Review of 39 Italian patients affected by Lesch – Nyhan disease

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INTRODUCTION

Complete deficiency of the activity of hypoxanthine phosphoribosyltransferase (HPRT) leads to the classic phenotype of Lesch-Nyhan syndrome (Lesch, 1964, Nyhan, 2005) All patients with HPRT deficiency have hyperuricemia and hyperuricosuria and are at risk for nephropathy, urinary tract stone disease, gouty arthritis, and tophaceous deposits. Those with the Lesch-Nyhan phenotype also have an impressive neurologic disorder and unusual self-mutilating behavior; this is the most common clinical picture of HPRT deficiency. It results regularly from major disruptions of the HPRT1 gene (OMIM308000) such as deletions, insertions, and stop codons, but it is also the most common consequence of single nucleotide substitutions. (Jinnah, 2000) Missense mutations that are more conservative lead to variant enzyme proteins with varying amounts of partial activity and to 2 phenotypes that correlate moderately well with amounts of enzyme activity found on assay in an intact cell system. (Page, 1981) Those with the greatest amounts of activity display hyperuricemia and gout or urinary tract stone disease without neurologic or behavioral abnormality. (Kogut, 1970) An intermediate group we have called "neurologic variants" appear neurologically identical to patients with classic Lesch-Nyhan disease, but intelligence is normal or near normal, and there are no abnormalities of behavior. (Bakay, 1979)

BACKGROUND

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 9 exons with a coding sequence of 654bp. Most HPRT-deficient patients present HPRT mRNA expression (Wilson 1986) and molecular diagnosis can be accomplished by cDNA sequencing (Davidson, 1989). In other cases, genomic DNA sequencing may be necessary (Gibbs, 1990). Documented mutations in HPRT deficiency show a high degree of heterogeneity in type and location within the gene: deletions, insertions, duplications, and point mutations have been described as the cause of HPRT deficiency. To date, more than 290 disease-associated mutations have been found (HGMD). Single point mutations are the main cause of partial deficiency of the enzyme, whereas Lesch-Nyhan syndrome is caused mainly by mutations that modify the size of the predicted protein (Torres, 2000). HPRT deficiency is inherited as an X-linked recessive trait. However, about 30% of patients' mothers are not somatic carriers, and these patients probably carry *de novo* mutations due to a germinal cell mutation.
Molecular diagnosis of HPRT defects allows for a faster and more accurate carrier and prenatal diagnosis.

PURPOSE AND HYPOTHESIS

The purpose of this study is offering an original contribution on molecular diagnosis of 39 Italian Lesch-Nyhan patients. Particular attention is given to the methods required to confirm the diagnosis.
Using DNA sequence analysis it is possible to detect mutations in the HPRT1 gene in the majority of males who display the full Lesch-Nyhan syndrome phenotype (Jinnah et al 2000, Jinnah et al 2004). On the other hand, considering that between 21% (Bertelli et al 2004) and 24% (Jinnah et al 2000) of mutations in HPRT1 are large deletions, additional methods are required for testing the potential healthy carrier females. Our work shows how RNA study combined with qPCR (Lapucci et al, 2006) is needed for a correct molecular diagnosis of LNS in female carriers and in some cases also in affected males.

PATIENTS AND METHODS

Patients
Blood samples were collected from 38 unrelated patients and their families after obtaining the informed consent, according to the Bioethics Laws of the European Union and Italy. The 39 patients came from 31 unrelated families. All patients had clinical manifestations and positive tests for HPRT enzyme deficiency.

DNA analysis
Each of the 9 exons of the HPRT1 gene was amplified by PCR, using specific oligonucleotide primers. PCR was performed in a final volume of 20 µl, containing 50 ng of genomic DNA, 10 pmol of each primer; 10 mM Tris-HCl pH 8.3, variable $MgCl_2$ concentration, depending on the exon amplified, 50 mM KCl, 1.5 units of *Taq* DNA polymerase (Gibco BRL, Gaithersburg, MD), 200 mM of each dNTP. The reaction mixture was subjected to 35-40 cycles of PCR in an automated thermal cycler (PCR TC-512 Techne). PCR products were purified with the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Pharmacia-GE Healthcare), and directly sequenced with the same primers as used for PCR, using the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems) on an automatic ABI PRISM 3130 sequencer (Applied Biosystems). DNA sequences were purified by AutoSeq G-50 kit (GE Healthcare, UK).

mRNA analysis
First-strand cDNA was synthesized using 1 µg of total RNA (DNase-treated) in a 20 µl reverse transcriptase reaction mixture.
1-2 micrograms of total RNA were incubated with 2 units of amplification-grade ribonuclease (RNase)-free DNase I (Gibco BRL) in buffer (final concentration: 20 mM Tris pH 8.3, 50 mM KCl, 2.5 mM MgCl2) in a final volume of 20 µl for 15 min at room temperature. DNase I was inactivated by the addition of 2 µl of 25 mM EDTA and heated to 65°C for 10 min. 2 uL of this mixture were used in a control PCR reaction to check for complete DNA elimination. In case a band of contaminating genomic DNA was recovered, digestion with DNase was repeated.RNA Dnase-treated was used in a final volume of 60 µl containing 60 units of placental RNase inhibitor (Rnasin, Eppendorff), 0.5 mM each dNTP, reverse transcriptase reaction buffer having the following composition: 50 mM Tris pH 8.3, 15 mM KCl, 3 mM MgCl2, 10 mM DTT, and 0.6 µg of random hexamers previously denaturated for 10 min at 65°C and cooled on ice for 5 min before added to reaction.
The mixture described was added of 200 U of M-MuLV Reverse Transcriptase (Fermentas) above and incubated at 25°C for 15 min, at 42°C for 60 min and finally heat inactivated at 70°C for 10 min. cDNA was purified with the GFXTM PCR DNA and Gel Band Purification Kit (Amersham Pharmacia-GE Healthcare). Aliquots of 2 ul of cDNA were used immediately in 25-µl PCR reactions.

