the QIAamp DNA Blood Midi kit (Qiagen, ON, Canada) according to the manufacturer's instructions. Total DNA of the participants and their parents when available was used as a template for amplification of the genomic sequences of *LRPPRC*. *LRRPRC* segments (including 38 exons and all exon–intron borders) were amplified as previously described [9]. Sequence analyses were performed using Big Dye terminator technology (ABI 3730xl) (Applied Biosystems, ON, Canada) and were analyzed using variant reporter software 2 (Applied Biosystems).

Table 1
Clinical and genetic characteristics of LSFC patients.

LSFC Patients	Sex	Age	Number of acidosis crisis	Clinical presentation	Mutation in the *LRPPRC* gene
1	M	25	>10	severe psychomotor delay, hypotonia, non autonomous	p.Ala354Val/p.Cys1,277Xdel8
2	M	25	0	severe psychomotor delay, hypotonia, non autonomous	p.Ala 354Val
3	F	23	1	severe psychomotor delay, hypotonia, non autonomous	
4	M	6	1	mild psychomotor delay, autonomous	
5	F	17	0	mild psychomotor delay, autonomous	
6	F	8	0	mild psychomotor delay, autonomous	
7	F	4	1	moderate psychomotor delay, autonomous	
8	F	21	3	moderate psychomotor delay, hypotonia, semi autonomous	
9	M	5	0	mild psychomotor delay	
10	M	2 months	1	NA	
11	M	12 WA	–	NA	
12	F	19 WA	–	NA	

WA: week of amenorrhea. NA: not applicable.

2.3. Cell culture

Skin fibroblasts were obtain from LSFC patients and controls as this tissue is easier to obtain than brain, liver or lung cells. Moreover, the respiratory chain complex IV in LSFC skin fibroblasts is decreased of 50% compared to control cell lines. It was therefore considered a good model for this study. Briefly, primary skin fibroblasts of LSFC participants and age matched control individuals were isolated from cutaneous biopsies and were grown in Dubelcco's Modified Essential Medium (DMEM) rich in glucose and enriched with 10% fetal bovine serum and 100 µl/ml penicillin and streptomycin. Cultures were maintained at 37 °C in a 5% humidified CO_2 atmosphere.

2.4. Microarray screening

RNA was isolated from 3×10^6 fibroblasts using RNeasy plus mini kit (Qiagen, Valencia, CA). Microarray analysis was performed with Affymetrix Genechip HG-U133plus2 microarrays containing 54,675 probe sets (Affymetrix, Santa Clara, CA). This chip offers coverage of nearly the entire mitochondrial and nuclear transcriptome, defined by over 47,000 transcripts which, in turn, represent approximately 39,000 genes (www.thermofisher.com). Hybridization and scanning of images were performed at the McGill University and Genome Quebec Innovation Centre (www.genomequebec.mcgill.ca). RNA processing steps (RNA extraction, probe labeling and chip hybridization) were performed in parallel

for each pair of control and LSFC samples to minimize technical variability. Nevertheless, the microarrays were performed in two different sets spaced out by 5 years because of the recruitment of new LSFC participants to increase the statistical power of the study. The raw image files (CEL format) generated from the analysis of the scanned image were used for the statistical analysis. The analysis was performed using several packages available in Bioconductor (http://www.bioconductor.org) which uses R language (http://www.R-project.org). We used Affy package to assess artifacts and variability among microarrays and we normalized the probe intensities with robust multi-array analysis (RMA), which includes background correction, quantile normalization, and median polish steps. As batch effects was a parameter to take into account in the microarrays, due to different hybridization dates, we used the inSilicoMerging package with the Empirical Bayes method (COMBAT) to adjust the variance through the microarrays. Finally, Smyth's moderated *t*-test in Limma package was used to identify the genes that were differentially expressed between the LSFC participants and the control individuals with a cut off of 1.5 for fold change (Flc) and 0.05 for *p* values.

2.5. Gene ontology and construction of protein-protein interaction network

To understand the functional alterations behind the gene changes in LSFC cells, we performed gene ontology (GO) analyses based on two bioinformatic tools: DAVID (http://david.abcc.ncifcrf.gov) and Panther gene list analysis (http://www.pantherdb.org/). Then, we used STRING database (https://string-db.org) to identify the protein-protein interaction (PPI) networks for both the higher- and under-expressed genes using a combined interaction score of > 0.4 for significant interaction [18] and visualized the results using the network visualization software Cytoscape [19].

2.6. Real-time PCR (qRT-PCR)

To validate differences in gene expression levels observed in microarrays, qRT-PCR was performed on a selected set of genes according to their known functions in mitochondrial activities or glucose metabolism. Four genes were selected: NADH dehydrogenase, subunit 6 (complex I) (*ND6*), NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4-like 2 (*NDUFA4L2*), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 (*PFKFB4*), and hairy and enhancer of split 1, (Drosophila) (*HES1*). Reverse transcription of RNA was performed using the qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, Maryland). TaqMan qRT-PCR reaction were performed in 100-wells discs using the Rotor-Gene 6000 (Qiagen/Corbett, Valencia, CA) with the Perfecta qPCR ToughMix (Quanta Biosciences) in a final volume of 20 µl. Each sample was run in triplicate with a negative control. Each gene expression measure was repeated twice. A standard curve was done with three serial dilutions in triplicate for each selected gene and for ribosomal protein L13a (*RPL13A*), which was selected as housekeeping gene [20]. Quantification obtained from standard curves of each gene was normalized to the relative amount of *RPL13A* according to the two standard curves method (Rotor-Gene 6 software (version 6.0)). Expression level of each selected gene was measured in the two groups. Data were expressed as mean ± standard error of the mean (SEM) and was compared by Student's *t*-test. A p value <0.05 was considered significant.

