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Short Report

Two novel mutations in a purine nucleoside phosphorylase (PNP)-deficient patient

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Purine nucleoside phosphorylase (PNP) deficiency is a rare autosomal recessive disease, which presents clinically as severe combined immunodeficiency (SCID). We report here two novel mutations in the PNP gene that result in SCID phenotype, in a single patient. The maternal-derived allele carries a C to T transition in exon 2 resulting in a premature stop codon at amino acid 57. The paternal-derived mutation is a G to A transition at position +1 in intron 3, causing a complete skipping of exon 3 and a reading frameshift at the exon 2–exon 4 junction. The predicted polypeptide encoded by the aberrantly spliced mRNA terminates prematurely after only 89 amino acids. Both mutations predict severely truncated proteins resulting in a complete deficiency of PNP enzymatic activity, yet the development of profound immunodeficiency in this patient is greatly delayed.

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Purine nucleoside phosphorylase (PNP) deficiency is a lethal autosomal recessive disease that was recognized, more than 20 years ago (1), as a cause of severe combined immunodeficiency (SCID). Clinically, PNP-deficient patients experience recurrent bacterial, viral, and fungal infections usually beginning early in life. Some patients, however, have been asymptomatic until several years of age (2). Other common features include neurological disorders, ranging from mild developmental delay to severe spasticity and mental retardation. There is also an increased incidence of autoimmune diseases including the most common manifestation of hemolytic anemia. Patients with PNP deficiency typically have profound T-cell deficiency with variable B-cell function (2).

The PNP protein is a homotrimeric enzyme of approximately 96000 Da (3) and is encoded by a single gene of 6 exons spanning 9 kb of human chromosome 14q13 (4, 5). PNP mRNA is translated into a 32-kDa protein consisting of 289 amino acids (6). Human PNP reversibly catalyzes the degradation of the purine nucleosides inosine and deoxyinosine to hypoxanthine and that of guanosine and deoxyguanosine to guanine (7). Although the enzyme is considered a housekeeping gene product and is found in most tissues of the body, it is highly expressed in lymphoid tissues (2). T-cell death in PNP deficiency is believed to result from the accumulation of excess intracellular levels of deoxyguanosine triphosphate. This metabolite inhibits the enzyme ribonuclease reductase and, thus, blocks DNA synthesis and cell division (8, 9).

Surprisingly, very few disease-causing mutations have been reported so far (10–15). Different mutations in the PNP gene produce proteins with variable degrees of enzymatic activity (16, 17), which correlate with the accumulation of nucleoside substrates (18) and to some degree with the clinical course (14). Retention of partial enzyme activity may lead in some patients, but not all, to less severe metabolic abnormalities, delayed presentation or milder immune dysfunction and clinical features (19).

In this report, we describe two novel mutations of the PNP gene. The maternal-derived allele carries a C to T transition in exon 2 resulting in a premature stop codon at amino acid 57, and, the paternal-derived mutation causes skipping of exon 3, as a result of a G to A transition at position +1 of intron 3. Translation of the aberrantly spliced mRNA results in a reading frameshift and the predicted polypeptide terminates at amino acid 89.

Materials and methods

Patient

The patient (R.R.) is a 4.5-year-old male, born to healthy parents of Caucasian (father) and Japanese (mother) origin with no previous history of PNP deficiency. His two older sisters (aged 7 and 9 years) are completely healthy. His past medical history is remarkable for the removal of umbilical cord at 7 months of age and a mild delay in gross motor development. He grew up without any severe infections, oral thrush, diarrhea, or failure to thrive. At the age of 8 months, he experienced an uneventful varicella infection. At the age of 26 months, he was referred to our clinic for immunological assessment after developing a prolonged parvovirus infection which induced pure red cell aplasia that required multiple blood transfusions.

Upon examination he was a normal looking, thriving child. He had no palpable lymph nodes or tonsils. Neurological evaluation revealed mild ataxia and a 6–7-month delay in gross and fine motor skills, as well as speech development. Laboratory investigations showed normal neutrophil count, anemia, and low lymphocyte count. Immunoglobulin (IgA, IgG, IgM) levels, isohemagglutinins and specific antibody titers to tetanus toxoid, polioviruses 1, 2, 3 were normal (Table 1). However, his lymphocyte markers, and *in vitro* responses to mitogens were markedly reduced (Table 1). ADA enzyme activity was normal while PNP enzyme activity was essentially undetectable according to standard methods (Table 2). Both parents, as well as the two healthy sisters, had approximately half the PNP activity as compared with normal controls (Table 2).

