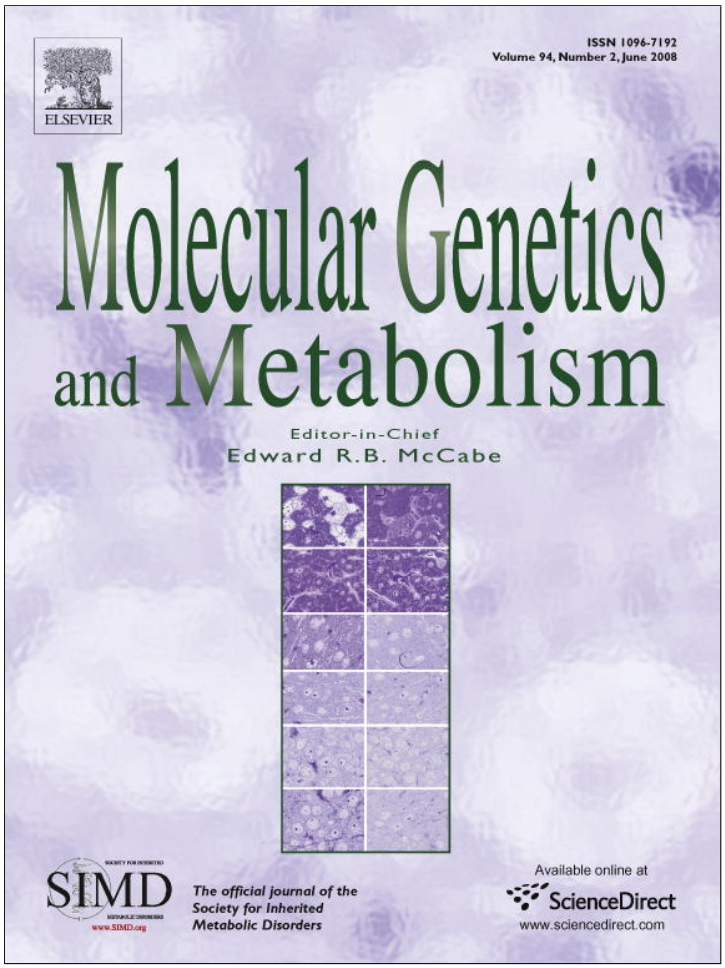

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Normal HPRT coding region in complete and partial HPRT deficiency

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Abstract

Lesch–Nyhan syndrome is an X-linked recessive inborn error of metabolism due to a virtually complete lack of hypoxanthine–guanine phosphoribosyltransferase (HPRT) activity (OMIM 300322). Partial deficiency of HPRT (OMIM 300323) is characterized by the effects of excess uric acid synthesis and a continuum spectrum of neurological manifestations, without the manifestations of full-blown Lesch–Nyhan syndrome. Both diseases have been associated with mutations in the HPRT gene. These mutations are heterogeneous and disperse throughout the entire HPRT gene. In 2005 Dawson et al. described, for the first time, an individual with gout in whom HPRT deficiency appeared to be due to a defect in gene regulation. In the present study we present four patients with partial HPRT deficiency and one patient with Lesch–Nyhan syndrome who showed a normal HPRT coding sequence and markedly decreased HPRT mRNA expression. This is the first report of a patient with Lesch–Nyhan syndrome due to a defect in HPRT gene expression regulation.

Keywords: Lesch–Nyhan; HPRT; Gene regulation; Real-time PCR; Hyperuricemia

Introduction

A virtually complete lack of hypoxanthine–guanine phosphoribosyltransferase (HPRT; EC 2.4.2.8) activity causes the Lesch–Nyhan syndrome (OMIM 300322) [1,2]. This syndrome is manifested by excessive purine production and characteristic neurological manifestations, including compulsive self-mutilation, choreoathetosis, dystonia and defects in attention and executive functions [3].

