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Functional analysis of 22 splice-site mutations in the *PHEX*, the causative gene in X-linked dominant hypophosphatemic rickets

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ABSTRACT

Context: X-linked hypophosphatemic rickets (XLH) is caused by inactivating mutations in the *PHEX* gene and is the most common form of hereditary rickets. The splice-site mutations account for 17% of all reported *PHEX* mutations. The functional consequence of these splice-site mutations has not been systemically investigated.

Objective: The current study was undertaken to functionally annotate previously reported 22 splice-site mutations in the *PHEX* gene.

Methods: *PHEX* mini-genes with different splice-site mutations were created by site-directed mutagenesis and expressed in HEK293 cells. The mRNA transcripts were analyzed by RT-PCR, cloning, and sequencing.

Results: These splicing mutations led to a variety of consequences, including exon skipping, intron retention, and activation of cryptic splice sites. Among 22 splice-site mutations, exon skipping was the most common event accounting for 73% (16/22). Non-canonical splice-site mutations could result in splicing errors to the same extent as canonical splice-site mutations such as c.436 + 3G > C, c.436 + 4A > C, c.436 + 6T > C, c.437-3C > G, c.850-3C > G, c.1080-3C > A, c.1482 + 5G > C, c.1586 + 6T > C, c.1645 + 5G > A, c.1645 + 6T > C, c.1701-16T > A, c.1768 + 5G > A, and c.1899 + 5G > A. Interestingly, non-canonical (c.436 + 6T > C and c.1586 + 6T > C) and canonical splice-site mutations (c.1769-1G > C) could generate partial splicing errors (both wild-type and mutant transcripts were detected), resulting in incomplete inactivation of *PHEX* gene, which may explain the mild disease phenotype reported previously, providing evidence of genotype-phenotype correlation. c.1645C > T (p.R549*) had no impact on pre-mRNA splicing although it is located next to canonical splice donor site GT.

Conclusions: Exon skipping is the most common outcome due to splice-site mutations. Both canonical and non-canonical splice-site mutations can result in either severe or mild RNA splicing defects, contributing to phenotype heterogeneity. Non-canonical splice-site mutations should not be overlooked in genetic screening especially those located within 50 bp from canonical splice site.

1. Introduction

Hereditary hypophosphatemic rickets (HR) is a group of renal phosphate wasting disorders, which can be divided into FGF23-dependent and FGF23-independent HR [1]. The FGF23-dependent HR includes X-linked HR (XLH, MIM 307800), also called X-linked dominant HR, autosomal dominant HR (ADHR, MIM 193100), autosomal recessive HR Type 1 (ARHR1, MIM 241520), and autosomal recessive HR Type 2 (ARHR2, MIM 613312). Each of the disorders is caused by mutations in the *PHEX*, *FGF23*, *DMP1*, and *ENPP1*, respectively. The FGF23-independent HR includes hereditary hypophosphatemic rickets with hypercalciuria (HHRH, MIM 241530), hypophosphatemic rickets with nephrolithiasis and osteoporosis type 1 (NPHLOP1, MIM 612286), hypophosphatemic rickets with nephrolithiasis and osteoporosis type 2 (NPHLOP2, MIM 612287), Dent disease 1 (MIM 300009), and Dent disease 2 (MIM 300555) [1]. The underlying genetic defects for FGF23-independent HR include mutations in the *SLC34A3*, *SLC34A1*, *SLC9A3R1*, *CLCN5*, and *OCRL*, respectively.

XLH is the most common form of HR with an incidence of approximately 1 in 20,000 live births due to inactivation mutations in the *PHEX* gene [2–4]. The *PHEX* gene is 219 kb long located on chromosome Xp22.1 and encodes for a 749 amino acid glycoprotein with 22

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exons [5]. Its protein regulates the expression of FGF23 (fibroblastic growth factor 23), a phosphaturic hormone (phosphatonin) that inhibits sodium-dependent phosphate uptake in the renal proximal tubule [6]. Among 467 *PHEX* mutations reported in the Human Gene Mutation Database (HGMD, http://www.hgmd.cf.ac.uk/ac/index.php, accessed April 21, 2019), 81 are splice-site mutations accounting for 17% (81/467) of all reported mutations. The remaining mutations are missense/nonsense mutations (174, 37%), small deletions (96, 21%), small insertions/duplications (54, 12%), gross deletions (42, 9%), gross insertions/duplications (6, 1%), small indels (6, 1%), regulatory substitutions (4, 1%), and complex rearrangements (4, 1%). Based on HGMD, *PHEX* mutations were found in 529 patients. Among them, 96 patients had splice-site mutations (18%). In our previous review of 82 patients with sporadic XLH who had de novo *PHEX* mutations, 16 patients (20%) carried splice-site mutations [7]. Thus, in general, the prevalence of *PHEX* splice-site mutation is approximately 18–20%. However, the prevalence of *PHEX* splice-site mutation may be higher in Turkish patients. Among 26 families we investigated previously for *PHEX* mutations [3,4,7,8], 8 families (31%) were found to have splice-site mutations.