At the age of 32 months he developed varicella zoster infection which was treated with acyclovir. Three months later, he experienced severe hemolytic anemia associated with mycoplasma pneumonia infection.

Table 1. Humoral and cell-mediated immunity

	PNP-deficient patient	Normal control
Humoral immunity		
IgG (g/l)	5.9	4.5–14.3
IgA (g/l)	3.7	0.2–1.0
IgM (g/l)	2.4	0.2–1.5
Tetanus toxoid titre (IU/ml)	0.14	>0.01
Poliovirus	1:32	>1:8
Isohemagglutinin titre	Anti B 1:16	>1:8
Lymphocyte subsets		
Absolute lymphocyte count ($\times 10^9$/l)	0.28–0.43	2.0–8.0
CD2 (%)	46	75–95
CD3 (%)	16	60–85
CD4 (%)	13	30–60
CD8 (%)	2	15–35
CD19 (%)	33	5–20
CD56 (%)	31	5–20
Lymphocyte proliferation (cpm)		
Background	713	1637
PHA	1235	40 458
ConA	1978	3316
SAC	2946	9533
SPA	8774	65 219

Abbreviations: cpm, counts per minute; PHA, phytohemagglutinin; ConA, concavalin A; SAC, staphylococcus protein A cown; SPA, staphylococcus protein A.

Table 2. PNP activity in patient, family members and control erythrocyte lysates

Source	PNP enzyme activity (nmol/min/10^6 cells)
Patient	Undetectable
Father	1239
Mother	1185
Sister 1	1278
Sister 2	963
Controls	2499–3735

RNA and DNA purification

DNA and RNA were isolated from peripheral blood mononuclear cells following Ficoll-Hypaqe gradient centrifugation. Cells were resuspended in Trizol reagent (Gibco, BRL) and the total RNA was extracted according to Chomczynski and Sacchi (20). DNA was isolated by proteinase K digestion of cells in lysis buffer (100 mM Tris pH 8.0, 5 mM EDTA, 0.2% SDS, 200 mM NaCl) at 37°C for 4 h. DNA was purified by two phenol/chloroform extractions followed by ethanol precipitation (21).

T-cell receptor Vβ repertoire

Total RNA was extracted from peripheral mononuclear cells as described above. RT-PCR was performed as previously described using a specific T-cell receptor (TCR) constant region primer for the reverse transcription reaction (22). For analysis of the TCR Vβ repertoire, 17 PCR amplification reactions were performed using the same constant region primer, together with one of 17 Vβ family-specific primers. Samples of the PCR products were run on a 0.8% agarose gel and visualized by ethidium bromide fluorescence.

PCR of PNP cDNA, subcloning and sequencing

Oligo(dT) primed first strand cDNA synthesis was performed on total RNA. The resulting cDNAs were used as substrates for PCR reactions using elongase (Gibco, Burlington, Canada).

PNP cDNA was amplified by a sense primer from −1 to +18 (CATGGAGAACGGATACACC), relative to initiation of translation in the normal PNP cDNA and an antisense primer from +852 to +870 (TCAACTGGCTTTGTCAGGG). The reaction cycle conditions were: denaturation at 94°C (30 s), annealing at 52°C (30 s), and extension at 68°C (90 s) for 30 cycles. The 870-bp PCR amplified PNP products were subcloned into a pUC19 vector and sequenced with Sequenase kit (Amersham, Buckinghamshire, UK).

PCR of genomic DNA

Genomic DNA was extracted from peripheral blood mononuclear cells as described above. To examine the mutation caused by the deletion of exon 3 we used the following primers to amplify the intron 2–exon 3 junction: a sense primer on intron 2 (TAATGCCTGGCTCTCTCA) and an antisense primer on exon 3 from nucleotides +221 to +238 (TCACACAGGCCCTGCCAT). Primers, used for PCR of exon 3–intron 3 junction, were a sense primer from nucleotides +221 to +238 (ATGGCAGGGCCTGTGTGA) with an antisense primer on exon 4 from nucleotides +321 to +342 (GGTGACTACCAGGGTGTCCACA). To confirm the C to T transition at position 172 we used genomic DNA for PCR amplification with a sense primer on exon 2 from nucleotide +35 to +57 (CACTGCAGAATGGCTTCTGTCT) together with an antisense primer on intron 2 (GAATTCCAAGAACATCCCTCAGT).