On the other hand, a partial deficiency of HPRT activity causes the Kelley Seegmiller syndrome or HPRT-related gout (OMIM 300323) [4]. Partial deficiency is characterized by the effects of excess uric acid synthesis in renal, articular and other tissues. Patients may present a continuum spectrum of neurological manifestations, without the manifestations of full-blown Lesch–Nyhan syndrome. The phenotypes produced by HPRT deficiency can be divided into four groups [5], and in the less severe forms, partial HPRT deficiency presents as hyperuricemia, hyperuricosuria, nephrolithiasis and gout without evident neurological manifestations [5].

Human HPRT is encoded by a single structural gene spanning approximately 45 Kb on the long arm of the X chromosome at Xq26 and consists of nine exons with a coding sequence of 654 bp [6]. Documented mutations in HPRT deficiency show a high degree of heterogeneity in type and location within the gene: deletions, insertions, duplications etc., and to date more than 300 disease-associated mutations have been described [7] (www.lesch-nyhan.org). Single point mutations are the main cause of partial HPRT deficient activity, whereas Lesch–Nyhan syndrome is mainly caused by mutations that alter the size of the predicted protein [8]. Dawson et al. [9] have described a partial HPRT deficient patient in whom neither a mutation in the genomic DNA nor in the cDNA was found.

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Patients and methods

Patients: Clinical presentation

Propositus 1 (P1): A 20-year-old male with a family history of gout (maternal grandfather), presented with hyperuricemia (16 mg/dl), and arthralgias that improved with colchicine therapy.

Propositus 2, 3 and 4 (P2, P3 and P4) were three male brothers 15-, 12- and 10-year-old, with a family history of hyperuricemia, gout and lithiasis. They presented asymtomatic hyperuricemia with an increased urinary uric acid/creatinine ratio. P2 was diagnosed as having an obsessive–compulsive behavior for which he was treated. On physical exam he showed slight neurological alterations, such as slight dystonia and dispraxia. P3 and P4 showed minimal neurological involvement.

Propositus 5 (LN1): A 2-year-old male with a normal family history, presented with hyperuricemia, nephrolithiasis, and psychomotor delay at 12 months of age.

Venous blood, both with EDTA and sodium heparin, was obtained from the patients for enzyme and molecular studies.

Enzyme assays

HPRT and adenine phosphoribosyltransferase (APRT) activities in erythrocyte lysates were determined by high performance liquid chromatography [10]. Residual HPRT activity was determined in intact erythrocytes as previously described [5].

Analysis of HPRT coding region

Total RNA was isolated from peripheral blood using the QIAamp RNA Blood Mini Kit (QIAGEN GmbH, D-40724, Hilden, Germany). A first-strand cDNA template was generated using the ImProm-II™ Reverse Transcriptase system (Promega, Promega Corporation, WI, USA) and oligo(dT) as a primer for RT-PCR. The entire coding region of the HPRT cDNA was amplified from the single strand cDNA by two-nested PCR (Fig. 1A) [8,11]. A 754 bp DNA fragment was obtained and sequenced by an automated sequencer using the BigDye® Terminator Cycle Sequencing kit (Applied Biosystems) in an ABI PRISM® 377 DNA Sequencer (Applied Biosystems).

Analysis of genomic DNA

The RNA-free genomic DNA samples were isolated from whole blood using a DNA Purification Kit (Puragene, Gentra systems, Minneapolis, MN 55447, USA). All nine exons of the human HPRT gene were amplified on eight separate DNA fragments of different lengths as previously described [8,12]. Both strands, forward and reverse, of the amplified DNA fragments were sequenced employing BigDye® Terminator Cycle Sequencing kit (Applied Biosystems) in an ABI PRISM® 377 DNA Sequencer (Applied Biosystems).