3. Results

3.1. Clinical and mutational diagnosis

Twelve LSFC affected individuals and twelve control participants cell lines were included in this study. LSFC participants were six females and six males aged from 12 weeks of amenorrhea to 25 years. Most participants, among the patients who were born, presented hypotonia, developmental delay, mild facial dysmorphism, and chronic well-compensated metabolic acidosis. Six patients (6/12, 50%) developed one or more acidosis crisis and have survived, exept one who died before the age of five years.

Mutational analysis of *LRPPRC* gene identified the homozygous founder mutation of missense type c.1,061 C > T transition in exon 9 predicting a missense p.Ala354Val in eleven LSFC individuals. One patient was compound heterozygous; he was heterozygous for the p. Ala354Val amino acid change and for n 8-nt deletion in exon 35 resulting in a premature stop at amino acid 1277 (Table 1).

3.2. Gene expression analysis

Transcriptional profiling of twelve LSFC affected individuals and twelve control participants fibroblasts was performed using the Affymetrix Genechip HG-U133plus2 chip platform. Microarray gene expression analysis showed significant differences in the expression of 84 genes between LSFC and control fibroblasts ($p < 0.05$ and Flc > 1.5). Four genes were mitochondrial and the others were nuclear. These differentially expressed genes were classified into eight clusters based on their main function: mitochondrial respiratory chain and energy production (5) , glucose and lipids metabolism (7) , oncogenesis (9) , immune response (10) , cell growth and apoptosis (15) , transcription (5) , signal transduction (6) , and 27 genes with other or not yet known function. Table 2 and Fig. 1 summarize the results of the microarray profiling. Table 3 shows the more and less expressed genes in each cluster. In total, 45 genes were higher expressed and 39 genes were under expressed.

Four genes were analyzed by qRT-PCR to confirm the gene expression data obtained from microarray analysis (*ND6, NDUFA4L2, PFKFB4* and *HES1*) on the twelve LSFC and twelve control fibroblasts. The qRT-PCR results were agreeing with the microarray data except for *ND6* which was found to be higher expressed in microarrays but under expressed in qRT-PCR results (Fig. 2).

3.3. Protein-protein interactions network of the differentially expressed genes

Functional annotation and pathway profiling of the differentially regulated genes, using DAVID, Panther and STRING online database, provided an overview of the molecular function of each gene and its potential involvement in biological and cellular processes. STRING PPI network showed a strong interaction between the protein NDUFA4L2 and proteins of the other dysregulated mitochondrial respiratory chain ND6, ND4, COX1, and COX3 (Fig. 3).

4. Discussion

Currently, the pathogenic mechanisms underlying LSFC disease remain unclear and no cure exists. The unique available option to reduce the high-energy demands of digestion is eating several small meals throughout the day. The responsible gene for LSFC disorder, *LRPPRC*, was discovered in 2003 [9]. The encoded protein LRPPRC belongs to the family of pentatricopeptide repeat proteins that is involved in post transcriptional mitochondrial gene expression. LRPPRC regulates the stability and handling of mature messenger RNAs. In mitochondria, LRPPRC forms a mitochondrial ribonucleoprotein complex with steroid receptor RNA activator (SRA) stem-loop interacting protein (SLIRP) [10]. This complex controls polyadenylated mRNAs and is required for mitochondrial mRNA stability [21]. As shown in Table 1, two *LRPPRC* mutations have been identified in the studied LSFC individuals: the transition c.1061C > T (p.Ala354Val) and p.Cys1277Xdel8. The missense variation p.Ala354Val is identified in 95% of the cases of LSFC in SLSJ. The carrier rate of this is variant in the SLSJ region is 1/23. A carrier-screening test for this founder mutation has become routinely

Table 2
List of genes differentially expressed in LSFC fibroblasts in comparison with healthy controls.

