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X-linked hypophosphatemia in Polish patients. 1. Mutations in the *PHEX* gene

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Abstract. We present twenty-nine *PHEX* gene mutations extending our previous work, giving it to a total of 37 different mutations identified in Polish patients with familial or sporadic X-linked hypophosphatemia. Deletions, insertions and nucleotide substitutions leading to frameshift (27%), stop codon (29%), splice site (24%), and missense mutations (20%) were found. The mutations are distributed along the gene; exons 3, 4, 11, 12, 14, 15, 17, 20 and 22 are regions with the most frequent mutation events. Four mutations, P534L, G579R, R549X and IVS15+1nt, recurred in three, four, two and three unrelated patients, respectively. They have also been detected in affected persons from other countries. Twenty-eight mutations are specific for Polish population and almost all of them are unique. Most of the identified mutations are expected to result in major changes in protein structure and/or function.

Key words: deletion, insertion, *PHEX* gene mutation, substitution, X-linked hypophosphatemia.

Introduction

X-linked hypophosphatemia (XLH; MIM#307800) is the predominant type of familial hypophosphatemic rickets (MCKUSICK 1994, RASMUSSEN, TENENHOUSE 1995). XLH is inherited as a dominant trait and has been mapped to the short arm of chromosome X in the region p22.1-p22.2 (READ et al. 1986, MACHLER et al. 1986, THAKKER et al. 1987, ROWE et al. 1994). In 1995 a gene responsible for XLH (named the *PEX* gene) was isolated and a partial nucleotide sequence of

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cDNA was presented (HYP CONSORTIUM 1995). In 1996-1997 the full length cDNA sequences of the *PEX*(human)/*Pex*(mouse) genes (DU et al. 1996, ROWE et al. 1997, FRANCIS et al. 1997, GRIEFF et al. 1997, GUO, QUARLES 1997, BECK et al. 1997) and structure of human gene (FRANCIS et al. 1997) were published.

The *PEX* gene (present name: *PHEX* gene) is supposed to encode a protein with homology to a family of metalloendopeptidases, which are involved in the degradation or activation of peptide hormones (HYP CONSORTIUM 1995). All members of the neutral endopeptidase family are type II integral membrane glycoproteins. They have a short cytoplasmic N-terminal region, a transmembrane domain and a large extracellular C-terminal domain which includes a zinc-binding motif. It was supposed that the *PHEX* endopeptidase takes part in proteolytic processing of a circulating factor (HYP CONSORTIUM 1995, NELSON et al. 1997), which has recently appeared to be a phosphaturic factor, MEPE (ROWE et al. 2000).

Up to now over one hundred and thirty different *PHEX* gene mutations (deletions, insertions, and substitutions) have been identified in XLH families originating from Europe, North Africa, North America and Asia (HYP CONSORTIUM 1995, ROWE et al. 1997, FRANCIS et al. 1997, HOLM et al. 1997, SUŁEK et al. 1998, DIXON et al. 1998, FILISETTI et al. 1999, TYYNISMAA et al. 2000, SABBAGH et al. 2000).

This study of Polish patients with XLH was undertaken to gain an understanding of the genesis of the disease and relations between genotype and phenotype. In the first part we present the *PHEX* gene mutations identified in a group of unrelated Polish patients and affected family members.

Material and methods

Thirty-five patients with XLH from different families (10 familial and 25 sporadic events) were studied. In many cases other affected members of a family were also examined.

Genomic DNA was isolated from peripheral blood leukocytes and *PHEX* gene exons (1-22) were amplified. Alterations in exon size and structure were identified by agarose or polyacrylamide gel electrophoresis and by single strand conformation polymorphism (SSCP) analysis – (ORITA et al. 1989) or heteroduplex analysis (HA) – (WHITE et al. 1992). The shifted fragments were sequenced by the dideoxy-chain termination method using a cycle sequencing kit (Amersham) and [^{35}S]αdATP (Amersham) or using fluorescent dyes and automated ABI PRISM 310 DNA Analyser (Perkin Elmer).