Real-time HPRT expression quantification

HPRT mRNA expression was quantified by Real-time PCR with the use of a relative quantification method [13]. We employed a housekeeping gene such as β-Actin as a reference gene and the results were

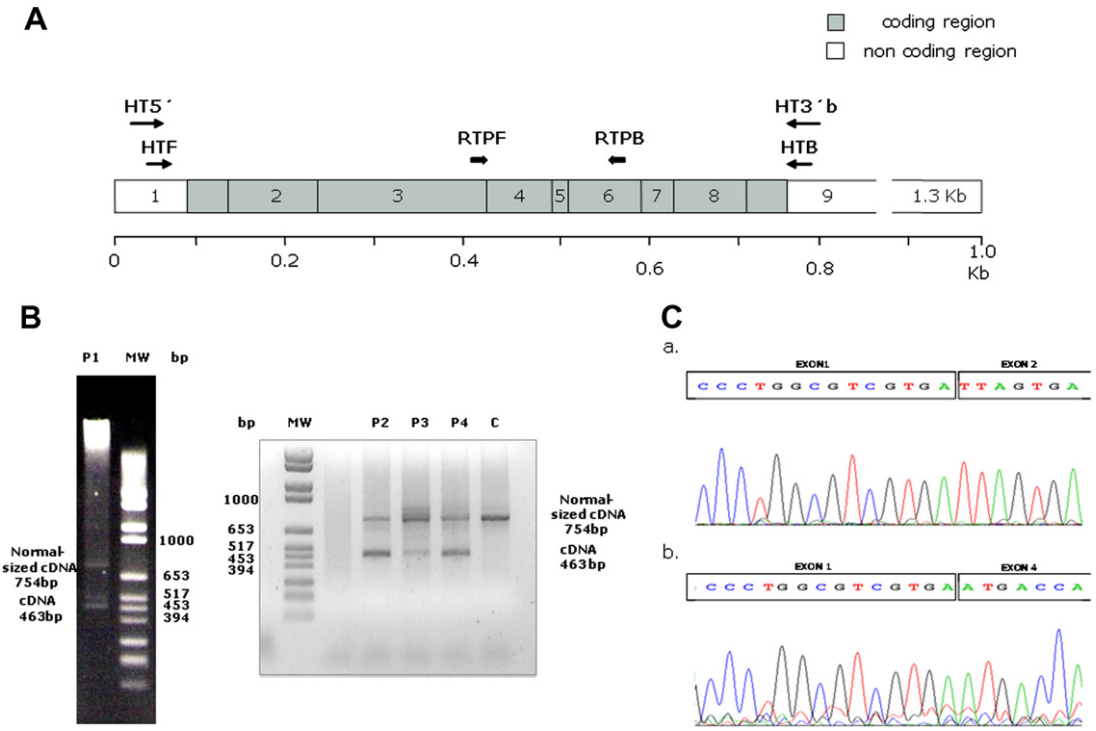

Fig. 1. (A) Specific primers for HPRT cDNA amplification and for Real-Time HPRT expression quantification. The entire coding region of the HPRT cDNA was amplified from the single strand cDNA by two-nested PCR (PCR1 and PCR2). **HT5′** (Previously described, 11) and **HT3′b**, 5′-AAG CTC TAC TAA GCA GAT GGC CAC AGA ACT-3′ (modified from 11) were used in PCR1 to amplified a 877 bp. Using 5 μl of PCR1 as template, **HTF**, 5′-TTC CTC CTC CTG AGC AGT C-3′ and **HTB**, 5′-TGG CGA TGT CAA TAG GAC TC-3′ primers were used in PCR2 to amplified a 754 bp fragment. For Real-time HPRT expression quantification primers **RTPF** and **RTPB** (Roche Diagnostics) were used. HPRT amplicon (181 bp) covered the end of exon 3, the complete exons 4 and 5, and most of exon 6. As the forward primer is located in exon 3 and the backward primer is located in exon 6, transcripts without exon 3 or 6 cannot be amplified. (B) HPRT cDNA from Patients P1, P2, P3, P4 and a control sample (C) obtained in PCR2. P1, P2, P3 and P4 show, in addition to the normal-sized cDNA (754 bp), several shorter cDNA fragments, the most abundant corresponding with a 463 bp fragment. (C) Chromatogram of (a) normal-sized cDNA (754 bp) and (b) the 463 bp cDNA, respectively.

expressed as a relative ratio of the HPRT expression to a reference target measured in the same sample material. To obtain the concentration of these two parameters a standard curve for each target was used.

