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IDENTIFICATION OF A NEW LESCH-NYHAN SYNDROME MUTATION ($HPRT_{BRASIL}$) AND ANALYSIS OF POTENTIALLY HETEROZYGOUS FEMALES

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ABSTRACT - The mutation in the hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) gene has been determined in two brothers affected with Lesch-Nyhan syndrome. Female members of the family who are at risk for being heterozygous carriers of the *HPRT* mutation were also studied to determine whether they carry the mutation. DNA sequencing revealed that the boys' mother is heterozygous for the mutation in her somatic cells, but that three maternal aunts are not heterozygous. Such carrier information is important for the future pregnancy plans of at-risk females. The mutation, an A→T transversion at cDNA base 590 (590A→T), results in an amino acid change of glutamic acid to valine at codon 197, and has not been reported previously in a Lesch-Nyhan syndrome male. This mutation is designated $HPRT_{Brasil}$.

KEY WORDS: Lesch-Nyhan syndrome, DNA, HPRT.

Identificação de uma nova mutação ($HPRT_{BRASIL}$) em uma família brasileira com a síndrome de Lesch-Nyhan e análise de mulheres potencialmente heterozigotas

RESUMO - Uma mutação no gene hipoxantina-guanina fosforibosiltransferase (HPRT) foi determinada em dois irmãos afetados pela síndrome de Lesch-Nyhan. O sequenciamento do cDNA do gene HPRT das mulheres desta família, potencialmente portadoras heterozigotas da mutação HPRT, que é uma transissão de A→T na base 590 do cDNA (590A→T), revelou que a mãe dos meninos é heterozigota para esta mutação, mas que três tias maternas não são heterozigotas. Este tipo de informação é importante no planejamento de gestações futuras, nas mulheres em risco. Como esta mutação ainda não havia sido relatada em indivíduos do sexo masculino na síndrome de Lesch-Nyhan, foi, portanto, denominada $HPRT_{Brazil}$.

PALAVRAS-CHAVE: síndrome de Lesch-Nyhan, DNA, HPRT.

Lesch-Nyhan syndrome, an inborn error of purine metabolism, is caused by loss of the enzyme hypoxanthine-guanine phosphoribosyltransferase due to mutations in the X-chromosome gene *HPRT*[1,2]. Because this is an X-chromosome linked disease, only males are generally affected and females can be heterozygous carriers. It has been estimated that one-third of Lesch-Nyhan syndrome males would represent new mutations[3]. Features of this syndrome are spastic cerebral palsy, choreathetosis, uric acid urinary stones and neurological disfunction including self-destructive biting of fingers and toes.

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A method for the diagnosis and the analysis of the *HPRT* mutations for Lesch-Nyhan syndrome using peripheral blood T-lymphocytes has been developed[4]. This method is based on the ability of *HPRT*-deficient T-lymphocytes to proliferate and form colonies in the presence of the cytotoxic purine analog 6-thioguanine[4]. Applying this T-lymphocyte cloning assay to Lesch-Nyhan syndrome families allows both diagnosis of the disease and analysis of the *HPRT* mutations responsible for the disease, as well as determination of the carrier status of at-risk females[5,6]. Here, we report the study of a Lesch-Nyhan syndrome family in which a new *HPRT* mutation was found, and the determination of the carrier status of at-risk females in the family. This new mutation is termed $HPRT_{Brasil}$, after the country of origin of the family.

METHOD

We analyzed two Brazilian caucasoid brothers, aged twelve and fourteen years old, and their mother. The first clinical manifestations on the boys became apparent during the third month of life in both of them, with psychomotor retardation and generalized muscular hypotonia. The younger brother started to present self mutilation (biting of lips and fingers) when he was three years old. The older one, however, presented this manifestation only at fourteen years old, usually biting his hands.

Neurological examination showed spasticity, chorioathetosis and signs of self mutilation. The laboratory tests showed hyperuricemia, urinary uric acid increased, and deficiency of the enzyme hypoxanthine-guanine phosphoribosyltransferase (HPRT) that belongs to purine metabolism.