The canonical splice donor GT and acceptor AG sites are present in 98.71% of mammalian genes [9]. The corresponding introns of *PHEX* vary in size from 998 to 34,687 bp and all of their splice-site sequences conform to the canonical GT–AG splicing rule using the U1–U2 snRNPs. Mutations in these canonical dinucleotides would always result in splicing errors from pre-mRNA transcripts into mature mRNA [10]. It has been reported that sequence variations surrounding the canonical splice sites may not cause splicing errors due to the fact that these exonic or intronic cis elements are weakly conserved and their alterations do not always disrupt the splicing processes [11]. There are 16 reported splice-site mutations in the *PHEX* gene that is located outside the canonical GT–AG consensus splicing junction such as c.436 + 3G > C, c.436 + 4A > C, c.436 + 6T > C, c.437-3C > G, and c.1701-16T > A. The consequence of these mutations on pre-mRNA splicing has not been verified.

In the present study, we systematically investigated the effect of 14 previously reported splice-site mutations on pre-mRNA splicing which are located outside the GT–AG dinucleotide splice donor or acceptor sites. As a control, 8 splice-site mutations at the canonical GT–AG consensus splicing junction were also investigated. Most of the mutations resulted in aberrant splicing such as exon skipping, activation of cryptic splice sites, and intron retention. Only one mutation (c.1645C > T) was found to have no impact on pre-mRNA splicing.

2. Materials and methods

2.1. PHEX minigene construction

Two *PHEX* minigenes (minigene-1 and minigene-2) were created by gene synthesis and subcloned into pcDNA3.1 vector (Invitrogen, CA). The minigene-1 contains exons 3–11 with 8 introns of 480 bp each. The minigene-2 contains exons 12–20 with 8 introns of 480 bp each as well (Sup Fig. 1). Eight mutant constructs were created by site-directed mutagenesis of minigene-1: c.436 + 2T > A, c.436 + 3G > C, c.436 + 4A > C, c.436 + 6T > C, c.437-3C > G, c.850-3C > G, c.1080-2A > C, and c.1080-3C > A. Fourteen mutant constructs were created by site-directed mutagenesis of minigene-2: c.1482 + 1G > C, c.1482 + 5G > C, c.1586 + 1G > A, c.1586 + 6T > C, c.1645C > T, c.1645 + 1G > A, c.1645 + 5G > A, c.1645 + 6T > C, c.1701-16T > A, c.1768 + 2T > G, c.1768 + 5G > A, c.1769-1G > C, c.1899 + 1G > T, c.1899 + 5G > A. The constructs were transfected into HEK293 cells and total RNA was extracted 48 h after transfection as described previously [11,12].

2.2. RT-PCR and sequencing analysis of PHEX minigene transcripts

Two micrograms of total RNA were reverse-transcribed into cDNA using Promega reverse transcription system (Promega, Madison, WI). To improve specificity, nested PCR was performed to amplify *PHEX* minigene transcripts using primer pairs indicated in Table 1. The PCR conditions were 95 °C for 5 min followed by 35 cycles of amplification (95 °C for 40s, 52 °C for 40s, and 72 °C for 40s). The resulting PCR products were analyzed by gel electrophoresis, and either directly sequenced using an automated ABI PRISM 3700 sequencer (Foster City, CA) or cloned into a TA vector (Invitrogen, CA). Individual clones were subsequently sequenced.

Splice site prediction.

Three web-based programs were used to predict whether splice-site mutation would cause exon skipping or cryptic splice-site activation: Alternative Splice Site Predictor (http://wangcomputing.com/assp/) [13], Human Splicing Finder Version 3.0 (http://www.umd.be/HSF3/) [14], Splice Site Prediction by Neural Network (http://www.fruitfly.org/seq_tools/splice.html) [15].