In addition to the studied patients, HPRT expression was quantified in 14 control subjects and in 11 HPRT-deficient patients (9 with Lesch–Nyhan syndrome and 2 with partial deficiency). In all subjects, total RNA was isolated from peripheral blood using the QIAamp RNA Blood Mini Kit (QIAGEN GmbH, D-40724, Hilden, Germany). A first-strand cDNA template was generated using ImProm-II™ Reverse Transcriptase (Promega, Promega Corporation, WI, USA) and 15-mer oligo(dT) as a primer for RT-PCR.

A control RNA was reverse transcribed and the cDNA was employed as calibrator. A standard curve for human β-Actin was constructed using serial dilutions of this calibrator. The calibrator sample was assigned a value of 100. Real-time PCR was performed in a Roche LightCycler using LC Fast Start DNA Master SYBR Green I (Roche) with 2 μl of cDNA as the template. A melting curve analysis was used to determine the melting temperature (T_m) of the amplified products so as to ensure its specificity.

LightCycler h-HPRT Housekeeping Gene Set Primer/Hybridization Probe mixture (Roche Diagnostics) was used to amplify a 181 bp fragment of HPRT cDNA (Fig. 1A). In vitro-transcribed human HPRT RNA (Roche Molecular Biochemicals) was employed to construct a standard curve for HPRT expression quantification. Real-time PCR was performed in a Roche LightCycler using LC Fast Start DNA Master Hybridation Probes (Roche) with 5 μl of cDNA as template.

Analysis of quantification data was made with the LightCycler software. This software only considers fluorescence values measured in the exponential growing phase of the PCR amplification process. The crossing point (Cp) is defined as the cycle numbers where fluorescence levels of all samples are the same, just above background. The Cp is automatically calculated by the LightCycler software by the "Second Derivative Maximum Method". This is achieved by a software algorithm that identified the first turning point of the fluorescence curve. This turning point corresponds to the first maximum of the second derivative curve. A standard curve is generated by plotting the Cp versus the logarithmus of the concentrations for each dilution of the calibrator sample or the standard. The software calculated a linear regression line through the data point and this allows interpolating Cp of any sample and calculating the respective concentration [13].

Analysis of HPRT regulatory genomic regions

A total of 1670 bp fragments corresponding to an HPRT promoter were amplified by PCR. Sequences of intron 1 and intron 2 linked to HPRT gene regulation in ES cells (980 bp 3′ to exon 1 corresponding to part of intron 1, and the total sequence of intron 2) [14] were also amplified by PCR. PCR primers employed and amplicon sizes are shown in Table 1. Both strands of the PCR products were then sequenced by an automated sequencer, ABI PRISM® 377 DNA Sequencer (Applied Biosystems).

Results and discussion

Enzymatic assays

HPRT activity in the hemolysate of P1, P2, P3 and P4 patients was decreased, being undetectable on the Lesch–Nyhan patient (LN1) (Table 2). Conversion of radioactive hypoxanthine into IMP at physiological phosphate concentration confirmed the diagnosis of partial HPRT deficiency in P1, P2, P3 and P4. However, the percentage of radioactive hypoxanthine converted into IMP at 18 mM Pi (phosphoribosyl-pyrophosphate, PRPP, enriched conditions) in intact erythrocytes was close to normal in these patients. No HPRT activity in intact erythrocytes could be detected in LN1 patient (Table 2).