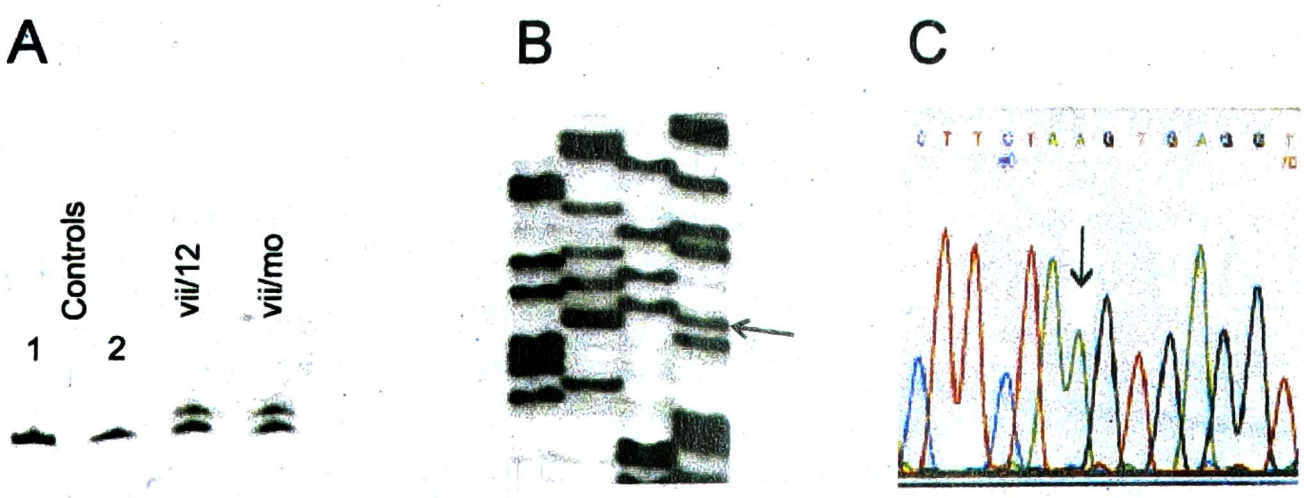

Figure 1. Detection of mutations in the *PHEX* gene. A. Pattern of heteroduplex analysis for exon 6 in girl vii/12 and her affected mother v. vontrol alleles. B. Sequence analysis in girl xviii/27. Arrow indicates presence of C (normal)and T (mutation) in codon 534 (exon 15). C. Automated sequence analysis in girl xxxiii/48 (1) and her affected father (2). frame indicates presence of duplicated sequence TGAC in exon 20

Results

Twenty-nine different *PHEX* gene mutations in 35 unrelated patients with familial or sporadic XLH were identified (Figures 1A, B, C). Including previously described results (HYP CONSORTIUM 1995, ROWE et al. 1997, SUŁEK et al. 1998), 37 different mutations have been recognised in 45 unrelated Polish patients (Table 1).

The identified mutations are nucleotide substitutions leading to missense mutations (four cases, in exons 12, 14, 15 and 17), stop codon mutations (twelve cases, in exons 1, 10, 11, 15, 16, 18, 20, 21 and 22) and donor splice site mutations (five cases, in introns 1, 4, 9, 15 and 17) or an acceptor splice site mutation (one case, in intron 20). Besides, insertions of one nucleotide (one case, in intron 17), four nucleotides (two cases, in exons 20 and 22) or thirty-one nucleotides (one case, in intron 19) and various deletions involving the loss of one nucleotide (three cases, in exons 4, 6 and 11), two nucleotides (one case, in exon 11), four nucleotides (one case, in intron 9), five nucleotides (two cases, in exons 3 and 12), six nucleotides (one case, in exon 9), fifteen nucleotides (one case, in exon 14), 104 nucleotides (one case, in exon 5/intron 5) and more than 50000 nucleotides (one case, including exons 2-4) were detected.

In order to test the possibility that three novel splice site mutations involving positions +3 and +5 (IVS17+3nt, IVS4+5nt, IVS17+5nt) and two missense mutations (V442F, R510P) were polymorphic insertion/substitutions, 80 alleles from unrelated individuals were tested by SSCP and HA analyses or by sequencing. No other DNA samples had base changes, which suggests that our patients are cases with true mutations.

Discussion

Our previous research and this study revealed thirty-seven different mutations distributed along the *PHEX* gene. Exons 3, 4, 11, 12, 14, 15, 17, 20, 22 and adjacent introns are the most frequent mutational event regions, with 2 or 3 different mutations in each. Most of the mutations are specific to Polish patients and most are unique and found in only one family.

