Molecular characterization and prenatal molecular evaluation of three fetuses in four unrelated Korean families with Lesch-Nyhan syndrome

Han-Wook Yoo and Gu-Hwan Kim

The Lesch-Nyhan syndrome which is caused by the deficiency of hypoxanthine guanine phosphoribosyltransferase is an X-linked recessive disorder characterized by hyperuricemia, choreoathetosis, mental retardation and compulsive self-injurious behavior. Clinical management of the patients with the Lesch-Nyhan syndrome is frustrating and requires burdensome medical treatment since it cripples the patient and shortens the life span by progression of neurological symptoms, but there are no cures or measures for relieving relentless natural course of the disease yet. Therefore, prenatal diagnosis of the affected fetus is important in genetic counselling for the family at high risk. In this study, four different mutations in the HPRT gene of four probands have been identified in four unrelated families; K215X, Q109X, nt.631 ΔA, and nt.289 ΔGT. Two mutations among them altered restriction enzyme sites; *Spe*I for Q109X and *Mae*I for nt.289 ΔGT. Based on their molecular defects, prenatal diagnoses of 3 the fetuses were successfully made between ninth and eleventh week of gestation by polymerase chain reaction (PCR), restriction digestion and DNA sequencing using cDNA obtained from chorionic villus samples (CVS). We predicted the outcome of all fetuses prenatally. Among the three fetuses two were male and one was female according to the identification made by PCR amplification of the sex determining region of the Y chromosome (SRY) gene. Each carried a wild type allele for the corresponding mutant allele. They were also tested postnatally for the mutations to be unaffected.

Keywords: Lesch-Nyhan syndrome, HPRT gene, Prenatal diagnosis, Mutation

INTRODUCTION

The Lesch-Nyhan syndrome, an X-linked recessive disorder, leads to hyperuricemia, choreoathetosis, mental retardation and compulsive self-injurious behavior (Lesch *et al.*, 1964). This disorder is caused by the complete or virtually complete absence of the activity of an enzyme involved in purine metabolism, hypoxanthine guanine phosphoribosyltransferase (HPRT, EC 2.4.2.8) (Seegmiller *et al.*, 1967). The HPRT gene lies on long arm of the X chromosome and contains nine exons distributed over 40kb of DNA, and four autosomal HPRT pseudogenes are known to be present within the human genome. This hinders DNA analysis (Patel *et al.*, 1986). This gene contains mRNA of 1.6kb and produces 217 amino acid enzyme (Wilson *et al.*, 1982; Jolly *et al.*, 1983).

When clinically suspected, the diagnosis of the Lesch-Nyhan syndrome can be made easily on the basis of hyperuricemia, choreoathetosis, and compulsive self-injurious behavior. However, prenatal diagnosis or detection of asymptomatic carriers has been problematic since biochemical assay for the HPRT enzyme activity is not valid to clarify the carrier status. Since the cDNA and protein of the human HPRT gene was elucidated (Seegmiller *et al.*, 1967; Patel *et al.*, 1986; Jolly *et al.*, 1983; Wilson *et al.*, 1982), direct identification of molecular defects has been facilitated. A large variety of genotypes have been reported (Sculley *et al.*, 1992), indicating genetic heterogeneity in the HPRT gene of Lesch-Nyhan patients as in other X-linked inherited disorders. Therefore, it is important to investigate the molecular defect in each individual family. Once the molecular defect is defined, accurate prenatal molecular diagnosis of the fetus can be made in the families at high risk. Otherwise, intragenic restriction fragment length polymorphism (RFLP) markers can be utilized for prenatal diagnosis of the selected families.

Department of Pediatrics, Asan Medical Center,
Ulsan University College of Medicine, Asan Institute for Life Sciences
Correspondence: Han-Wook Yoo, Department of Pediatrics,
Asan Medical Center, Ulsan University College of Medicine,
388-1 Poongnap-Dong, Songpa-Gu, Seoul 138-736, Korea,
Tel: 82-2-224-3374, Fax:82-2-473-3725, E-mail: hwyoo@www.amc.seoul.kr

In this study, we characterized molecular defects in the HPRT gene of four unrelated Korean families with Lesch-Nyhan syndrome patients. We applied these results to monitor the molecular status of fetuses in these families.