The reaction cycle conditions in these experiments were: 94°C (30 s), 52°C (30 s), and 68°C (60 s) for 35 cycles.

Results and discussion

The patient's clinical manifestations of multiple viral infections, lymphopenia, hemolytic anemia, and neurological symptoms were consistent with PNP deficiency. Assessment of the immune system revealed a normal number of B cells with normal function, commonly observed in these patients (Table 1). In contrast, cell-mediated immunity was profoundly affected. The patient T-cell subsets demonstrated reduced $CD2^+$, $CD3^+$ expressing cells, as well as, proportionally low $CD4^+$ and $CD8^+$ cells. The proliferative response of lymphocytes to mitogens was low, but not absent (Table 1). The absence of PNP enzyme activity in red blood cell lysate, obtained from the patient, clearly established the diagnosis of PNP deficiency SCID (Table 2). Both parents, as well as the two healthy siblings, exhibited reduced PNP activity in erythrocytes (Table 2), suggesting a carrier status.

Normal lymphoid tissues containing T cells have a wide range of PNP activity. High levels have been measured in mature peripheral T cells and in the spleen (23, 24) while in contrast, a lower level of activity was identified in the thymus (23). Within the thymus, medullary thymocytes express higher levels of PNP than cortical thymocytes (24, 25). Cowan et al. (26) have shown that T, B, and NK cells have approximately the same level of PNP activity per mg protein.

The exact mechanism by which PNP deficiency affects T-cell development is not entirely clear. However, some insight was provided by studying PNP-deficient mice, which were recently generated in our laboratory by gene targeting (16, 27). $PNP^{-/-}$ mice show increased apoptosis of double-positive $CD4^+CD8^+$ thymocytes markedly reducing the number of more mature single positive T cells. The effect is executed by the accumulation of deoxyguanosine. The relatively low threshold of double-positive progenitor cells to deoxyguanosine is probably because of their low level of Bcl-2 expression. It can therefore be argued that accumulation of deoxyguanosine in $CD4^+CD8^+$ positive cells accelerates negative selection by enhancing activation-induced apoptosis. Less dramatic changes were found in thymuses of partially PNP-deficient mice created by germ line mutagenesis (28), in which residual PNP activity varied from 1 to 5%.

In light of these findings in $PNP^{-/-}$ mice, it was important to study the repertoire of T cells in human PNP deficiency. To examine whether the patient's PNP-deficient T cells undergo a normal selection of the TCR, we analyzed peripheral blood mononuclear cells for Vβ transcripts by RT-PCR using 17 Vβ family-specific primers. Surprisingly, the patient's lymphocytes were found to express the full repertoire of 17 TCR Vβ families (Fig. 1), suggesting that PNP deficiency in humans does not result in a complete block of T-cell rearrangement nor does it cause a massive apoptotic event in the thymus. It remains unclear whether this discrepancy between mouse $PNP^{-/-}$ and human PNP deficiency is a result of species-specific sensitivity of $CD4^+CD8^+$ thymocytes or, because of some residual PNP activity in the patient (although below detection in our assay).

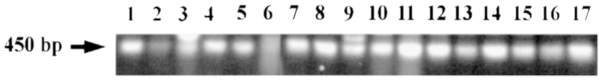


Fig. 1. Expression of TCR Vβ repertoire in patient's PNP-deficient T cells. PCR products of all 17 TCR Vβ families were found at the expected fragment size (around 450 bp), showing a normal TCR repertoire in the PNP-deficient peripheral T cells.

To elucidate the genetic basis for PNP deficiency in the patient, DNA and total RNA were isolated from the peripheral blood mononuclear cells of the patient and family members. The patient's poly A+ mRNA was reverse transcribed and the resulting cDNA used as substrates for PCR reactions with primers corresponding to the 5′ and 3′ ends of the translation coding region of the normal PNP gene. Surprisingly, the PCR product was found to consist of two bands (Fig. 2). One band was 870 bp in size, exactly as expected for a normal PNP cDNA sequence. However, the second band was approximately 100 bp shorter. A separate PCR amplification of the patient's, both parents', and normal control cDNA demonstrated the presence of two bands in only the patient and paternal samples, suggesting a deletion of approximately 100 bp in one of the paternal-derived alleles (Fig. 2, lanes 2 and 3). In contrast, both the control and maternal PCR products migrated as expected (Fig. 2, lanes 1 and 4). Both patient PCR products were cloned and sequenced.