Clusters	Probe set	ACCNUM	Gene Symbol[a]	Gene name	Cytoband[b]	p	Flc[c]	Function[d]
Mitochondrial respiratory chain and energy production	1553538_s_at	–	*COX1*	cytochrome *c* oxidase subunit I	M	4.73E-07	−2.15	complex IV subunit[1]
	238199_x_at	–	*COX3*	cytochrome *c* oxidase III	M	1.96E-13	−3.22	complex IV subunit[2]
	224372_at	NC_012920.1	*ND4*	NADH Dehydrogenase Subunit 4	M	1.69E-06	−1.57	complex I subunit[3]
	1553575_at	–	**ND6*	NADH dehydrogenase, subunit 6 (complex I)	M	1.81E-05	1.75	complex I subunit[4]
	218484_at	NM_020142	**NDUFA4L2*	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4-like 2	12q13.3	0.04	2.02	complex I inhibition in hypoxia[5]
Glucose and lipid metabolism	202672_s_at	NM_001030287	*ATF3*	activating transcription factor 3	1q32.3	0.042	1.94	regulation of metabolic homeostasis [6]
	203394_s_at	NM_005524	**HES1*	hairy and enhancer of split 1, (Drosophila)	3q28-q29	0.032	2.14	alpha-glucosidase activator[7]
	209581_at	NM_0011282	*PLA2G16*	phospholipase A2, group XVI	11q12.3	0.016	1.79	phospholipase [8]
	243296_at		*NAMPT*	nicotinamide phosphoribosyltransferase	7q22.3	0.041	1.53	regulation/ reprogramming of cellular metabolism[9]
	228499_at	NM_004567	**PFKFB4*	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	3p22-p21	0.03	1.55	activator of glycolysis enzyme [10]
	203767_s_at	AI122754	STS	steroid sulfatase	Xp22.31	0.036	−1.61	steroid metabolism[11]
	205825_at	NM_000439	PCSK1	proprotein convertase subtilisin/kexin type 1	5q15	0.045	−2.16	regulation of glucose homeostasis and food intake[12]
Oncogenesis	225557_at	NM_033027	*CSRNP1*	cysteine-serine-rich nuclear protein 1	3p22	0.007	1.60	tumor suppressor[13]
	202768_at	NM_0011141	*FOSB*	FBJ murine osteosarcoma viral oncogene homolog B	19q13.32	0.015	2.76	reduction of Fos and Jun proteins[14]
	201631_s_at	NM_003897	*IER3*	immediate early response 3	6p21.3	0.011	1.69	immune regulation and tumorigenesis[15]
	206377_at	NM_001452	*FOXF2*	forkhead box F2	6p25.3	0.015	−1.58	regulation of gene expression in embryonic development, tumoreginicity[16]
	212543_at	NM_001624	*AIM1*	absent in melanoma 1	6q21	0.015	−1.65	melanoma suppression[17]
	204320_at	NM_0011907	*COL11A1*	collagen, type XI, alpha 1	1p21	0.019	3.79	stimulation of cancer progression[18]
	201005_at	NM_001769	*CD9*	CD9 molecule	12p13.3	0.022	−1.76	tumor cell motility and adhesion[19]
	202149_at	NM_0011423	*NEDD9*	neural precursor cell expressed, developmentally down-regulated 9	6p25-p24	0.007	2.2	support of oncogenic signaling[20]
	202081_at	NM_004907	*IER2*	immediate early response 2	19p13.2	0.001	1.56	may be involved in the regulation of tumor progression and metastasis[21]
Inflammation and immune response	229487_at	NM_024007	*EBF1*	early B-cell factor 1	5q34	0.024	−1.61	activation of the B cell lineage program[22]
	201044_x_at	NM_004417	*DUSP1*	dual specificity phosphatase 1	5q34	0.021	1.6	regulation of anti-inflammatory genes[23]
	214240_at	NM015973	*GAL*	galanin prepropeptide	11q13.3	0.024	−1.55	skin immunity[24]
	205266_at	NM_002309	*LIF*	leukemia inhibitory factor (cholinergic differentiation factor)	22q12.2	0.029	1.53	anti-inflammatory and pro-gestational activities[25]
	223217_s_at	NM_001005474	*NFKBIZ*	kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	3p12-q12	0.021	1.57	inflammatory and immune response[26]
	238013_at	NM_021623	*PLEKHA2*	pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 2	8p11.22	0.024	1.57	B-cell activation[27]
	39402_at	M15330	IL1B	interleukin 1 beta	2q14.1	0.034	1.53	key mediator of the inflammatory response[28]
	226757_at	AA131041	IFIT2	interferon induced protein with tetratricopeptide repeats 2	10q23.31	0.047	−1.61	antiviral immune response and innate immunity[29]
	229450_at	AI075407	IFIT3	interferon induced protein with tetratricopeptide repeats 3	10q23.31	0.029	−1.54	antiviral immune response and innate immunity[29]
	1553142_at	NM_153218	LACC1	laccase domain containing 1	13q14.11	0.041	−1.63	cytokine secretion and bacterial clearance[30]
Cell growth and apoptosis	222108_at	NM_181847	*AMIGO2*	adhesion molecule with Ig-like domain 2	12q13.11	0.046	1.56	apoptosis inhibition [31]

(*continued on next page*)

Table 2 (*continued*)