MATERIALS AND METHODS

Subjects and clinical diagnosis of Lesch-Nyhan syndrome

The clinical diagnosis has been established in four male probands from four unrelated Korean families on the basis of undetectable HPRT activity in red blood cells and typical clinical features such as self-injurious behavior, hyperuricemia, and neurologic dysfunctions including choreoathetosis spasticity. All probands have positive family history for Lesch-Nyhan syndrome.

Culture of skin fibroblasts

Primary skin fibroblasts of probands were grown to confluence in T-25 culture flask, and were cultivated in DMEM (Gibco-BRL, Gaithersburg, USA) plus 10% Fetal calf serum (FCS) (Gibco-BRL, Gaithersburg, USA).

Total RNA isolation

Total RNA was isolated from chorionic villi from fetuses and from fibroblast from probands. After treatment with RNAzol, which contains guanidium- thiocyanide and phenol solutions, RNA was precipitated with isopropanol according to manufacturer's procedures (Bioprobe Systems, Montreuil-Sous-Bois, France).

Genomic DNA isolation

Genomic DNA was isolated from chorionic villi from fetuses. After treatment with 0.5% sodium dodecyl sulfate and proteinase K, genomic DNA was extracted using phenol and chloroform and precipitated with cold ethanol according to standard procedures (Aldridge *et al.*, 1984).

Preparation of cDNA

With oligo $(dT)_{12\text{-}18}$ as a primer cDNA was synthesized from total RNA. For each reverse transcriptations, reaction mixture contained 50 mmol/L Tris-HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L $MgCl_2$, 10mmol/L dithiothreitol, 50 μg/ml oligo $(dT)_{12\text{-}18}$, and 100 unit of M-MLV reverse transcriptase (Promega Biotech, Madison, WI, USA). Reactions were performed at 37°C for 1.5 h and terminated by adding EDTA.

Amplification of the HPRT gene and sex determining region of the Y chromosome (SRY) gene using genomic DNA from CVS

To determine the fetal sex, PCR of the DNA binding region of the SRY located at the centromeric region of the Y chromosome were carried out using a set of primers. For the SRY gene, the 270bp sequence representing the DNA binding domain of the SRY gene was amplified with primers: SRY-1F 5'-CAG TGT GAA ACG GGA GAA ACA GT-3', SRY-AR 5'-CTT CCG ACG AGG TCG ATA CTT ATA-3' (Nakagome *et al.*, 1991; Warburton *et al.*, 1991). Amplification of all cDNA of HPRT gene was performed using two pairs of synthetic oligonucleotide primers designed to span all cDNA: LN-1 5'-CAC CGG CTT CCT CCT CCT GA-3', LN-2 5'-CCA ATT ACT TTT ATG TCC CC-3', LN-3 5'-CAG ACT GAA GAG CTA TTG TA-3', LN-4 5'-TTA CTG GCG ATG TCA ATA GG-3'. After an initial denaturation of the template DNA, amplification were performed for 35 cycles which consisted of denaturation at 94°C for 1 min, annealing at 49°C for 1 min, and extension at 72°C for 2 min. For each reaction, the 50 μl amplification mixture contained 200 ng of prepared cDNA as template, 1 μmol/L each of sense and antisense primers, 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.8), 1.5 mmol/L $MgCl_2$, 0.1% Triton X-100, 50 μmol/L dNTPs, 2 units of Taq DNA polymerase (Promega Biotech, Madison, WI, USA). The reaction was performed in a thermocycler (Perkin-Elmer Cetus, Norwalk, CT, USA).

Direct sequencing of double stranded PCR product

PCR product (10 μl of each) was electrophoresed on 1.8% agarose gel to examine whether the targeted template was specifically amplified. When a single specific PCR product was amplified, double stranded DNA sequencing of each PCR product was performed directly without purification using sense and antisense primers and a Sequenase version 2.0 kit (United States Biochemical, Cleveland, OH, USA) according to manufacturer's instructions with the following modifications. The original amount of template for individual sequencing reaction ranged from 5 to 10 μl of each PCR product. Sequenase DNA polymerase and inorganic pyrophosphatase were mixed together (1 volume of each) and diluted with 6 volumes of glycerol enzyme dilution buffer [20 mmol/L Tris-HCl (pH 7.5), 2mmol/L dithiothreitol, 0.1 mmol/L EDTA, 50% glycerol]. The labelling reaction mixture was electrophoresed on 8% denaturing polyacrylamide gel in 0.8x glycerol tolerant gel buffer (20× buffer is 216 g Tris base, 72 g taurine, 4 g EDTA in liter H_2O).