T-lymphocyte isolation and culture

Peripheral blood samples were collected in vacutainer tubes containing sodium heparin and shipped by overnight carrier to the University of Vermont. (Alternatively, for genomic DNA analysis, DNA was extracted from peripheral blood samples collected in EDTA and shipped to Vermont). The samples were collected after informed consent was obtained. The mononuclear cell fraction was isolated by centrifugation over Histopaque and the cells plated for the T-lymphocyte cloning assay, as described previously[4]. Briefly, cells are plated in 96-well microtiter plates in medium RPMI 1640 containing 20% medium HL-1, 5% defined calf bovine serum (Hyclone, Logan, Utah, USA), 10% T-cell growth medium containing interleukin-1 and interleukin-2, 0.25 μg/ml phytohemagglutinin, and 1 x 10^4 irradiated (90 Gy) *HPRT* mutant human lymphoblastoid "feeder" cells in the absence or presence of 10 μM 6-thioguanine (TG). Cells from the affected males were plated at 1, 2, 5, 10 or 100 cells/well in the absence or presence of TG. Cells from their mother were plated at 2, 5, 10, 10^2 and 10^3 cells/well in the absence of TG and at 10^2, 10^3 and 10^4 cells/well in the presence of TG. Cells from the males were also plated at 1 x 10^5 cells/ml in 2 ml wells in the same medium containing no addition, hypoxanthine and thymidine (HT), hypoxanthine, aminospterin and thymidine (HAT) or TG. Wild type $HPRT^+$ cells grow in the absence of TG, in the presence of HT or of HAT, but not in the presence of TG. Mutant $HPRT^-$ cells grow in the absence of TG and in the presence of either TG or HT, but not in the presence of HAT. Thus, growth in the presence of TG, but not in HAT, is a rapid screen for *HPRT* mutant cells. *HPRT* mutant cells from a Lesch-Nyhan syndrome male should clone equally well in the absence and presence of TG and yield a mutant frequency of 1.0. Cells from a non-carrier female will contain a frequency of *HPRT* mutant cells of 1-20 x 10^{-6} [4]. Cells from a *HPRT* heterozygous female will contain *HPRT* mutant cells at a frequency of 0.1-5.0%. The frequency in carrier females is less than the 50% expected from random X-chromosome inactivation because of negative selection against mutant cells during hematopoietic stem cell proliferation[7]. In previous studies, carrier females showed *HPRT* mutant frequencies in the range of 1-5%[5,6].

HPRT mutation analysis

The *HPRT* gene contains approximately 42,000 base pairs in genomic DNA, has nine exons and a mRNA of approximately 1,800 bases and a protein coding sequence of 657 bases (initiation codon AUG to termination UAA). The entire gene has been sequenced and primers developed for polymerase chain reaction (PCR) amplification of the nine exons in genomic DNA and the mRNA[8,9]. Recent review articles have summarized the known *HPRT* mutations responsible for Lesch-Nyhan syndrome. Approximately 80 different mutations have been reported[12,13]. This wealth of information allows for rapid analysis of the *HPRT* gene by cDNA sequencing for point mutations and genomic PCR analysis for deletion mutations.

Cultured T-lymphocytes from the affected males and their mother were used for DNA analysis. Samples of 1 x 10^4 cells were centrifuged in 0.5 ml microfuge tubes, flash frozen in liquid nitrogen and stored at -70 C. Cell lysates were prepared and first strand cDNA synthesized by reverse transcriptase as described by Yang et al[8]. Two rounds of PCR amplification were performed with *HPRT* specific oligonucleotide primers as described previously[6]. The first round employed 30 cycles with primers 3 and 4b (*HPRT* base -60 to -41, and 769 to 746, respectively), and the second round 30 cycles with primers B and 4 (*HPRT* base -36 to -17, and 702 to 721, respectively). The cDNA products were sequenced directly by use of a Taq Dyedeoxy Terminator Cycle Sequencing Kit (Perkin Elmer - ABI) and an automated ABI sequencer Model 373A with *HPRT* primers B and A (*HPRT* bases -36 to -17 and 701 to 682, respectively). This is essentially the method originally described by Gibbs et al[9].

For genomic DNA sequencing, the primers developed to amplify *HPRT* exons 7 and 8 were used[10]. These primers are numbered according to the genomic *HPRT* gene sequence[11]. The primers are 38667 to 38691 (sense) and 40199 to 40176 (antisense). The DNA was sequenced with the antisense primer. Exon 8 is genomic bases 40033 to 40109.