3. Results

The consequence of splice-site mutation on pre-mRNA splicing was evaluated by RT-PCR and sequencing analysis of transcripts produced by *PHEX* minigene-1 or -2. Among minigene-1 mutants (Table 2), c.436 + 2T > A, c.436 + 3G > C, and c.436 + 4A > C all resulted in exon 4 skipping, c.850-3C > G resulted in exon 8 skipping, and c.1080-2A > C and c.1080-3C > A caused exon 10 skipping (Fig. 1). Interestingly, c.437-3C > G caused activation of an adjacent cryptic splice acceptor site AG located in exon 5 and led to in-frame deletion of AAG (p.K146TdelA) (Fig. 2A and B). As shown in Fig. 3A, c.436 + 6T > C could not result in complete exon 4 skipping: about 40% wild-type transcripts were still present, which may lead to mild phenotype. Indeed, the clinical presentations of patients who carried the mutation (mother and her two sons) were very mild [16].

Among minigene-2 mutants (Table 2), c.1482 + 1G > C and c.1482 + 5G > C resulted in exon 13 skipping, c.1768 + 2T > G and c.1768 + 5G > A resulted in exon 17 skipping, and c.1899 + 1G > T and

Table 1
Primer sequences used to amplify *PHEX* minigene cDNA.

Primers	Sequence	Location	Comments
Primer 1 (forward)	5′-GTAAATCTGTCTGTGGATCCTT-3′	Exon 3	Primers 1 and 3 for amplification of minigene-1 cDNA including exons 3–6.
Primer 2 (forward internal)	5′-TCGCTTGTGATGGCTGGATAAG-3′	Exon 3	Primers 2 and 4 for nested PCR
Primer 3 (reverse)	5′-ACTTGGCTTCTGTACTGTTATC-3′	Exon 6	
Primer 4 (reverse internal)	5′-GGTAGTCTTCCCTCACGGCCA-3′	Exon 6	
Primer 5 (forward)	5′-GATAACAGTACAGAAGCCAAGT-3′	Exon 6	Primers 5 and 7 for amplification of minigene-1 cDNA including exons 6–11.
Primer 6 (forward internal)	5′-TGGCCGTGAGGGAAGACTACC-3′	Exon 6	Primer 6 for nested PCR with primer 7
Primer 7 (reverse)	5′-AGGCAGCAAAGTTGTGGTCC-3′	Exon 11	
Primer 8 (forward)	5′-GAGTGGATGGATGCAGGAACGA-3′	Exon 12	Primers 8 and 10 for amplification of minigene-2 cDNA including exons 12–20
Primer 9 (forward internal)	5′-AACGAAAAGGAAAGCCAAAG-3	Exon 12	Primer 9 for nested PCR with primer 10
Primer 10 (reverse)	5′-GCTCCTCAAGTCCCTGCCTTCT-3′	Exon 20	

Table 2
Effect of splice-site mutation on pre-mRNA splicing.

PHEX minigene	Mutant	Location	Consequence	Severity of phenotype[a]
Minigene-1	c.436 + 2T > A	Splice donor site in intron 4	Exon 4 skipping	+ + + [33]
	c.436 + 3G > C	Splice donor site in intron 4	Exon 4 skipping	+ + + [32]
	c.436 + 4A > C	Splice donor site in intron 4	Exon 4 skipping	+ + + [34]
	c.436 + 6T > C	Splice donor site in intron 4	Exon 4 skipping (60%) Wt (40%)	+ [16]
	c.437-3C > G	Splice acceptor site in intron 4	In-frame deletion of AAG (p.K146TdelA) due to activation of adjacent cryptic splice acceptor site in E5	+ + + [34]
	c.850-3C > G	Splice acceptor site in intron 7	Exon 8 skipping	+ + + [35]
	c.1080-2A > C	Splice acceptor site in intron 9	Exon 10 skipping	+ + [36]
	c.1080-3C > A	Splice acceptor site in intron 9	Exon 10 skipping	+ + + [37]
Minigene-2	c.1482 + 1G > C	Splice donor site in intron 13	Exon 13 skipping	+ + + [30]
	c.1482 + 5G > C	Splice donor site in intron 13	Exon 13 skipping	Unknown [38]
	c.1586 + 1G > A	Splice donor site in intron 14	E14 skipping (60%) Partial E14 skipping (40%)	+ + + [30,39]
	c.1586 + 6T > C	Splice donor site in intron 14	E14 skipping (60%) Wt (40%)	Unknown [17]
	c.1645C > T	Splice donor site in exon 15	No splicing error but create a premature stop codon TGA	+ + + [24]
	c.1645 + 1G > A	Splice donor site in intron 15	72 bp intron 15 retention due to activation of cryptic splice donor site in intron 15	+ + + [33]
	c.1645 + 5G > A	Splice donor site in intron 15	72 bp intron 15 retention due to activation of cryptic splice donor site in intron 15	+ + + [30]
	c.1645 + 6T > C	Splice donor site in intron 15	72 bp intron 15 retention due to activation of cryptic splice donor site in intron 15	Unknown [17]
	c.1701-16T > A	Splice acceptor site in intron 16	Exon 17 skipping	Unknown [24]
	c.1768 + 2T > G	Splice donor site in intron 17	Exon 17 skipping	+ + + [40]
	c.1768 + 5G > A	Splice donor site in intron 17	Exon 17 skipping	+ + [31]
	c.1769-1G > C	Splice acceptor site in intron 17	Exon 17 skipping plus GTAG deletion in exon 18 (70%) Exon 17 and 18 skipping (10%) Wt (20%)	Unknown [41] + + by c.1769-1G > A [42]
	c.1899 + 1G > T	Splice donor site in intron 18	Exon 18 skipping	Unknown [41] + + + by c.1899 + 2T > A [43]
	c.1899 + 5G > A	Splice donor site in intron 18	Exon 18 skipping	+ + + [34]