All identified deletions, except the large one spread over 50 kb (patient iii/4, HYP CONSORTIUM 1995), are oligonucleotide or small polynucleotide deletions. ROWE et al. (1997), FRANCIS et al. (1997) and DIXON et al. (1998) also described numerous deletions, mainly involving the loss of one or several exons.

Two of the deletions identified by us covered six and fifteen nucleotides that do not change the correct reading frame. In the first case the loss of three amino acids (Asn, Val, Val) in positions 338-340 and translation of a methionine residue, unspecific in that place, is expected. Valine-340 is conserved in all members of the metalloendopeptidase family (NEP, ECE, KELL) (ROWE et al. 1997) and any

Table 1. Various *PHEX* gene mutations in Polish patients with X-linked hypophosphatemia

Patient	Inheri-tance	Sex	Muta-tion type	Change in genomic DNA	Change in polypeptide	Reference	Comment
xxxvii/60	familial	F	Missense mutation	G1324→T, ex 12, cod 442: GTT→TTT	Val(V)→Phe(F)	this study	spec./nov.
xvii/26	sporadic	F		G1529→C, ex14, cod 510: CGC→CCC	Arg(R)→Pro(P)	this study	spec./nov.
xviii/27	familial	F		C1601→T, ex 15, cod 534: CCG→CTG	Pro(P)→Leu(L)	this study	rec.
xix/29	sporadic	F				this study	rec.
xx/30	sporadic	F				this study	rec.
xxvi/38	familial	M		G1735→A, ex 17, cod 579: GGA→AGA	Gly(G)→Arg(R)	ROWE et al.1997 (as pat. POZN)	rec.
xxvii/40	familial	M				SUŁEK et al.1998 (as pat. ZS61)	rec.
xxviii/42	sporadic	F				this study	rec.
xxix/43	sporadic	M				this study	rec.
i/1	familial	M	Stop codon mutation	C58→T, ex 1, cod 20: CGA→TGA	Arg(R)→Ter(X)	ROWE et al.1997 (as pat. TH241)	rec.
xi/18	sporadic	F		G1103→A, ex 10, cod 368: TGG→TAG	Trp(W)→Ter(X)	this study	spec./nov.
xxxviii/61	sporadic	M		C1180→T, ex 11, cod 394: CAG→TAG	Gln(Q)→Ter(X)	this study	spec./nov.
xiii/20	familial	M		G1209→A, ex 11, cod 403: TGG→TGA	Trp(W)→Ter(X)	ROWE et al.1997 (as pat. M.)	spec.
xiv/23	sporadic	F		C1282→T, ex 11, cod 428: CAG→TAG	Gln(Q)→Ter(X)	this study	spec./nov.
xxi/31	sporadic	F		C1645→T, ex 15/16, cod 549: CGA→TGA	Arg(R)→Ter(X)	this study	rec.
xxxix/62	familial	F				this study	rec.
xxv/35	familial	F		G1684→T, ex 16, cod 562: GGA→TGA	Gly(G)→Ter(X)	this study	spec./nov.