Hypoxanthine salvage by intact erythrocytes, in PRPP enriched conditions, in the range of 66–97% has been previously described in partial HPRT-deficient patients with no neurological involvement [15]. However, no molecular analysis of the HPRT gene has been reported in these patients. This fact suggests that some functional enzyme exist in these patients that is able to transform hypoxanthine into IMP in the most favorable circumstances. In our experience, only one other partial HPRT-deficient patient was able to convert 54% hypoxanthine into IMP, and he presented a point mutation resulting in an amino-

Table 2
Enzyme activities in haemolysates and in intact erythrocytes

Patient	HPRT hemolysate (nmol/h/mg Hgb)	APRT hemolyate (nmol/h/mg Hgb)	% IMP 1 mM	%IMP 18 mM
P1	5	40	3.0	64.0
P2	6	38	3.0	97.8
P3	4	46	3.6	97.7
P4	4	41	4.9	97.4
LN1	<0.01	60	ND	<1%

The enzyme activity in haemolysate was expressed as nmol of IMP synthesized in 60 min per mg of haemoglobin. Enzyme activity in intact erythrocytes was expressed as the percentage of radioactive hypoxanthine transformed into IMP, in 5 or 40 min, at phosphate concentrations of 1 or 18 mM, respectively.
Normal values (means ± SD) for HPRT = 82 ± 6 nmol/h/mg hemoglobin; APRT = 26 ± 5 nmol/h/mg hemoglobin; % IMP 1 mM = 14 ± 5%; % IMP 18 mM = 98 ± 0.8%.

Table 1
Primers employed for Promotor and Intronic sequences amplification

	Primers		Size of the fragment amplified (bp)
	Forward	Reverse	
Promoter1 (−1200 to 1670)	5′-TCGTAAAACTCTTCATGGCAGT	5′-AGGTCAGGTAAGCAAGCAACA	470
Promoter2 (−1310 to 715)	5′-AGAGGGAGGCCACCAACTAT	5′-GCAGGTGGATCACTCAAGGT	595
Promoter3 (−753 to 232)	5′-GGCTGGTCTCGTACTCCTGA	5′-CCTACCAGTTTGCAGGCTCA	521
Intron 1 A (167–980)	5′-GGGTTCGGCTTTACGTCAC	5′-GGTGATTCCTGCCCTTAAAA	813
Intron 2 A (13260–13778)	5′-AAACATCAGCAGCTGTTCTGAG	5′-TGCATAGCCAGTGCTTGAGA	518
Intron 2 B (13761–14383)	5′-CAAGCACTGGCTATGCATGT	5′-ACTTTGGGAGGCTGGGTAAC	622
Intron 2 C (14211–14839)	5′-GTGCAGTGCAGCAGAATGAT	5′-CAACCTGGGCAATACAGTGA	628

acid substitution in the PRPP-binding domain of the enzyme [8].

Analysis of HPRT coding region

A 754 bp fragment of HPRT cDNA sequence (normal size) was obtained in all patients. P1, P2, P3 and P4 showed, in addition to normal size cDNA, several shorter cDNA fragments, the most abundant corresponding with a 463 bp fragment (Fig. 1B). These fragments were not found in the LN1 patient. No mutation was detected in the sequence of the normal-size cDNA fragment from any patient. The 463 bp shorter fragments were purified and sequenced, corresponding to an exon 2 and 3 deletion (Fig. 1C).

Many mutations compromising the splicing of the HPRT mRNA may cause multiple cDNA products [16,17]. Moreover, both decreased normal and abnormal mRNA have been detected in partial HPRT deficient patients with splice mutations [17–19]. Exon 2 and 3 exclusion is quite common with mutations in the 3′ splice sequence of introns 1 and 2 and the 5′ splice sequence of intron 3, and these mutations may be associated with multiple mRNA products [16]. Nonsense mutations in exons may also lead to low levels of mRNA and multiple mRNAs. At least three nonsense mutations in exon 3 have been reported to result in cDNA products with exon 2 and 3 excluded [16]. All three created chain terminating stop codons which probably results in an unstable mRNA, meanwhile the exclusion of exon $2 + 3$ results in an in-frame mRNA. Normal sequence of HPRT mRNA has not been described in Lesch–Nyhan patients.

Analysis of the genomic HPRT DNA

No mutation was found in the nine exons, with its intronic flanking sequences, of patients P1, P2, P3, P4 and LN1, although PCR amplification and sequencing were repeated several times. No change was found in 3′ splice sequence of intron 1 and 2 and the 5′ splice sequence in intron 3 that would explain the shorter fragments found in P1, P2, P3 and P4, nor in exon 3 coding sequence.