+ (mild): no history of osteotomies and bone deformities, no dental abscesses.
+ + (moderate): mild-moderate bone deformities, < 3 dental abscesses.
+ + + (severe): history of osteotomies and/or severe bone deformities, > 3 dental abscesses.
[a] Severity of phenotype is from original report and references are provided in bracket.

c.1899 + 5G > A caused exon 18 skipping (Fig. 1). Although we expected that c.1768 + 2T > G, or c.1768 + 5G > A may result in exon 17 skipping, we did not expect that c.1701-16T > A would result in the same splicing error as c.1768 + 2T > G, or c.1768 + 5G > A since c.1701-16T > A is located 14 bp from canonical splice donor site GT in intron 17 (Fig. 1). As shown in Fig. 3B, c.1586 + 1G > A caused both partial and complete exon 14 skipping. The partial exon 14 skipping is due to activation of a cryptic splice donor site GC present in exon 14. Similar to c.436 + 6T > C, c.1586 + 6T > C could not result in complete exon 14 skipping: about 40% wild-type transcripts were present (Fig. 3B). Although phenotype of the patient was not described in the original study [17], we expected mild phenotype of the patient. c.1645 + 1G > A, c.1645 + 5G > A, and c.1645 + 6T > C activated a cryptic splice donor site located 70 bp downstream from the canonical splice donor site GT in intron 15, resulting in 72 bp intron 15 retention (Fig. 2C). This is predicted to cause frameshift and premature stop codon 20 amino acids from R549 (p.R549Cfs*20). c.1645C > T is located next to the canonical splice donor site GT at the exon-intron15 junction (Sup. Fig. 1). Although c.1645C > T had no effect on pre-mRNA splicing, it created a premature stop codon TGA (Fig. 2C). As shown in Fig. 3C, c.1769-1G > C results in three major RT-PCR products. After cloning and sequencing of these products, the majority of them (360 bp fragment, 70%) were caused by activation of a cryptic splice acceptor site AG in exon 18, resulting in exon 17 skipping plus deletion of GTAG in exon 18. The 230 bp cDNA fragment (10% of RT-PCR products) was derived from skipping of exon 17 and 18. The 460 bp cDNA fragment was wild-type and accounted for about 20% of RT-PCR products, indicating that c.1769-1G > C could not result in complete inactivation of *PHEX* and retention of partial PHEX function is expected.

Finally, we used three splice prediction programs to predict whether splice-site mutation would cause exon skipping or cryptic splice-site activation. None of the programs could reliably predict the outcome of splice-site mutations.