Clusters	Probe set	ACCNUM	Gene Symbol[a]	Gene name	Cytoband[b]	p	Flc[c]	Function[d]
	202094_at	NM_001012270	*BIRC5*	baculoviral IAP repeat containing 5	17q25	0.033	−1.74	apoptosis inhibition[32]
	201147_s_at	NM_000362	*TIMP3*	TIMP metallopeptidase inhibitor 3	22q12.3	0.036	−1.54	apoptosis regulation[33]
	201170_s_at	NM_003670	*BHLHE40*	basic helix-loop-helix family, member e40	3p26	0.002	1.57	chondrocytes differentiation[34]
	201473_at	NM_002229	*JUNB*	jun B proto-oncogene	19p13.2	< 0.001	1.92	control of cell growth and differentiation[35]
	209189_at	NM_005252	*FOS*	FBJ murine osteosarcoma viral oncogene homolog	14q24.3	0.024	2.36	bone growth[36–37]
	242138_at	NM_001038493	*DLX1*	distal-less homeobox 1	2q32	0.016	−1.83	production of forebrain GABAergic interneurons[38]
	212327_at	NM_0011127	*LIMCH1*	LIM and calponin homology domains 1	4p13	0.027	2.14	non muscle myosin-II regulation and cell migration supression[39]
	220559_at	NM_001426	*EN1*	engrailed homeobox 1	12q23.3	0.025	1.64	regulation in early development[40]
	202202_s_at	NM_0011052	*LAMA4*	laminin, alpha 4	6q21	0.01	−1.53	constituent of basement membranes[41]
	201116_s_at	NM_001873	*CPE*	carboxypeptidase E	4q32.3	0.032	2.18	involved in the processing of the majority of neuropeptides and peptide hormones[42]
	200962_at	NM_001098577	*RPL31*	ribosomal protein L31	2q11.2	0.010	1.82	component of the 60S subunit[43]
	45714_at	AA436930	HCFC1R1	host cell factor C1 regulator 1	16p13.3	0.015	1.5	cell cycle regulation[44]
	222118_at	AK023669	CENPN	centromere protein N	16q23.2	0.038	−1.56	cell cycle regulation[45]
	223038_s_at	BG479856	SINHCAF	SIN3-HDAC complex associated factor	12p11.21	0.046	1.52	cell cycle regulation[46]
Transcription	228531_at	NM_001193307	*SMAD9*	sterile alpha motif domain containing 9	7q21.2	0.015	−1.60	transcriptional regulation in BMP signaling[47]
	231292_at	NM_0010083	*EID3*	EP300 interacting inhibitor of differentiation 3	12q23.3	0.026	−1.62	transcriptional control of testicular tissue[48]
	202935_s_at	NM_000346	*SOX9*	SRY (sex determining region Y)-box 9	17q23	0.031	2.59	transcription factor[49]
	206373_at	NM_003412	*ZIC1*	Zic family member 1 (odd-paired homolog, Drosophila)	3q24	0.014	3.03	transcription factor, differentiation and growth [50]
	201693_s_at	NM_001964	*EGR1*	early growth response 1	5q31.1	0.001	2.31	regulation of gene transcription[51]
Signal transduction	1558280_s_at	NM_004815	*ARHGAP29*	Rho GTPase activating protein 29	1p22.1-p21.3	0.014	−1.62	regulation of the RhoA-LIMK-cofilin pathway[52]
	207135_at	NM_000621	*HTR2A*	5-hydroxytryptamine (serotonin) receptor 2A	13q14-q21	0.039	1.8	serotonin receptor[53]
	221467_at	NM_005912	*MC4R*	melanocortin 4 receptor	18q22	0.004	−1.98	key regulator of energy homeostasis, food intake and body weight[54]
	225647_s_at	NM_0011141	*CTSC*	cathepsin C	11q14.2	0.002	−3.13	activation of granule serine proteases[55]
	227697_at	NM_003955	*SOCS3*	suppressor of cytokine signaling 3	17q25.3	0.018	1.57	suppressor of cytokine signaling[56]
	204338_s_at	NM_005613	RGS4	regulator of G protein signaling 4	1q23.3	0.043	1.89	cell Signaling[57]
Other functions	236532_at	NM_207645	*C11orf87*	chromosome 11 open reading frame 87	11q22.3	0.025	−2.09	not known
	235888_at	NR027026	*GUSPB1*	glucuronidase, beta pseudogene 1	5p14.3	0.004	1.51	not known
	238452_at	NM_001002901	*FCRLB*	Fc receptor-like B	1q23.3	0.021	−1.72	not known
	237075_at	AI191591	*ACTR3-AS1*	ACTR3 antisense RNA 1	2q14.1	0.001	1.67	not known
	223453_s_at	BC005096	*ATL3*	atlastin GTPase 3	11q13.1	0.005	−1.6	GTPase[58]
	1561141_at	AF086258	LINC02544	long intergenic non-protein coding RNA 2544	6q27	0.015	1.94	not known
	235874_at	AL574912	PRSS35	serine protease 35	6q14.2	0.016	1.7	not known
	241014_at	H09620	FLG-AS1	FLG antisense RNA 1	1q21.3	0.017	−1.56	not known
	229523_at	N66694	TMEM200C	transmembrane protein 200C	18p11.31	0.017	1.53	not known
	217220_at	AL050153	LOC100287387	uncharacterized LOC100287387	2q37.3	0.019	−1.59	not known
	230097_at	AI207338	GART	phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase	21q22.11	0.02	−1.52	purine synthesis[59]
	239229_at	AI342246	PHEX	phosphate regulating endopeptidase homolog X-linked	Xp22.11	0.03	−1.69	not known
	229656_s_at	AA236463	EML6	EMAP like 6	2p16.1	0.03	−1.62	not known

(*continued on next page*)

Table 2 (*continued*)