Restriction enzyme analysis

The amplified PCR product of the first part of HPRT cDNA was digested with *Mae*I enzyme at 45°C, the secondary part of HPRT cDNA with *Spe*I at 37°C for 2 h to monitor the inheritance of mutations in fetuses in the appropriate families.

RESULTS

Family JJH

In this family, we found a C to T transition in a codon 109 in exon 3, causing a substitution of a stop codon for an glutamine in a female obligate heterogyzote. This mutation eliminated *Spe*I recognition site. The pregnancy was monitored by cDNA analysis of the fetus using CVS at the tenth week of gestational age. The cDNA covering exon 3 was amplified and digested with *Spe*I. The pattern of restriction fragment lengths indicated that the fetus was unaffected since the 415 bp PCR product was completely digested generating 391 bp and 24 bp fragments (Fig. 1). PCR amplification for the SRY gene was positive (Fig. 4). Therefore, the fetus was a male hemizygote for the normal allele.

Family PYM

The cDNA analysis of the HPRT gene in the proband revealed a GT deletion in exon 3 making a frameshift mutation. This base deletion creates a new *Mae*I recognition site. The pattern of restriction fragment lengths indicated that the fetus was unaffected since the 355 bp PCR product was completely digested, generating 288 bp and 65 bp fragments (Fig.2). PCR amplification for the SRY gene was positive (Fig. 4). Therefore, the fetus was a male hemizygote for the normal allele.

Family MKJ

An A deletion was found at nucleotide 631 in the codon 211, encoding an threonine residue in exon 9, which resulted in a frameshift mutation making a stop in the codon 250. Evaluation of molecular status in a fetus was performed by direct sequencing of the exon 9 of the HPRT cDNA obtained from CVS, since this mutation does not alter any restriction site. The result revealed that mutation site of A was normal in the fetus cDNA (Fig. 3). PCR amplification for the SRY gene was negative (Fig. 4). Therefore, the fetus was a female homozygote for the normal allele.

Family KDH

In this family, we identified an A to T transition at nucleotide 643, resulting in a nonsense mutation replacing a lysine residue with a stop codon (K215X) in the codon 215 in exon 9 (Fig. 5). This mutation terminates the synthesis of the enzyme prematurely.

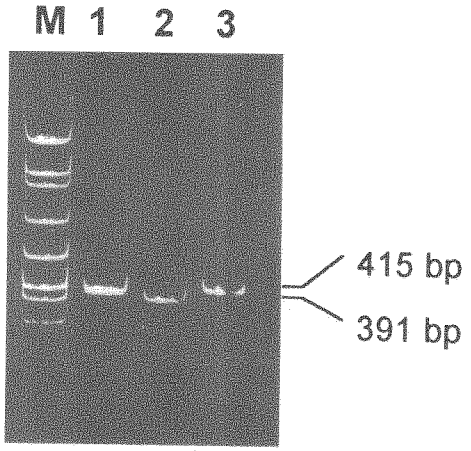

Fig. 1. Prenatal monitoring of the fetus for the presence of the mutation by *Spe*I digestion analysis in the family of JJH. Lane 1 is *Spe*I digested PCR product from a normal control, 415 bp; Lane 2 is *Spe*I digested PCR product from the proband, 391 bp; Lane 3 is *Spe*I digested PCR product from the fetus, 415 bp, indicating that fetus carries the normal allele resistant to *Spe*I. The pGEM DNA marker (Promega) was used as a size marker.