4. Discussion

In the present study, we have characterized 22 previously reported

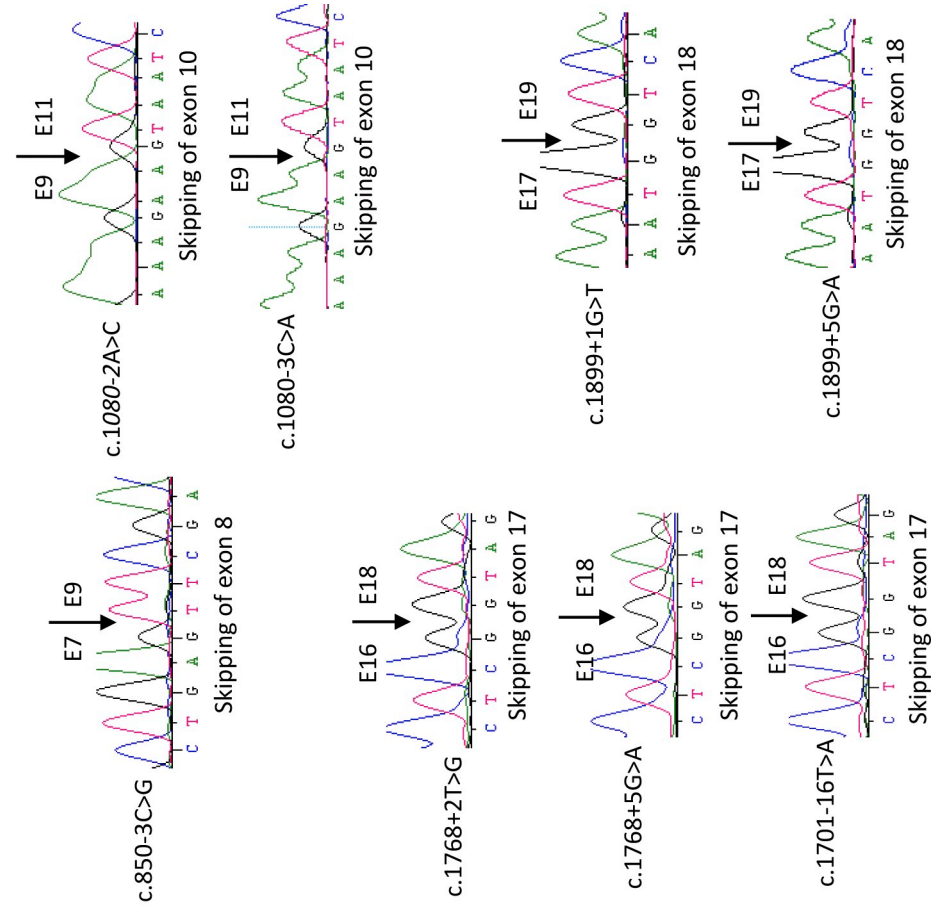


Fig. 1. Sequence analysis of *PHEX* minigene transcripts derived from exon skipping. Total RNA was extracted from Hek293 cells transfected with different minigene constructs. RT-PCR products were run on a 1.2% agarose gel. Single cDNA fragments were amplified from RT-PCR and subsequently sequenced. Sequence electropherograms are presented to show mutant and surrounding Wt sequence. Other regions of Wt sequence are chopped. Exon skipping is shown and exon boundary is indicated by an arrow.