Clusters	Probe set	ACCNUM	Gene Symbol[a]	Gene name	Cytoband[b]	p	Flc[c]	Function[d]
	229222_at	AI123815	ACSS3	acyl-CoA synthetase short chain family member 3	12q21.31	0.031	−1.62	not known
	204984_at	NM_001448	GPC4	glypican 4	Xq26.2	0.032	1.51	not known
	1568720_at	BC018100	ZNF506	zinc finger protein 506	19p13.11	0.033	1.58	not known
	218959_at	NM_017409	HOXC10	homeobox C10	2q13.13	0.034	2.12	not known
	219230_at	NM_018286	TMEM100	transmembrane protein 100	17q22	0.039	−1.64	not known
	1553654_at	NM_153262	SYT14	synaptotagmin 14	1q32.2	0.041	−1.57	not known
	201531_at	NM_003407	ZFP36	ZFP36 ring finger protein	19q13.2	0.042	1.55	not known
	219686_at	NM_018401	STK32B	serine/threonine kinase 32B	4p16.2	0.046	−1.61	not known
	233947_s_at	U47671	TBX5-AS1	TBX5 antisense RNA 1	12q24.21	0.047	−2.16	not known
	221900_at	AI806793	COL8A2	collagen type VIII alpha 2 chain	1p34.3	0.047	1.62	Not known
	210839_s_at	D45421	ENPP2	ectonucleotide pyrophosphatase/ phosphodiesterase 2	8q24.12	0.048	−1.57	not known
	222803_at	AI871620	PRTFDC1	phosphoribosyl transferase domain containing 1	10p12.1	0.048	−1.5	not known
	227928_at	AI224977	PARPBP	PARP1 binding protein	12q23.2	0.049	−1.5	not known
	235085_at	BF739767	PRAG1	PEAK1 related, kinase-activating pseudokinase 1	8p23.1	0.036	1.59	not known

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[a] Genes marked by an asterisk were selected to be tested by real-time PCR (qRT-PCR).

[b] Gene location obtained from National Center for Biotechnology Information public database (http://www.ncbi.nlm.nih.gov).

[c] Fold-changes (Flc) are indicated for each probe set significantly more or less expressed between LSFC and control fibroblasts ($p < 0.05$; absolute Flc > 1.5). Positive data indicate that the genes are more expressed by LSFC fibroblasts; negative data indicate that the genes are less expressed by LSFC fibroblasts.

[d] References that allow classification of differentially expressed genes in function categories:

offered to couples with SLSJ ancestry. It was shown that LSFC fibroblasts present several mitochondrial functional abnormalities including reduced mitochondrial membrane potential, fragmentation of the mitochondrial network, and impaired OXPHOS capacity [13]. In mice harboring an hepatocyte-specific inactivation of *Lrpprc*, it was observed an alteration of the mitochondrial pemeability transition pore and of the lipid composition of mitochondrial membranes [22].

Little is currently known about the sequences of biological pathways altered in LSFC patients. We conducted a microarray gene expression of twelve LSFC patient primary fibroblasts compared to twelve control ones paired for age and sex in order to better understand the functional impact of *LRPPRC* gene mutations and the molecular mechanisms linking the *LRPPRC* mutations to the LSFC disorder.

The microarray gene expression analysis showed 84 significant differentially expressed genes (p value<0.05 and Flc > 1.5) between the LSFC and control cells lines. These genes are implicated in several cellular deregulated processes including the mitochondrial respiratory chain and energy production, glucose and lipid metabolism, oncogenesis, cell growth and apoptosis, inflammation and immune response, signaling transduction and transcription. Considering the mitochondrial type of LSFC disorder and the known *LRPPRC* role in mitochondrial mRNA stability, we think that the potential altered genes, related to the LSFC disorder, are those implicated in the mitochondrial function. This cluster includes two genes encoding for complex IV subunits (*COX1* and *COX3*), two genes encoding for complex I subunits (*ND4* and *ND6*) and *NDUFA4L2* gene whose function and association with respiratory chain complexes remains obscure. *COX1*, *COX3*, *ND6* and *ND4* were under expressed, whereas *NDUFA4L2* is higher expressed in LSFC cells.

These results are partially in agreement with the previous study of Xu et al. (2004) showing that *LRPPRC* is required for the expression of *COX1* and *COX3* [14].

NDUFA4L2 is expressed two times more in LSFC fibroblasts compared to control fibroblasts ($p = 0.04$; Flc = 2.02). NDUFA4L2 protein is the target of the hypoxia inducible factor-1 (*HIF-1*) gene, which is activated in low oxygen conditions. It has been shown that *NDUFA4L2,* in hypoxic conditions, inhibits electron transport chain (ETC) activity and this reduces mitochondria oxygen consumption, which limits intracellular reactive oxygen species production and plays an important role in the control of glycolysis and glucose oxidation [23] [24]. Consequently, *NDUFA4L2* can mediate the function of oxidative phosphorylation and reactive oxygen species (ROS) production in mitochondria. In the case of LSFC patients for which we observed an increase of *NDUFA4L2* expression, we hypothesize that COX deficiency could lead to relative hypoxia similar to the one induced by HIF-1. Consequently, *NDUFA4L2* expression is induced which could counterbalance the oxygen decrease by preventing the overloading of the respiratory chain, thus resulting in metabolic acidosis.