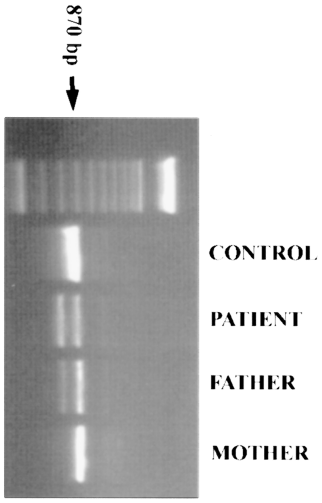


Fig. 2. PCR products of PNP cDNA amplification. The amplification was carried out using primers corresponding to the 5′ and 3′ ends of the normal PNP cDNA. The results demonstrate the presence of two bands in only the patient and paternal samples (lanes 2 and 3). In contrast, the control and maternal PCR products migrated as expected (lanes 1 and 4). The results indicate that the patient carries a deletion of approximately 100 bp in the paternal-derived PNP allele.

Five of 12 patient clones which contained the normal length PCR product were found to carry a C to T transition in exon 2 at position 172 (Fig. 3A). The same point mutation was found in three of seven maternal clones, confirming that this mutation is indeed maternally derived. Furthermore, using a sense primer in exon 2 together with an antisense primer in intron 2, the same mutation was identified in the patient's genomic DNA. This point mutation leads to a premature stop codon after 57 amino acids probably leading to a translation of a severely truncated protein (Fig. 3B).

Recently, X-ray crystallography has been used to study the three-dimensional structure of human PNP (29). The enzyme is a symmetric trimer consisting of three identical subunits. Each subunit contains an eight-stranded mixed β-sheet and a five-stranded mixed β-sheet, which join to form a distorted β-barrel structure. The sheets are flanked by seven α-helices. The active site of the PNP enzyme is located near the interface of two subunits within the trimer. A total of eight segments appear to be involved in substrate binding; one of these comes from an adjacent subunit (29). The patient's mutation, which places a premature stop codon at position 57, results in the ablation of 7 of 8 amino acid residue segments and is involved in the active site of PNP enzyme, undoubtedly severing its activity.

A review of the previously described point mutations in the PNP gene (see Table 3) has shown that most (75%) are transition mutations. These results are consistent with a review of 139 published point mutations in disease genes, showing that 63% are mutations of this type (30). However, most reported PNP gene mutations so far are not in the CpG sequence (CG→TG or CG→CA), which is known to be a 'hot spot' for mutations in the vertebrate genome. Krawczak et al. showed that 32% of all point mutations in disease genes occur in this sequence. This represents a 12-fold higher frequency than predicted by random expectation, consistent with a chemical model of mutation via methylation-mediated deamination (31). The C to T transition in our PNP-deficient patient is only the second published mutation in the GC 'hot spot' in this gene.

In the remaining 7 of 12 patient clones, corresponding to the shorter PCR product (paternal allele), the sequence was found to be identical to the published PNP cDNA, with the exception of a complete deletion of exon 3 (103 bp) (Fig. 4A). The defect responsible for the complete skipping of exon 3 was thought to reside in or around the intron–exon boundaries of exon 3. To analyze this possibility, DNA fragments encoding the intron