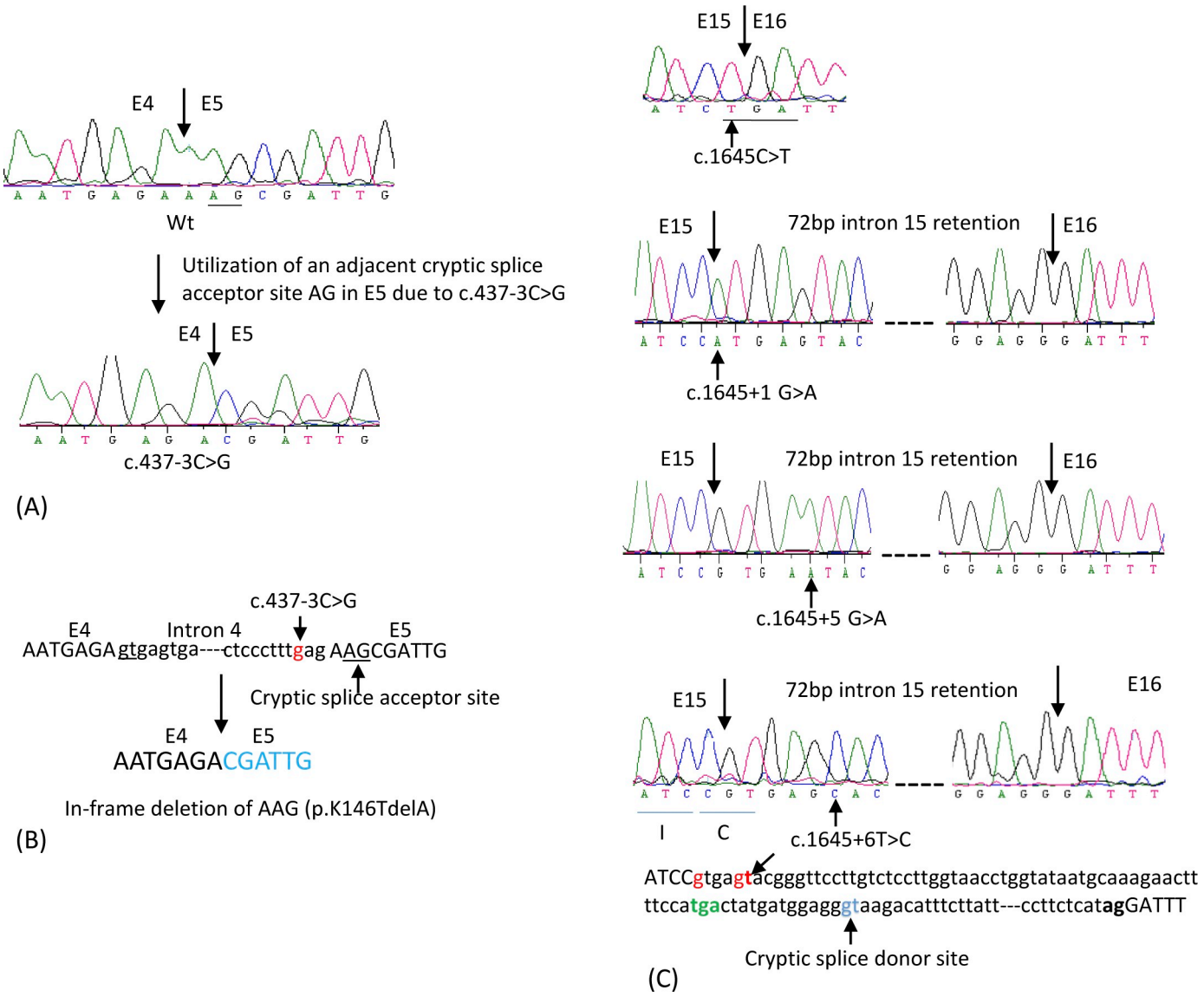
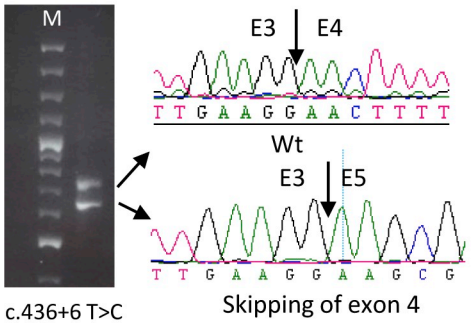


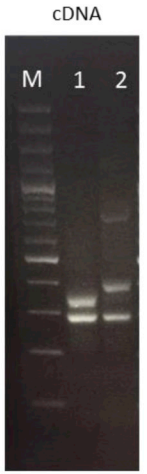
Fig. 2. Sequence analysis of *PHEX* minigene transcripts derived from utilization of cryptic splice site. Single cDNA fragments were amplified from RT-PCR and directly sequenced. Sequence electropherograms are presented to show mutant and surrounding Wt sequence. Other regions of Wt sequence are chopped. (A) c.437-3C > G results in utilization of adjacent cryptic splice acceptor site AG in exon 5 and in-frame deletion of AAG (p.K146TdelA). The cryptic splice acceptor site is underlined. (B) Schematic diagram showing the location of c.437-3C > G mutation (indicated in red) and its surrounding DNA sequence. (C) Partial intron 15 retention due to c.1645 + 1G > A, c.1645 + 5G > A, or c.1645 + 6T > C mutation resulting in frameshift and premature stop codon 20 amino acids from R549 (p.R549Cfs*20). The three splice donor site mutations, premature stop codon, and a cryptic splice donor site are indicated in red, green, and blue color, respectively in a schematic diagram showing partial exons 15–16 (in upper case) and intron 15 (in lower case). The sideway arrow indicates the c.1645 + 6T position. Normal splice acceptor site AG in intron 15 is indicated in bold lower case. c.1645C > T has no effect on pre-mRNA splicing, but created a premature stop codon TGA (indicated by an arrow). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