Moreover, other researchers showed that loss of *LRPPRC* function in

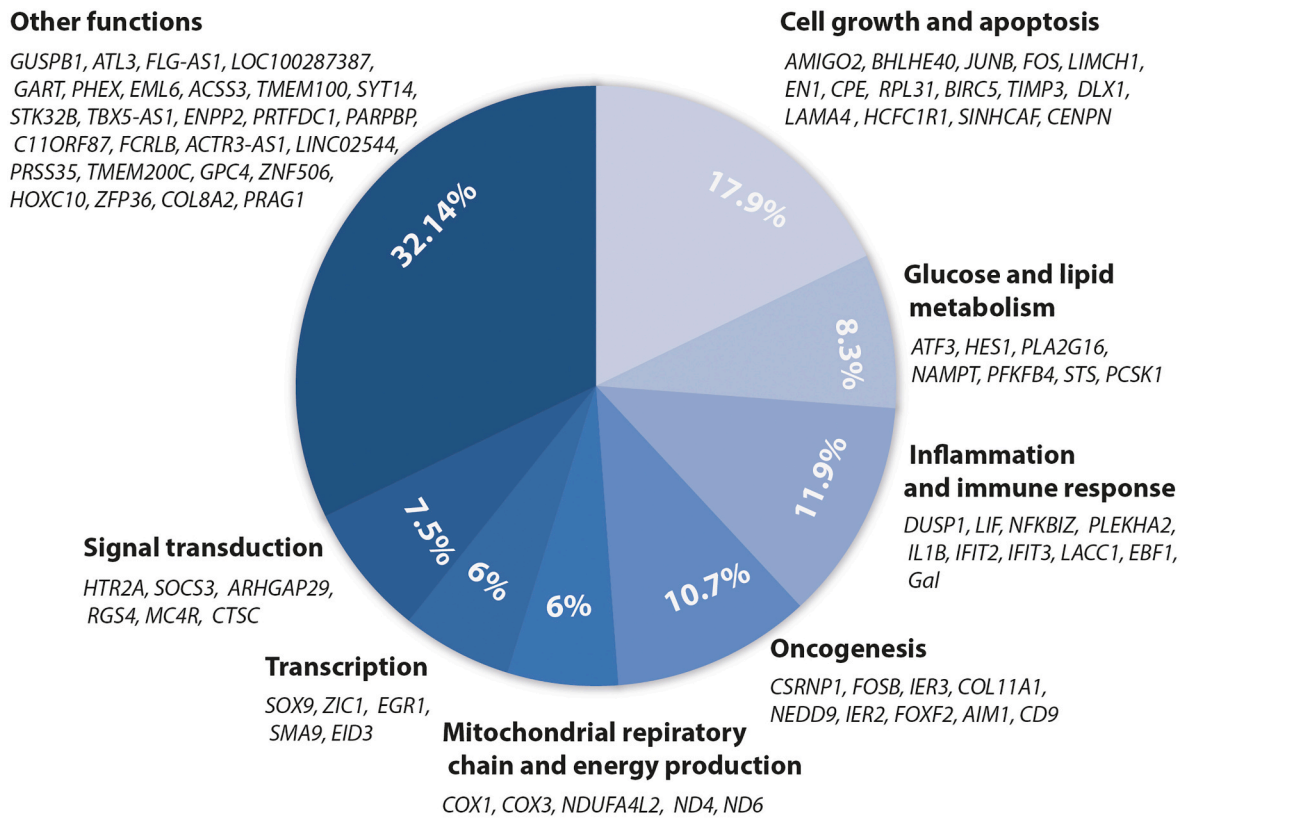

Fig. 1. Differentially expressed genes clusters according to their molecular function Comparison of gene expression profile of twelve paired LSFC and controls cell lines (fibroblasts) by microarrays showed a set of 84 significant differentially expressed genes (Flc $\geq$ 1.5 and $p \leq 0.05$). Based on the molecular function of these genes, they were classified on seven clusters: mitochondrial respiratory chain and energy production (5), glucose and lipids metabolism (7), oncogenesis (9), immune response (10), cell growth and apoptosis (15), transcription (5), signal transduction (6), and 27 genes with other not yet known function.

Table 3
List of higher and under expressed genes in LSFC patients.

Clusters	Higher expressed genes	Under expressed genes
Mitochondrial respiratory chain and energy production	*ND6, NDUFA4L2*	*COX1, COX3, ND4*
Glucose and lipid metabolism	*ATF3, HES1, PLA2G16, NAMPT, PFKFB4*	*STS, PCSK1*
Oncogenesis	*CSRNP1, FOSB, IER3, COL11A1, NEDD9, IER2*	*FOXF2, AIM1, CD9*
Inflammation and immune response	*DUSP1, LIF, NFKBIZ, PLEKHA2, IL1B*	*EBF1, Gal, IFIT2, IFIT3, LACC1*
Cell growth and apoptosis	*AMIGO2, BHLHE40, JUNB, FOS, LIMCH1, EN1, CPE, RPL31, HCFC1R11, SINHCAF*	*BIRC5, TIMP3, DLX1, LAMA4, CENPN*
Transcription	*SOX9, ZIC1, EGR1*	*SMA9, EID3*
Signal transduction	*HTR2A, SOCS3, RGS4*	*ARHGAP29, MC4R, CTSC*
Other functions	*GUSPB1, ACTR3-AS1, LINC02544, PRSS35, TMEM200C, GPC4, ZNF506, HOXC10, ZFP36, COL8A2, PRAG1*	*C11orf87, FCRLB, ATL3, FLG-AS1, LOC100287387, GART, PHEX, EML6, ACSS3, TMEM100, SYT14, STK32B, TBX5-AS1, ENPP2, PRTFDC1, PARPBP*