xxxii/46	familial	F		G1881→A, ex 18, cod 627: TGG→TGA	Trp(W)→Ter(X)	this study	spec./nov.
xL/63	sporadic	F		C2044→T, ex 20, cod 682: CAG→TAG	Gln(Q)→Ter(X)	this study	spec./nov.
xLi/64	sporadic	F		C2104→T, ex 21, cod 702: CGA→TGA	Arg(R)→Ter(X)	this study	rec.
xLii/65	sporadic	F		C2182→T, ex 22, cod 728:CAG→TAG	Gln(Q)→Ter(X)	this study	spec./nov.
xxxv/51	familial	M		C2239→T, ex 22, cod 747: CGA→TGA	Arg(R)→Ter(X)	this study	rec.
iii/4	familial	M	Deletion, insertion – frameshift mutation	~50 kb del, ex 2-4 , cod: 40-145	trunc: protein without 106 aa in position 40-145	HYP 1995 (as pat. TK11)	spec.
iv/7	sporadic	M		A201-A205 del, ex 3, cod: 67-69	trunc.: 76 aa protein with 10 unspecific aa	ROWE et al.1997 (as pat. RG175)	spec.
v/8	sporadic	M		C412 del, ex 4, cod: 138	trunc.: 142 aa protein with 5 unspecific aa	this study	spec./nov.
vi/9	familial	M		104 bp del, ex 5, cod: 204-221 and IVS5+(1-50) nt	trunc.: probably at least 160 aa protein with several unspecific aa	ROWE et al.1997 (as pat. MW180)	spec.
vii/12	familial	F		C677 del, ex 6, cod: 226	trunc.: 230 aa protein with 5 unspecific aa	this study	spec./nov.
viii/14	sporadic	F		A1013-G1018 del, ex 9, cod: 338-340	trunc.: translation of unspecific Met in place of deleted 3aa	this study	spec./nov.
xii/19	sporadic	F		G1185 del, ex 11, cod: 395	trunc.: 406 aa protein with 11 unspecific aa	this study	spec./nov.
xLiii/66	sporadic	M		G1270-A1271 del, ex 11, cod: 424	trunc.: 429 aa protein with 6 unspecific aa	this study	spec./nov.
xv/24	sporadic	M		A1400-G1404 del, ex 12, cod: 467-468	trunc.: 480 aa protein with 14 unspecific aa	ROWE et al.1997 (as pat. PY)	spec.
xvi/25	sporadic	F		A1523-T1537 del, ex 14, cod: 508-512	trunc.: deletion of 5 aa	this study	spec./nov.
xxxiii/48	familial	F		1990 ins TGAC (dupl), ex 20, cod: 664	trunc.: 663 aa protein	this study	rec.
xxxvi/57	familial	M		2245 ins ACTC (dupl), ex 22, cod: 749	longer 793 aa protein with 45 unspecific aa	this study	spec./nov.

ii/3	sporadic	M	Splice site mutation	IVS1, 118+1 nt: g→a	probably loss of the entire protein	SUŁEK et al.1998 (as pat. JM455)	spec.
xLiv/67	sporadic	F		IVS4, 436+5 nt: g→t	trunc.: probably protein without 29 aa (L118 - K146)	this study	spec./nov.
ix/15	familial	M		IVS9, 1079+1 nt: g→a	trunc.: probably 313 aa protein with 2 unspecific aa	this study	spec./nov.
x/16	familial	M		IVS9, 1079+(2-5) nt: taag del	trunc.: probably 313 aa protein with 2 unspecific aa	SUŁEK et al.1998 (as pat. JJ46)	spec.
xxii/32	sporadic	F		IVS15, 1645+1 nt: g→a	trunc.: probably 560 aa protein with 32 unspecific aa	this study	rec.
xxiii/33	sporadic	F				this study	
xxiv/34	sporadic	F				this study	
xxx/44	sporadic	F		IVS17, 1768+3 nt: ins t	probably several types of the protein, including normal and truncated forms	this study	spec./nov.
xxxi/45	sporadic	M		IVS17, 1768+5 nt: g→a	probably several types of the protein, including normal and truncated forms	this study	spec./nov.
xLv/68	sporadic	F		IVS19, 1965+3 nt: ins 31 nt : dupl /1936-(1965+1)/ nt	trunc.: probably protein without 22 aa (V634-R655)	this study	spec./nov.
xxxiv/50	sporadic	M		IVS20, 2071-1 nt: g→a	trunc.: probably 695 aa protein with 5 unspecific aa	this study	spec./nov.

Abbreviations used: ex = exon, cod = codon, del = deletion, ins = insertion, dupl = duplication, aa = amino acid, trunc = truncation, spec. = specific, nov. = novel, rec. = recurrent.

mutation involving that amino acid may influence *PHEX* enzymatic activity. In the second case the deletion of five unconserved amino acids (Gln, Thr, Arg, Lys, Tyr) in positions 508-512 and some structural changes of the *PHEX* protein are predicted.

The two other deletions are situated in the intron (patient x/16) or at the boundary exon/intron (patient vi/9), and are responsible for loss of specific donor splice site sequences. In patient x/16 a 4 bp deletion located in intron 9 in position from +2 to +5 would result in out-of-phase exon 9 skipping, and in patient vi/9 a 104bp deletion in exon 5/intron 5 would result in out-of-phase exon 5 skipping, followed by frameshift of the mis-spliced exon sequences. Similarly, a 4 bp deletion in donor sequence (position from +3 to +6) of intron 14 was described by HOLM et al. (1997) in a boy with XLH.