We then looked for a splicing mutation that could explain HPRT deficiency and, in partial patients, the shorter cDNA fragment. No splicing mutation was found in a 100 bp intronic region flanking the exonic sequences. Sege-Peterson et al. [18] have described a partial HPRT deficient patient in whom two types of transcripts were found; one normal HPRT mRNA and one in which the first 49 nucleotides of exon 6 were removed originating a truncated protein. Sequence analysis of a 322 bp fragment spanning the 3′ splice site of exon 6 failed to reveal any change in 100 bp preceding exon 6. Thus, the cause of the abnormal splicing in that mutant remains unclear.

Along these lines, Valentine and Heflich [20] have observed that many nonsense HPRT mutants from Chinese hamster ovary (CHO) cells contain greatly reduced concentrations of the major normally spliced HPRT mRNA and minor HPRT mRNA species lacking one or more exons. They verified, with a protocol designed to specifically detect exon-deleted mRNAs, that exon-deleted mRNAs are normal constituents of CHO cells, but that they are not detected in wild-parental cells because their amplification is suppressed by the relatively high concentration of normal HPRT mRNA. In view that no splice mutation could be found, we can assume that the exons 2 and 3 deleted mRNA obtained in our partial patients may be normal constituents of human cells, and that a decrease in normal mRNA expression could explain their presence. A decreased normal mRNA expression could also explain HPRT deficiency in the LN1 patient in whom no mutation in the codificant sequence was found. To confirm the decreased HPRT mRNA expression we performed a Real-time PCR assay.

HPRT expression

HPRT and β-Actin mRNA concentrations in each sample were calculated from their respective standard curves. HPRT expression by Real-time PCR in each sample was expressed as the ratio HPRT/β-Actin concentration.

Control subjects did not show a great variability in HPRT expression (595 ± 190, range from 358 to 925). A significant positive correlation was found between HPRT expression and β-Actin expression ($r = 0.991$; $p < 0.0001$) (Fig. 2A). Two partial HPRT-deficient patients, unlike patients P1, P2, P3 and P4, showed normal HPRT expression ratio (661 and 865, respectively). Five Lesch–Nyhan patients also showed a normal HPRT/β-Actin ratio, whereas the other 4 Lesch–Nyhan patients other than LN1 showed a decreased HPRT expression (207, 40, 12 and 64) (Fig. 2B). Wilson et al. have previously found, by Northern analysis, essentially normal concentrations of HPRT mRNA in 12 out of 15 Lesch–Nyhan patients and in 8 out of 9 gouty patients [21].

In contrast, all patients included in this report showed a significantly decreased HPRT/β-Actin ratio ($P1 = 9.8$; $P2 = 54$; $P3 = 28$; $P4 = 21$ and $LN1 = 32$; 1.6–9% of control HPRT/β-Actin expression). Thus, taken together these results show that HPRT deficiency in the examined patients may be due to a markedly decreased HPRT mRNA expression that could be due to a defect in gene regulatory sequences.

HPRT regulatory sequences

Mutations in gene regulatory sequences have been described as a cause of human disease. Hemophilia B Leyden type is associated with mutations in the promoter sequence of the clotting factor IX gene [22]. In an individual with hereditary thrombophilia due to a type I protein C deficiency, a mutation in the promoter of protein C gene (–14 T → C) has been described, associated with disruption of a binding site for hepatocyte nuclear factor 1 [23].