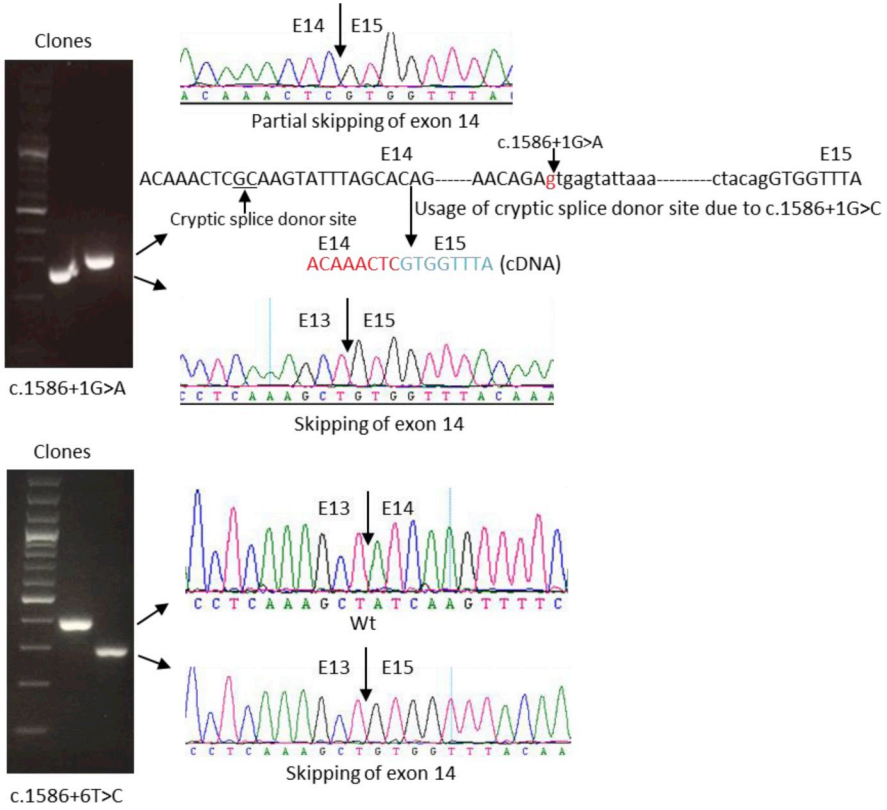
splice-site mutations in the *PHEX* gene. Exon skipping is the most common consequence from splice-site mutations. We have also shown that non-canonical splice-site mutations can sometimes result in splicing errors to the same extent as canonical splice-site mutations. Furthermore, non-canonical splice-site mutations can cause partial splicing errors, which may contribute to the mild phenotype of the disease.

Although splice-site mutations account for 17% of all reported *PHEX* mutations, they may represent up to ~50% of disease-causing mutations in genes with a large number of introns [18,19]. The molecular consequence of a splice-site mutation includes, by order of frequency, exon skipping, activation of cryptic splice sites, and intron retention [20]. Exon skipping and aberrant splice-site activation account for most splice-site mutations in higher eukaryotes [21]. The outcomes may have different phenotypic consequences and are often challenging to predict without analysis of RNA samples from affected individuals. Without patients' RNA samples, one can still perform RNA analysis by cloning and expressing the defected gene. Since the *PHEX* gene is 219 kb long with 21 introns including 12 introns larger than 5 kb, it would be impossible to clone and express the whole gene to study splice mutations. By creating two *PHEX* minigenes followed by site-directed mutagenesis and RT-PCR-sequencing analysis, we were able to evaluate the consequence of splice mutations, which could not be accurately predicted by current available programs. This is due to the fact that nucleotide sequences surrounding the canonical splice sites are weakly conserved and their mutations do not always or only partially disrupt the splicing processes. In contrast to the general belief that canonical splice-site mutations usually result in complete splicing errors, we have demonstrated that partial splicing errors can also be produced by canonical splice-site mutations such as c.1769-1G > C, which would

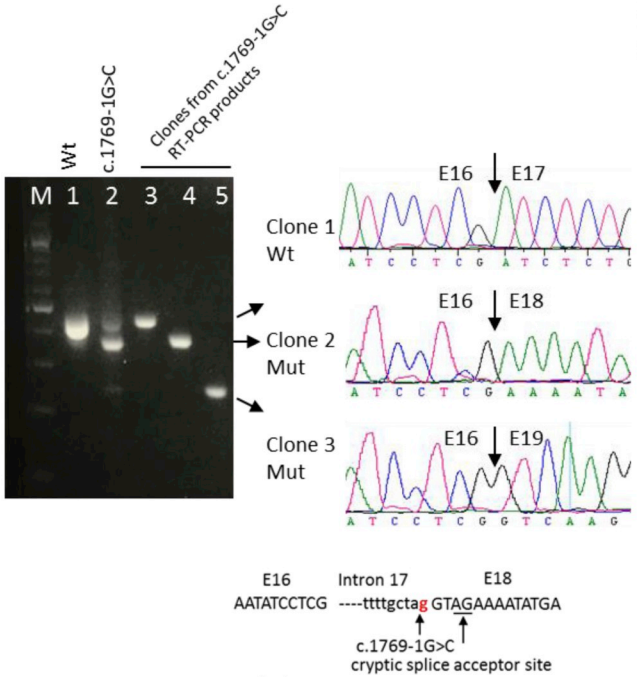

M
E3
E4
T T G A A G G A A C T T T T
Wt
E3
E5
T T G A A G G A A G C G
c.436+6 T>C
Skipping of exon 4