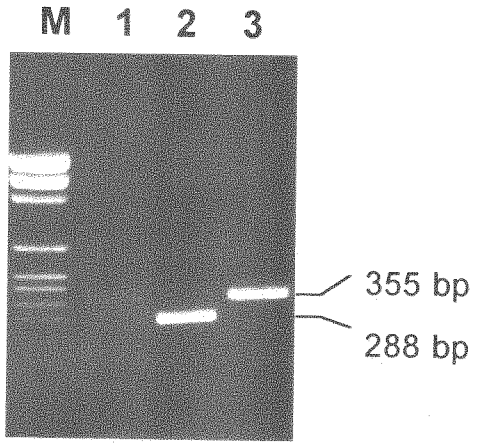

Fig. 2. Prenatal monitoring of the fetus for the presence of the mutation by *Mae*I digestion analysis in the family of PYM. Lane 1 is *Mae*I digested PCR product from a normal control, 355bp; Lane 2 is *Mae*I digested PCR product from the proband, 288bp; Lane 3 is *Mae*I digested PCR product from the fetus, 355 bp, indicating that the fetus carries the normal allele uncleaved by *Mae*I. The pGEM DNA marker (Promega) was used as a size marker.

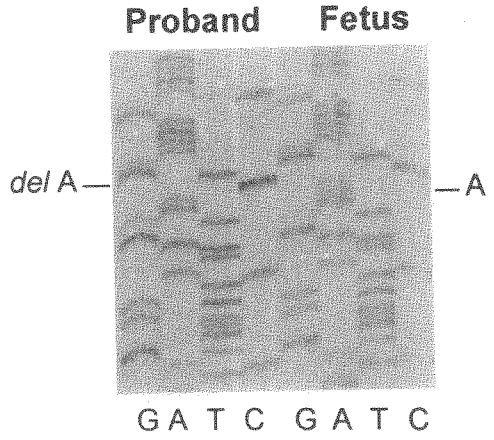

Fig. 3. Partial nucleotide sequences of PCR-amplified cDNA of the HPRT gene of the family MKJ. When compared with the proband, hemizygous for A deletion in codon 211, causing frameshift mutation (nt.631 ΔA), the partial nucleotide sequences of cDNA of the HPRT gene obtained from CVS of the fetus was completely normal for this mutated site.

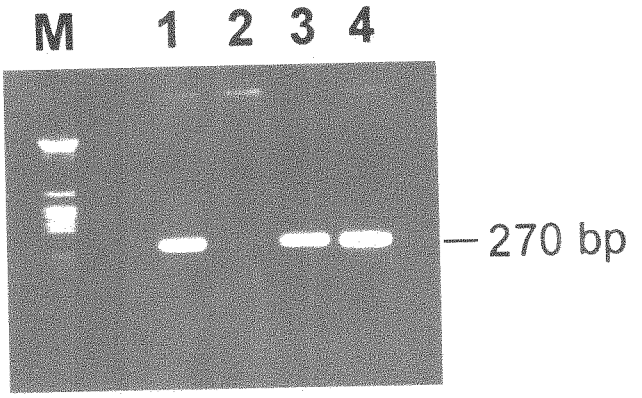

Fig. 4. PCR amplification for the SRY gene of CVS obtained from fetuses. Lane 1 is a male control, showing 270 bp PCR product derived from the SRY gene; Lane 2 is the fetus from the family MKJ that shows no PCR product, indicating the fetus is a female. The 270 bp PCR product is shown in lane 3 and 4, which are fetuses from the family JJH and PYM respectively, indicating both males.

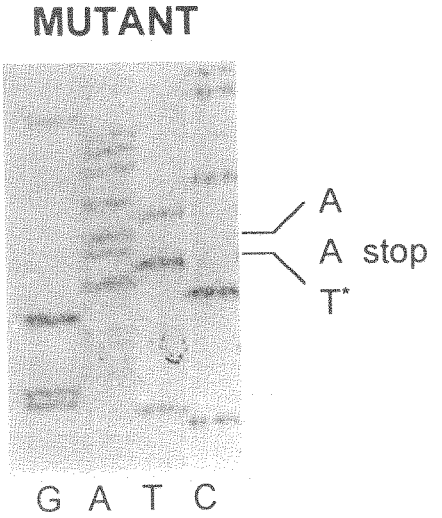

Fig. 5. Partial nucleotide sequences of PCR-amplified cDNA of the HPRT gene from the proband KDH. This novel nonsense mutation results from a A to T transition at nucleotide 643 in exon 9, replacing a lysine residue with a stop codon in the codon 215 (K215X).