RESULTS

The *HPRT* mutant frequency was determined with cells from the two affected males and their mother. Both males showed similar cell cloning in the absence and presence of TG, consistent with the Lesch-Nyhan syndrome diagnosis. Their mother showed an *HPRT* mutant frequency of 3.9%, consistent with her being a heterozygous carrier (Table 1). Sequencing of cDNA from males revealed a single base substitution at cDNA base 590 in exon 8. This is the only difference from the published wild type *HPRT* coding sequence[9]. This 590A→T transversion changes codon 197 from G<u>A</u>A to G<u>T</u>A, resulting in the amino acid change 197glu→val. Sequencing of cDNA from the mother's cells grown in the absence of TG showed the wild type A at cDNA base 590. Sequencing of cDNA from the mother's TG resistant mutant cells showed the 590A→T transversion mutation. To absolutely confirm the heterozygous nature of the mother and to test the feasibility of direct DNA analysis for testing at risk females in the family, the exon 8 region of genomic DNA was sequenced. The DNA sequence from the males encompassing genomic bases 40151 to 39801 showed only a single change from the normal sequence[11]. This was an A to T change at base 40090. This 40090A→T mutation is the base designated cDNA base 590. The mother's exon 8 sequence showed both an A and a T at genomic base 40090, confirming that she is heterozygous for the mutation in both her somatic and germinal cells.

Table 1. Mutation analysis in $HPRT_{Brasil}$ Family.

		HPRT Alterations[2]	
Individual	Mutant Frequency[1]	cDNA	Genomic DNA
Affected Male 1	1.0	590A→T	40090A→T
Affected Male 2	1.0	590A→T	40090A→T
Mother	3.9 x 10^{-2}	590A→T	40090A and A→T (carrier)
Aunt1	-	-	40090A only (non-carrier)
Aunt 2	-	-	40090A only (non-carrier)
Aunt 3	-	-	40090A only (non-carrier)
Control	4.2 -8.3 x 10^{-6}	590A	40090A only

[1]*HPRT* mutant frequency by T-lymphocyte cloning assay; MF, ratio of cloning efficiency in the presence of 10 μM TG to cloning efficiency in the absence of TG.
[2] *HPRT* exon 8: (586) AAT G<u>A</u>A TAC (594)→AAT G<u>T</u>A TAC, 197 glu→197 val

Table 2: Amino acid changes resulting from single base substitution mutations in the HPRT gene cDNA bases 571-606 (codons 191-202).

cDNA	(571)											(606)
Bases	TAT	GCC	CTT	GAC	TAT	AAT	GAA	TAC	TTC	AGG	GAT	TTG
Amino	(191)											(202)
Acids	tyr	ala	leu	asp	tyr	asn	glu	tyr	phe	arg	asp	leu
Lesch-Nyhan syndrome				asn tyr			val ($HPRT_{Brasil}$)		val		tyr	
Gout				glu	cys					gly asn		
Somatic												
TG^r	TAA	asp	arg	ala	TAA	asp	TAA		ser	gly	asn	TAG
		pro		asn	asp	his	gly		tyr	lys	gly	met
		ser		his	asn		lys			net	tyr	phe
		thr		tyr			val			tyr	val	trp
				val								

An important aspect of Lesch-Nyhan syndrome mutation analysis is the ability to apply these methods to heterozygote detection in order to determine the carrier status of at-risk females in the family. DNA from three maternal aunts of the affected boys was used to determine the sequence of the genomic region containing exon 8. All three showed only an A at 40090, indicating that none is a carrier.

DISCUSSION

This 590A→T mutation in exon 8 results in a change of codon 197 from GAA to GTA, and an amino acid change of 197 glu→val. This single base substitution reduces the *HPRT* enzyme activity to very low levels and allows the cells to grow in the presence of 6-thioguanine, normally a cytotoxic purine analogue. A mutation at base 590 in codon 197 has not been reported previously in a Lesch-Nyhan syndrome patient[12,13]. As shown in Table 2, mutations in this region of the *HPRT* gene (codons 191-202) have been reported both in Lesch-Nyhan syndrome and partial *HPRT* deficiency (gout). In addition, many mutations have been found in this region of the gene in somatic cell *HPRT* mutations which were isolated as TG resistant (TG^r) mutants. One of these includes the same 197 glu→val change observed in $HPRT_{Brasil}$[14].

Lastly, the 590 A→T mutation creates a TA dinucleotide repeat rich region in exon 8 (TATAATGAATA→TATAATGTATA), suggesting that the mutation might have been induced by slippage during DNA replication in this region of repeated sequences.

Data generated from this type of studies may have significant implications for diagnosis and prognosis in Lesch-Nyhan syndrome patients and their relatives. The aim of future therapies is to slow or stop the progression of Lesch-Nyhan syndrome in affected persons. Knowledge of the molecular basis of the disease in the population should help families at risk and improve genetic counseling.

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