LSFC fibroblasts displayed primarily a COX deficiency and a global reduction in the steady-state levels of all mitochondrial mRNAs except *ND3* and *ND6* [10,11]. Indeed, *ND6* mRNA lacks poly A tail that is why its steady-state level was shown to not be changed in the absence of LRPPRC in the mouse heart [21].The present microarrays expression results showed a variable expression of *ND6* gene in LSFC fibroblasts compared to control ones. We think that *ND6* expression may be variable

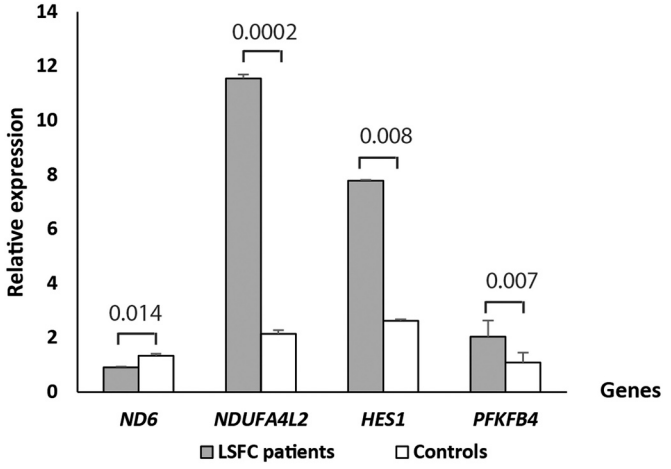

Fig. 2. Expression of the four selected genes using real-time PCR (qRT-PCR). NADH dehydrogenase, subunit 6 (complex I) (*ND6*), NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4-like 2 (*NDUFA4L2*), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 (*PFKFB4*), and hairy and enhancer of split 1, (Drosophila) (*HES1*) mRNA was extracted from skin fibroblasts of LSFC (gray bars) and paired controls (white bars) individuals. Measure of the mRNA expression by real-time RT-PCR was done twice in triplicate with negative control and normalized to *RPL13A* expression using two-standard curves method. Data are expressed as mean + SEM values. *NDUFA4L2*, *PFKFB4*, and *HES1* mRNA level are significantly ($p < 0.05$) higher in LSFC skin fibroblasts participants compared with controls.

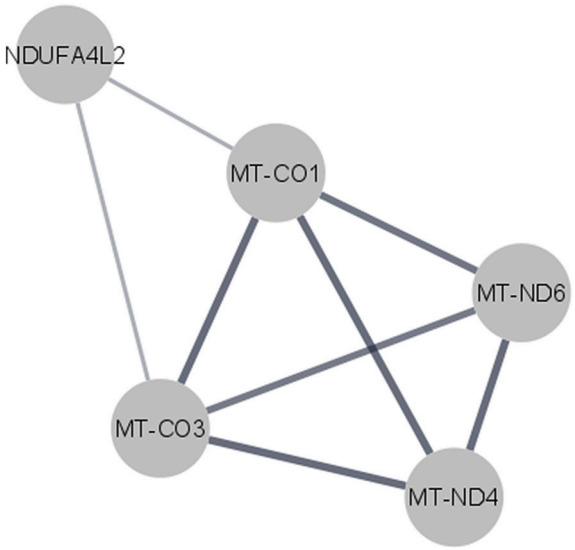

Fig. 3. Protein protein interactions network. Network analysis of dysregulated genes was performed using STRING database, considering a combined interaction score > 0.4 cut off for significant interaction. A strong interaction between the differentially expressed genes of the mitochondrial and energy production cluster was observed.

between heart and fibroblasts specially that it was shown that usually heart is less affected in LSFC patients [10,12]. Nevertheless, *ND6* gene was higher expressed in microarrays (p = 3.34E-05; Flc = 1.72) and under expressed in qRT-PCR (p = 0.014; Flc = 1.46). This contradiction could be explained by the fact that the microarrays were performed in two different sets spaced out by 5 years and were not all carried out at the same time nor by the same manipulator.

STRING showed strong interactions between *COX1*, *COX3*, *ND4*, *ND6* and *NDUFA4L2*, such interaction is crucial to induce an adaptive response of mitochondria in LSFC cells (Fig. 3). This is in agreement with a previously study showing that LSFC fibroblasts preserved ATP levels in basal conditions, suggesting the activation of a compensatory mechanism [13].

Based on the present microarray results analysis, we hypothesize that in LSFC fibroblasts, LRPPRC loss causes a COX deficiency by decreasing its two subunits COX1 and COX3. This could lead to relative hypoxia that induced the expression of *NDUFA4L2*. *NDUFA4L2* attenuates mitochondrial oxygen consumption by ETC inhibition via the decreasing expression of ND4 subunit. This reduces function of the transcription/translation mitochondrial machinery, and limits the intracellular reactive oxygen species production under low-oxygen conditions [23] (Fig. 4).