All of the identified deletions, except in patients viii/14 and xvi/25, probably result in frameshift and truncated forms of the *PHEX* protein with several C-terminal unspecific amino acids.

Splice-site mutations were identified in eleven unrelated patients with XLH. In six cases they resulted from transitions or transversions in the first or second position of conserved 5' or 3' intron sequences and they were predicted to result in out-of-phase adjacent exon skipping followed by stop codon. According to NAKAI and SAMAMOTO (1994) only a small number of mutations of this type are associated with activation of cryptic donor or acceptor splice sites. In two cases (girl xLiv/67 and boy xxxi/45) the nucleotide substitution is located in intron at position +5. This position should be occupied by guanidine and its substitution usually results in formation of several types of transcripts, including a fraction of the normal form. Two donor splice site mutations resulted from insertions of one nucleotide (girl xxx/44) or 31 nucleotides (girl xLv/68) at position +3. It is expected that in the first case a few types of transcript will be formed, and in the second case mainly transcript without exon 19 will be produced.

One mutation in the set of different splice site mutations involved an acceptor splice site sequence in intron 20 (patient xxxiv/50). As a consequence of the mutation, skipping of exon 21 with a subsequent frameshift and stop codon was predicted. The truncated protein should harbour the catalytic domains in exon 17 and 19.

Insertions are the least frequent types of mutations in the *PHEX* gene. Of the four identified insertions, two were located in introns and two in the coding sequence. Two insertions were duplications of preceeding 4 bp sequences in exons 20 and 22. In the first case a duplication started at position 1990 and contained four nucleotides (TGAC) repeated from codons 662/663. The inserted fragment put a stop codon (TGA) in position 664. The same duplication was described by FRANCIS et al. (1997) A second insertion was a duplication of an ACTC fragment at the C-terminal sequence of exon 22. In consequence, a longer protein with 793 amino acids is predicted to be formed. ROWE et al. (1997) presented only two small insertions, FRANCIS et al. (1997) three small insertions and DIXON et al.

(1998) three small insertions and two deletional-insertions in groups of 43, 26 and 31 different mutations, respectively.

In our set of 37 different mutations, twelve nonsense mutations in exons 1, 10, 11, 15/16, 18, 20, 21 and 22 were identified. Three distinct stop codons were situated in exon 11. All stop codons resulted from nucleotide substitutions, in nine cases from C→T transitions, in two cases from G→A transitions and in one case from G→T transversion. Four of the stop codon mutations, R20X, R549X, R702X and R747X, were recurrent mutations and – excluding our cases – have been identified, respectively, in three, two and five familial or sporadic XLH events from Europe, North Africa, North America and Asia (ROWE et al. 1997, FRANCIS et al. 1997, HOLM et al. 1997, DIXON et al. 1998).

Four different missense mutations (V442F, R510P, P534L and G579R) were identified in nine unrelated Polish patients. Two of them (P534L and G579R) were the most frequent mutations, observed, respectively, in three and four of our sporadic or familial XLH cases, and in addition, respectively, in eight and six cases from other European countries, North Africa and North America (ROWE et al. 1997, FRANCIS et al. 1997, HOLM et al. 1997, DIXON et al. 1998). HOLM et al. (1997) reported that codon 579 was the site of another mutation (G→T), leading to the Gly-579→Val substitution. Glycine-579 is directly adjacent to the zinc-binding motif and it is conserved in all except one endopeptidase (ROWE et al. 1997). It may play an important role in substrate specificity or enzyme function. Replacing the glycine with the basic positively charged arginine reduces the hydrophobicity of the region and probably decreases *PHEX* enzymatic activity.

The other two missense mutations (V442F, R510P) were specific to the Polish population. They were identified in the unconserved regions of the *PHEX* gene. The presence of a phenylalanine in position 442 or a proline in position 510 has never been documented in any member of the metalloendopeptidase family (ROWE et al. 1997).

It can be concluded that all our nonsense mutations, as well as splice site mutations and most of the deletions which lead to stop codons downstream, presumably result in a truncated protein without catalytic and substrate-binding domains. In cases of small in-frame deletions and missense mutations, the residual level of *PHEX* enzymatic activity would depend on the conservation and degree of structural damage of the mutated regions. We believe that all the *PHEX* gene mutations identified by us are pathogenic because they are present only in affected persons, and in familial cases they co-segregate with clinical symptoms of the disease.

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