DISCUSSION

The clinical features of the Lesch-Nyhan syndrome are mental retardation, spasticity, choreoathetosis, uric acid urinary stones and compulsive self destructive biting of fingers and lips, which results from a complete deficiency of the purine salvage enzyme, hypoxanthine guanine phosphoribosyltransferase (HPRT) (Rossiter *et al.*, 1995). A number of clinical signs that precede the onset of self mutilation are evident in patients with Lesch-Nyhan syndrome. Neurological abnormalities may become apparent within the first year as choreoathetosis and spasticity development. The first signs of hyperuricemia may be uric acid crystalluria. Despite these early signs, the clinical diagnosis of the Lesch-Nyhan syndrome is rarely made before self-mutilating behavior begins unless there is previous family history of the disorder (Nyhan, 1973). The family history is positively documented in about 2/3 of probands. Its onset warrants immediate measurements of cellular HPRT activity and genetic analysis. In this study, the clinical diagnosis has been made easily in 4 probands who presented aforementioned signs. Their HPRT activity were completely absent (data not shown).

Though biochemical analysis can also be utilized to detect the presence or absence of HPRT activity in cells or tissues that normally express the enzyme, DNA-based mutation detection techniques can be used accurately for the diagnosis of affected males and for the determination of carrier status of asymptomatic females prenatally or postnatally. Mutations at the HPRT locus in the Lesch-Nyhan syndrome are so heterogeneous that almost every affected family carries its own mutation. About 12% of mutations are major gene rearrangement detectable by Southern blot analysis or multiplex PCR of each exon, and the remainders are point mutations or other minor alterations (Wison *et al.*, 1986).

We have described four different mutations in four unrelated families (K215X, Q109X, nt.631 ΔA, and nt.289 ΔGT). Three of the mutations, Q109X, nt.631 ΔA, and

nt.289 ΔGT, have been reported previously (Ki-Joong *et al.*, 1997). However, the mutation K215X is a novel mutation that has not been described previously. Among them, two of the mutations (Q109X, nt.289 ΔGT) were associated with alterations of restriction enzyme sites (*Spe*I, *Mae*I). Based on the alterations of these sites, prediction of the specific mutation in the individual family has been successfully carried out in 2 fetuses. Since the other mutation (nt.631 ΔA) didn't change a recognition site, prenatal evaluation of the fetus was achieved successfully by direct DNA sequencing reaction using cDNA from CVS in this family. So far, at least four autosomal HPRT pseudogenes are known to be present within the human genome (Patel *et al.*, 1984). This may hamper mutation analysis at the genomic DNA level. In this study, we employed cDNA analysis for the mutation detection using CVS. A large variety of genotypes have been recognized in the HPRT gene of patients with Lesch-Nyhan syndrome, including intragenic deletions and point mutations without predominance of a single particular genotype (Sculley DG *et al.*, 1992; Tarle SA *et al.*, 1991). Since genetic heterogeneity is considerable in this disorder, it is important to investigate the molecular defect in each individual family. Although this approach is labor intensive, involving numerous sequencing, the direct detection of the nucleotide sequence alteration becomes technically feasible on a routine basis by automation of nucleotide sequencing or PCR-SSCP (single strand conformation polymorphism) screening.

Even though the specific mutation is not known, mutant HPRT alleles can be followed within a family by association with readily identifiable DNA polymorphisms. There are one intragenic polymorphic site (*Bam*HI) and two extragenic polymorphisms (*Taq* I) (Nussbaum *et al.*, 1983; Renwick *et al.*, 1991). In addition, the polymorphic short tandem repeats (CTAT)n lies between exon 3 and 4, and 11 alleles have been observed. This tandem repeat polymorphism is much more informative than the three RFLPs (Edwards *et al.*, 1991). The PCR amplification using primers flanking the polymorphic (CTAT)n sequence within the human HPRT gene results in different sized fragments according to the number of repeat units.

This study report is the first of its kind in Korea that prenatal molecular evaluation has been successfully undertaken by direct investigation of specific mutations in fetuses at high risk whose families were not informative of the intragenic RFLP markers.

ACKNOWLEDGEMENT

This research project was partly supported by the Grant No. HMP-96-M-2-0022 from the Health Technology Planning and Evaluation Board of the Korean Government.