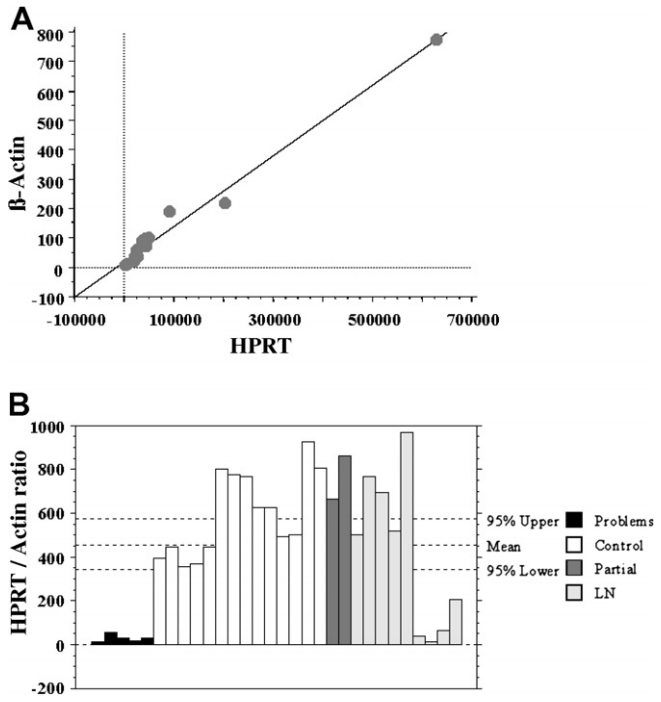

Fig. 2. HPRT mRNA expression by Real-time PCR. The HPRT and β-Actin mRNA concentrations of each sample were calculated from their respective standard curves. HPRT expression by Real-time PCR in each sample was expressed as the ratio HPRT/β-Actin. (A) Correlation between HPRT and β-Actin expression in control samples. There was a significant positive correlation between HPRT expression and β-Actin expression ($r = 0.991$; $p < 0.0001$). (B) HPRT/β-Actin expression in *Problems*: P1, P2, P3, P4 and LN1 patients (black bars), *Controls* (white bars), *Partial patients* (dark gray bars) and Lesch–Nyhan *patients* (gray bars). Bars represent individual values. Mean and 95% confidence interval is represented for control values.

Moreover, in Wilson disease, a 15 nt deletion from position –440 to position –427, 5′ to the translation start site of the Wilson disease gene has been found in patients from the Sardinia population. This mutated sequence presents a transcriptional activity in expression assays of approximately 25% as compared to the normal sequence [24]. The low expression of HPRT mRNA in the studied patients, along with the absence of mutations either in the genomic DNA or in cDNA, suggests that the deficiency of the enzyme could be caused by mutations in regulating elements that determine gene expression, such as promoter and intronic sequences.

Both strands of the 1670 bp fragment of the HPRT promoter, intron 2 and intron 1 sequences were analyzed several times, and found to be normal in all studied patients.

In the promoter of patient LN1 we found a T to G transition, located 321 nucleotides upstream of the A of initiation codon. The patient's mother and several control subjects were homozygous for this transition, which is considered a polymorphism.

The HPRT promoter has common elements with regions of the promoter of other housekeeping genes as well as with some viral promoters [25]. It lacks the consensus sequences TATA and CAAT located 80 bp upstream of the start codon ATG in many eukaryotic genes transcribed by the RNA polymerase II. In addition, it has many GC-rich regions and contains, separated by very little distance, copies of the repetition GGGCGG, which agrees with the consensus-binding site of the Sp family to transcriptional activators.

With respect to the regulating capacity of introns, LH Reids et al. [14] argues for the existence of sequences in introns 1 and 2 that influence the expression of the HPRT gene in ES cells. The sequences located in intron 1 act like enhancers of the expression of HPRT gene. On the other hand, the sequences located in intron 2 are necessary for effective expression of the gene. However, we could not find any mutation in these sequences that would explain the decreased HPRT mRNA expression. Perhaps more distant regulatory elements could be implicated in their HPRT deficiency.

In summary, this report illustrates that complete and partial HPRT deficiency may be attributed to a defect in gene regulation, causing decreased HPRT expression. HPRT expression by Real-time PCR is, particularly in these cases, a useful method for the molecular diagnosis of HPRT deficiency. This is the first report of a patient with Lesch–Nyhan syndrome due to a defect in HPRT gene expression regulation. The cause of the defect in gene regulation could not be determined. Unknown distant regulatory elements could be implicated in the HPRT expression defect.

Acknowledgments

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