Real Time PCR for HPRT1 deletions detection
Quantitative PCR analysis was performed in a 96-well optical reaction plate using an ABI Prism 7000 sequence detector (Applied Biosystems). Oligonucleotide primers were designed with Primer Express. Amplification of FANCB gene was used as reference. Reactions were performed in a final volume of 25 uL containing 2× SYBR Green PCR Master Mix (Applied Biosystems, USA), 300 nM of each primer, and 5 to 40 ng genomic DNA, according to serial dilution protocol. *FANCB* gene was chosen as genomic reference because it is localized on chromosome arm Xp but is far enough from *HPRT* not to be compromised by its deletions. Oligonucleotides were designed with Primer Express software (Applied Biosystems) and tested for specificity using NCBI's BLAST tool. In each assay, 5 normal controls and no-template controls were included. Each sample was run in triplicate, and each experiment was repeated 4 times.
PCR conditions were: 2 min at 50°C, 2 min at 95°C, and 50 cycles of 15 sec at 95°C and 30 sec at 60°C. The target and the reference genes were amplified in separate wells. All reactions were performed in triplicate. Reaction mix, without DNA, was used as negative control in each run. A standard curve for each target was used. A standard curve for human FANCB was constructed using serial dilutions of this calibrator. The 2-DDCt method was used to quantify gene expression compared with FANCB DNA.

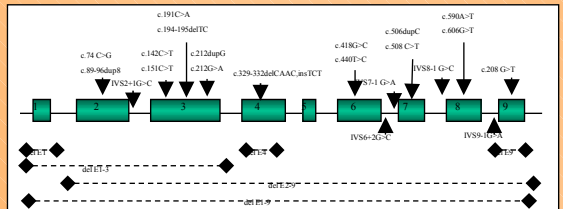
RESULTS

ID	aa change	mutation	ID	aa change	mutation
1		**IVS7-1G>A**	21	p.Gly71Asp	**c.212G>A**
2	p.Arg169X	**c.508C>T**	22		**del E1-9**
3		**del E2-9**	23		**IVS9-1G>A**
4	p.Gly70Trp	**c.208G>T**	24	p.Gly140Arg	**c.418G>C**
5		**c.329-332delCAAC,insTCT**	25		**del E9**
6		**del E1-3**	26		**del E9**
7	p.Pro25Arg	**c.74C>G**	27		**c.212dupG**
8		**IVS8-1G>C**	28	p.Leu147Pro	**c.440T>C**
9		**c.212dupG**	29		**IVS6+2**
10	p.Arg51X	**c.151C>T**	30		**IVS6+2**
11	p.Arg51X	**c.151C>T**	31		**IVS6+2**
12		**c.506dupC**	32		**IVS6+2**
13	p.Ala64Asp	**c.191C>A**	33		**IVS6+2**
14		**del E1**	34		**del E1-9**
15		**del E1**	35	p.Leu147Pro	**c.440T>C**
16		**IVS2+1G>C**	36		**RNA level exclusion E4-5**
17		**del E4**	37		**RNA level exclusion E4-5**
18	p.Leu202Phe	**c.606G>T**	38		**c.89_96dup8**
19	p.Glu197Val	**c.590A>T**	39	p.Leu48Phe	**c.142C>T**
20		**c.194-195delTC**			

CONCLUSIONS

In our cohort of 39 Italian Lesch-Nyhan patients we found 9 different missense mutations in 10 patients (25.6%), 2 different nonsense mutations in 3 patients (7.7%), 1 small deletion (2.6%), 3 different small duplications in 4 patients (10.3%), 1 small insertion-deletion (2.6%), 5 different splice site mutations in 9 patients (23.1%), 6 different gross deletions in 9 patients (23.1%) and 1 exclusion of exons 4 and 5 at RNA level in 2 patients (5,1%). Mutations were spread along the entire gene region.
The correct molecular diagnosis required the use of three different methods. In 37 (95%) of the 39 affected males genomic DNA sequencing was sufficient. However in 2 cases (patient 36 and patient 37) DNA sequencing failed to identify the disease causing mutation and only mRNA level studies were able to show a transcript missing exons 4 and 5. Additional studies of the intronic regions in these patients are currently underway in order to establish the origin of this "alternative splicing" pattern.
Normalizing the number of female carriers to the number of patients we could say that 72% of the carrier females were diagnosed by genomic DNA sequencing, 5% were identified only by RNA level studies and 23% were identified by qPCR.
Our conclusion is that a combination of DNA sequencing and RNA study is required for a correct diagnosis of LNS in affected males and that a CNV method like qPCR or MLPA is of primary importance for the recognition of carrier females.
Molecular level testing for LNS is used in patients as confirmation of the clinical and biochemical diagnoses and is of primary importance in carrier detection and prenatal diagnosis.

	Test method	Detection rate
Affected males	DNA Sequencing	95%
	RNA Studies	5%
Carrier females	DNA Sequencing	72%
	RNA Studies	5%
	qPCR	23%



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