REFERENCES

Aldridge, J., Kunkel, L. and Bruns, G. (1984) A strategy to reveal high frequency RFLPS along the human X chromosome. *Am J Hum Genet.* 36: 546-564

Edwards, A., Civitello, A., Hammond, H. A. and Caskey, C. T. (1991) DNA typing and genetic mapping with trimeric and tetramenric tandem repeats. *Am J Hum Genet* 49: 746-756

Jolly, D. J., Okayama, H., Berg, P., Esty, A. C., Filpula, D., Bohlen, P., Johnson, G. G., Shively, J. E., Hunkapillar, T. and Friedmann, T. (1983) Isolation and charaterization of a full-lengh expressible cDNA for human hypoxanthine phosphoribosyl transferase. *Proc Natl Acad Sci USA* 80: 477-81.

Ki-Joong, K., Yamada, Y., Suzumoru, K., Choi, Y., Yang, S. W., Cheong, H. I., Hwang, Y. S., Goto, H. and Ogasawara, N. (1997) Molecular Analysis of hypoxanthine guanine phosphoribosyl-transferase (HPRT) gene in five Korean families with Lesch-Nyhan syndrome. *JKMS* 12: 332-9

Lesch, M. and Nyhan, W. L. (1964) A familiar disorder of uric acid and central nervous system functions. *Am J Med* 36: 561-70

Nakagome, Y., Seki, S., Fukutani, K., Nagafuchi, S., Nakahori, Y. and Tamura, T. (1991) PCR detection of distal Yp sequences in an XX true hermaphrodite. *Am J Med Genet* 41: 112-4

Nussbaum, R. L., Crowder, W. E., Nyhan, W. L. and Caskey, C. T. (1983) A three allele restriction fragment length polymorphism at the hypoxanthine phosphoribosyl transferase locus in man. *Proc Natl Acad Sci USA* 80: 4035-4039

Nyhan, W. L. (1973) The Lesch-Nyhan syndrome. *Annu Rev Med* 24: 41-52

Patel, P. I., Feamsion, P. E., Caskey, C. T. and Chinault, C. A. (1986) Fine structure of the human hypoxanthine guanine phosphoribo-syltransferase gene. *Mol Cell Biol* 6: 393-403

Patel, P. I., Nussbaum, R. L., Framson, P. E., Ledbetter, D. H., Caskey, C. T. and Chinault, A. C. (1984) Organization of the HPRT gene and related sequences in the human genome. *Somat Cell Mol Genet* 10: 483-493

Renwick, P., Raybould, M., Birley, A. and Hulten, M. (1991) RFLP in the region of the HPRT locus. *Nucleic Acids Res* 19: 4576-4582

Rossiter, B. J. F. and Caskey, C. T. (1995) Hypoxanthine-guanine phos-phoribosyl transferase deficiency: Lesch-Nyhan syndrome and gout, In *The metabolic and molecular bases of inherited disease*, 7th Edn., (Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle D. eds.), pp. 1679-1706, McGraw-Hill, New York

Sculley, D. G., Dawson, P. A., Emmerson, B. T. and Gordon, R. B. (1992) A review of the molecular basis of hypoxanthine guanine phosphoribosyltransferase (HPRT) deficiency. *Hum Genet* 90: 195-207

Seegmiller, J. X., Rosenbloom, F. M. and Kelly, W. N. (1967) Enzyme defect associated with a sex-linked human neurological disorder and excessive purine synthesis. *Science* 155: 1682-4

Tarle, S. A., Davidson, B. L., Wu, V. C., Zidar, F. J., Seegmiller, J.

E., Kelley, W. N. and Palella, T. D. (1991) Determination of the mutations responsible for the Lesch-Nyhan syndrome in 17 subjects. *Genomics* 10: 499-501

Warburton, P. E., Greig, G. M., Haaf, T. and Willard, H. F. (1991) PCR amplification of chromosome specific alpha satellite DNA: Definition of centromeric STS markers and polymorphic analysis. *Genomics* 11: 325-33.

Wilson, J. M., Stout, J. T., Palella, T. D., Davidson, B. L., Kelley, W. N. and Caskey, C. T. (1986) A molecular survey of hypoxanthine guanine phophoribosyl transferase deficiency in man. *J Clin Invest* 77: 188-195

Wilson, J. M., Tarr, G. E., Mahoney, W. C. and Kelly, W. N. (1982) Human hypoxanthin guanine phosphoribosyltransferase. Complete amino acid sequence of the erythrocyte enzyme. *J Biol Chem* 257: 10978-85