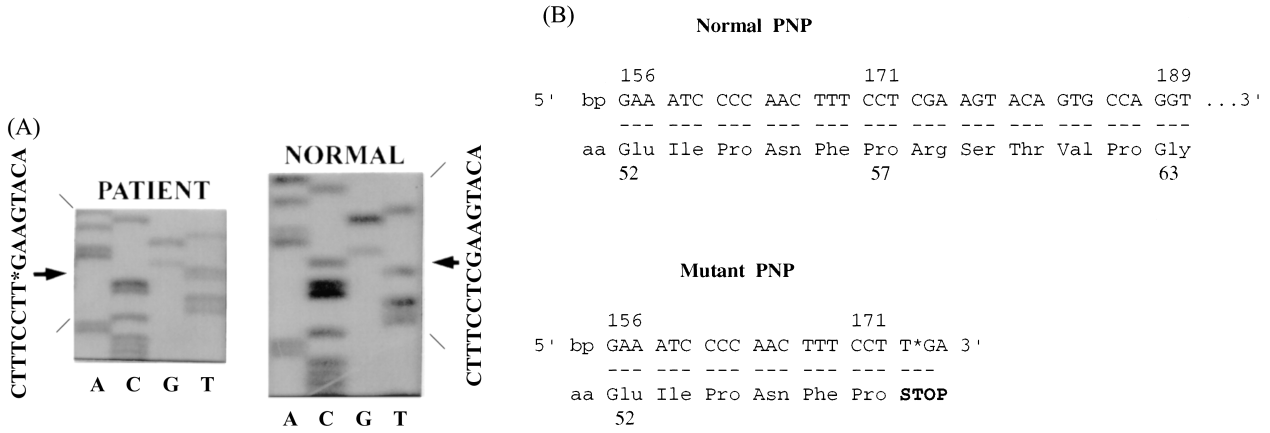


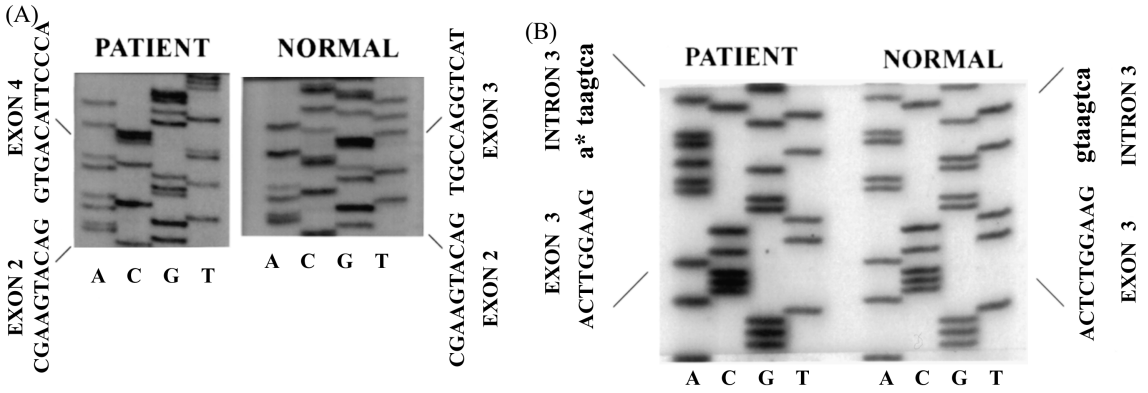
Fig. 3. Point mutation in the maternal-derived PNP allele. (A) Sequencing of patient clones, containing the normal length PNP PCR product, revealed a C to T transition in exon 2 at position 172. (B) Predicted amino acid sequence. Comparison between the normal and mutant gene shows that the C to T transition creates a premature stop codon at position 57.

2–exon 3 junction and exon 3–intron 3 junction were generated from patient genomic DNA by PCR. Sequence analysis of these genomic DNA fragments revealed a transition from G to A at position +1 of intron 3 in one of the PNP alleles (Fig. 4B), which is part of the invariant GT dinucleotide found at the 5′ end of introns. No other changes in the boundaries of exon 3 were found. This single point mutation appears to result in the skipping of exon 3 during PNP pre-mRNA processing. The skipping of exon 3 causes a reading frameshift at the exon 2–exon 4 junction, and translation of this mRNA would result in an addition of 29 non-relevant amino acid sequences downstream of the frameshift. Moreover, the predicted polypeptide encoded by the aberrant

Table 3. Mutations in PNP

Patient #	Age[a]	Neurologic features	Enzyme activity	Nucleotide change and location	Mutation
1	Early	ND[b]	0%	265 G→A exon 3	E 89 K
2	Early	encephalitis	<0.3%	181 G→T exon 2 701 G→C exon 6	aberrant splice→PS[c] R 234 P
3	Early	ataxia	0%	383 A→G exon 4 701 G→C exon 6	D 128 G R 234 P
4	Early	motor retardation	0%	575 A→G exon 5 730 del 1 exon 6	Y 192 C frame shift→PS[c]
5	ND[b]	ND[b]	ND[b]	265 G→A exon 3 520 G→C exon 5	E 89 K A 174 P
6	ND[b]	ND[b]	ND[b]	569 G→T exon 5 Homozygous	G 190 V
7	ND[b]	ND[b]	ND[b]	385 del 3 exon 4 701 G→C exon 6	ΔI 129 R 234 P
8	Late	cerebral palsy	3%	−18Δ(3′)G→A intron 3 701 G→C exon 6	aberrant splice→PS[c] R 234 P
9	Early	ND[b]	1–2%	70 C→T exon 2 Homozygous	R 24 stop
10	Late	motor retardation, ataxia	0%	172 C→T exon 2 +1∇(5′) G→A intron 3	R 58 stop frame shift→PS[c]

[a]Age: early, significant infection prior to 2 years of age; late, significant infection after 2 years of age.
[b]ND: not described.
[c]PS: premature stop.
Δ−18: 18 nucleotides upstream of the 3′ end of intron 3.
∇+1: 1 nucleotides downstream of the 5′ end of intron 3.
Data from references: (10–15).