Mitochondrial respiration is crucial for cellular metabolic function. In normal cells, LRPPRC promotes fatty acid uptake and oxidation of hepatocytes by increasing oxidative phosphorylation activity, which limit blood lipid level and interdicts non-alcoholic fatty liver disease (NAFLD) in mice [25]. In LSFC disorder, many perturbations were observed in fatty acid metabolism in mitochondria [26] as well as a lipid dyshomeostasis [27]. Indeed, loss of LRPPRC caused oxidative phosphorylation deficiency and decreased the capacity to oxidize fatty acids. In the present work, we observed higher expression of several genes involved in lipid and glucose metabolism as *PFKFB4* gene encoding for an activator of glycolysis enzyme and *PLA2G16*, a phospholipase. The increased expression of glycolytic and lipidic genes may in part represent a biochemical adaptation to compensate for the loss of mitochondrial ATP production by enhancing glycolytic ATP production. Previous studies have shown increased expression of genes involved in glycolysis in mitochondrial DNA mutant cells [28,29]. The higher expression of these genes may in part lead to a metabolic switch away from

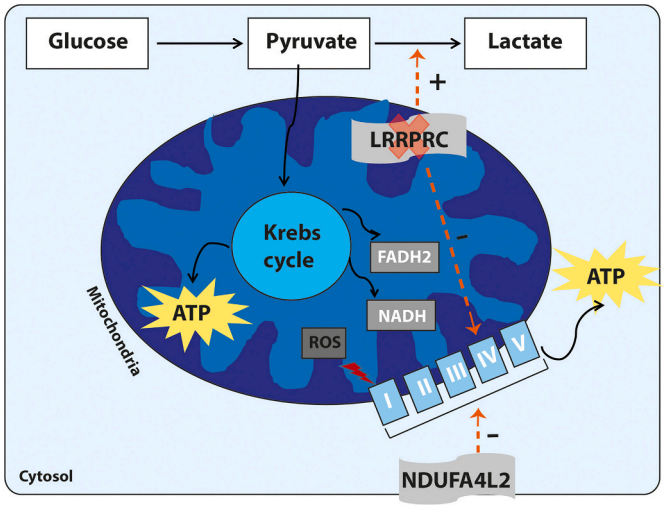

Fig. 4. Depiction of the respiratory chain defects in LSFC patients. The five mitochondrial complexes are shown embedded in the inner mitochondrial membrane and called I, II, III, IV, and V. Loss of LRPPRC decreases the activity of the mitochondrial complex IV that results in accumulation of reactive oxygen species (ROS) in the mitochondria. As an adaptative mechanism, cells switch away from mitochondrial ATP production toward glycolysis, a necessary adaptation to the loss of mitochondrial respiratory capacity in LSFC cells leading to increasing level of blood lactic acid. This will cause hypoxia condition that increases the expression of the *NDUFA4L2* gene. *NDUFA4L2* decreases oxygen consumption by inhibiting the electron transport chain activity.

mitochondria toward glycolysis, a necessary adaptation to the loss of mitochondrial respiratory capacity in LSFC cells.

Non-targeted lipidomic analysis was also performed and thirty-three distinct lipids were shown to be altered in H-Lrpprc$^{-/-}$ mice mitochondria indicating that LRPPRC deficiency leads to changes in the lipid composition of mitochondrial membranes [22].

The present LSFC gene expression profile analysis showed also a dysregulation of the expression of several genes involved in tumor progression and cancer. This is not surprising as recent studies have shown that LRPPRC expression increases in various cancer tissues and tumor cell lines, including prostate cancer [30–32], gastric cancer [16], and lung adenocarcinoma [16,33]. Further experiments are needed to explore the eventual implication of these oncogenesis genes in LSFC disorder.

We also observed the altered expression of genes involved in cell growth and apoptosis, inflammation and immune response, transcription and transduction signaling. These pathways are in majority a result of the mitochondrial respiratory chain defect. It was reported that the reactive oxygen species are a major activator of apoptosis that has been linked with oxidative stress in acute respiratory distress syndrome, chronic obstructive pulmonary disease (COPD and lung fibrosis [34–38]. Interestingly, a close link was observed between oxidative stress and inflammatory responses [38].

5. Conclusion

In summary, the present study used global high-throughput microarray analysis together with bioinformatics-assisted functional clustering to identify the expression profile in LSFC patients cell lines. Our data demonstrates that LSFC fibroblasts present a series of adaptations to potentially overcome the decrease in mitochondrial respiration. A set of interesting differentially expressed genes in LSFC patients was identified. Specifically, genes involved in the mitochondrial chain respiratory, seem to be directly involved in the LSFC disease. The present work provides a better understanding of the biological pathways altered in LSFC disorder. Nevertheless, the downregulation of *LRPPRC* expression

is tissue specific, that is why, these data cannot be extrapolated to other tissues such as brain and liver, which have different energetic metabolism. Further functional gene expression studies in these tissue cells are required to strengthen the significance of our findings in the biology of LSFC disorder.

Author contributions

CL build and manage the LSFC biobank, design the study and reach the financial support, supervise trainee and research staff, edit paper and approval the final version. CM is a pediatrician involved in patient recruitment and sampling and revised the paper. JT performed experiments and participated in data analysis. MB participated in data analysis and interpretation. MB and JT wrote the first draft of the manuscript.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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