(C)

Normal PNP

```
   162                  Exon - 2  Exon - 3          198
5' bp CCC AAC TTT CCT CGA AGT ACA G  TG CCA GGT CAT GCT GGC
   --- --- --- --- --- --- --- -  -- --- --- --- --- ---
   aa Pro Asn Phe Pro Arg Ser Thr V  al Pro Gly His Ala Gly
   54                                                  66

                                                      243
   CGA CTG GTG TTT GGG TTC CTG AAT GGC AGG GCC TGT GTG ATG
   --- --- --- --- --- --- --- --- --- --- --- --- --- ---
   Arg Leu Val Phe Gly Phe Leu Asn Gly Arg Ala Cys Val Met
                                                       81

                                          270
   ATG CAG GGC AGG TTC CAC ATG TAT GAA GGG ...3'
   --- --- --- --- --- --- --- --- --- ---
   Met Gln Gly Arg Phe His Met Tyr Glu Gly
                                           90
```

Mutant PNP

```
   162                  Exon - 2  Exon - 4
5' bp CCC AAC TTT CCT CGA AGT ACA G  GT GAC ATT CCC AGT GAG
   --- --- --- --- --- --- --- -  -- --- --- --- --- ---
   aa Pro Asn Phe Pro Arg Ser Thr G  ly Asp Ile Pro Ser Glu
   54

   GGT TTT CCA CCT TCT GGG TGT GGA CAC CCT GGT AGT CAC CAA
   --- --- --- --- --- --- --- --- --- --- --- --- --- ---
   Gly Phe Pro Pro Ser Gly Cys Gly His Pro Gly Ser His Gln


   TGC AGC AGG AGG GCT GAA CCC CAA GTT TGA 3'
   --- --- --- --- --- --- --- --- --- ---
   Cys Ser Arg Arg Ala Glu Pro Gln Val STOP
```

Fig. 4. An exon 3 skipping, paternal-derived PNP mutation. (A) Sequencing of the short PNP PCR product revealed the complete absence of exon 3 and that exon 2 was precisely joined to exon 4. (B) Sequencing of the exon 3–intron 3 boundary revealed a G to A transition at position + 1 of intron 3, resulting in skipping of exon 3 during PNP pre-mRNA processing. (C) Predicted amino acid sequence. Comparison between the normal and mutant gene shows that the G to A transition causes exon 3 deletion and a reading frameshift at the exon 2–exon 4 junction, resulting in a completely different amino acid sequence downstream from the site of the aberrantly spliced mRNA (indicated by underlined sequence). Translation of the mutant gene terminates after 89 amino acids, 29 residues downstream of the exon 2–exon 4 junction.

mRNA terminates at 89 amino acids (Fig. 4C). This severely truncated polypeptide is unlikely to be functional, as the active site of PNP enzyme would be completely abrogated (29).

It is estimated that up to 15% of point mutations causing human genetic disease result in an mRNA splicing defect (21). The most common mutations occur in the 5′ splice site, of which 60% are involved in the invariant GT dinucleotide. A nonrandom distribution of mutation was found, with a higher frequency at position +1 (G) and +2 (T) than at −1 and −2 of the preceding exon (30, 31). Indeed, our patient exhibits this more frequently reported mutation that causes exon skipping. In contrast, a previously reported PNP defect, caused by skipping of exon 2, had a mutation at position −1 of the skipped exon (11).

Our findings expand the surprisingly low number of PNP-deficient patients characterized at the genetic level. We report here two novel mutations of PNP, which presumably originate from the different genetic backgrounds, Caucasian and Japanese. The first mutation is a C to T transition resulting in a premature stop codon at amino acid 57. The second mutation is a G to A transition at position +1 in intron 3, causing a complete skipping of exon 3 and a reading frameshift at exon 2–exon 4 junction. Both mutations likely lead to a complete alteration of the PNP enzyme active site, yet the development of profound immunodeficiency in this patient is greatly delayed. It is therefore conceivable that some residual activity of PNP, which is not detected by our assay or, alternatively other factors such as exposure to various pathogens, may explain the milder phenotype and delayed presentation in this patient.

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