(A)


cDNA
M
1
2
1: c.1586+1G>A
2: c.1586+6T>C


Clones
E14
E15
A C A A A C T C G T G G T T T A
Partial skipping of exon 14
c.1586+1G>A
E14
E15
ACAAACTCGCAAGTATTTAGCACAG------AACAGAgtgagtattaaa---------ctacagGTGGTTTA
Cryptic splice donor site
Usage of cryptic splice donor site due to c.1586+1G>C
E14
E15
ACAAACTCGTGGTTTA (cDNA)
E13
E15
C C T C A A A G C T G T G G T T T A C A A A
Skipping of exon 14
c.1586+1G>A
Clones
E13
E14
C C T C A A A G C T A T C A A G T T T T C
Wt
E13
E15
C C T C A A A G C T G T G G T T T A C A A
Skipping of exon 14
c.1586+6T>C

(B)


Wt
c.1769-1G>C
Clones from c.1769-1G>C RT-PCR products
M 1 2 3 4 5
Clone 1
Wt
E16
E17
A T C C T C G A T C T C T C
Clone 2
Mut
E16
E18
A T C C T C G A A A A T A
Clone 3
Mut
E16
E19
A T C C T C G G T C A A G
E16
Intron 17
E18
AATATCCTCG
----ttttgctag GTAGAAAATATGA
c.1769-1G>C
cryptic splice acceptor site

(C)

Fig. 3. Sequence analysis of *PHEX* minigene transcripts containing both Wt and mutant cDNA fragments. cDNA fragments from RT-PCR were cloned into a TA vector and at least 20 individual clones were sequenced to determine the ratio of mutant and wild-type clones. Agarose gels are presented showing Wt and mutant cDNA fragments from RT-PCR. The extra blank margins are chopped to save space. Sequence electropherograms are presented to show mutant and surrounding Wt sequence. Other regions of Wt sequence are chopped. (A) c.436 + 6T > C generates both Wt and mutant (exon 4 skipping) transcripts. M: 100bp DNA ladder. (B) c.1586 + 1G > A results in two mutant cDNA fragments (330 bp and 290 bp) whereas c.1586 + 6T > C produces both Wt (390 bp) and mutant (290 bp) cDNA fragments. The 330 bp fragment is derived from partial exon 14 skipping due to usage of a cryptic splice donor site GC (underlined). The 290 bp fragment results from exon 14 skipping. (C) c.1769-1G > C results in three cDNA fragments including Wt (clone 1, 460 bp) and two mutant fragments (clone 2, 360 bp, and clone 3, 230 bp). The 360 bp mutant fragment results from utilization of a cryptic splice acceptor site AG in exon 18, leading to exon 17 skipping and deletion of GTAG in exon 18. The cryptic splice acceptor site is underlined and indicated by an arrow in the schematic diagram showing the location of the mutation (indicated in red) and its surrounding DNA sequence. Upper case letter indicates exon and lower case letter for intron. The 230 bp cDNA fragment is derived from skipping of exon 17 and 18. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

result in incomplete inactivation of *PHEX* and retention of partial PHEX function.

Most disease-causing splice-site mutations are single nucleotide substitutions located either in the canonical splice donor sites GT (+1/+2) or acceptor sites AG (−1/−2) [22]. However, pathological splice-site mutations have also been found in other positions, which can be close or fairly distant from canonical GT-AG dinucleotides to cause aberrant splicing by disruption of interactions with U1 snRNP, U6 snRNP, and U2AF 35 or 65 in the splicing process [16,23,24]. For example, a point mutation in intron 32 (c.2701-25T > G) results in aberrant splicing of *COL5A1* gene (exon 33 skipping) [25]. This mutation lies 2 bp upstream of a highly conserved adenosine branch point, which is 20–50 bp upstream of acceptor site and is involved in lariat formation. The branch-site sequence is highly conserved in the Yeast (UACUAAC) and more degenerative in the human (yUnAy), where the underlined adenosine is the branch point and the lowercase pyrimidines (y = U or C) are not as well conserved as the uppercase U and A [26]. The c.1701-16T > A in the *PHEX* gene is close to the branch-point sequence and may disrupt lariat formation as well. A recent computational model has predicted substantial aberrant splicing due to intronic variants located > 30 nucleotides away from the splice site and found that known disease variants alter splicing nine times more often than common variants [27].

The c.1586 + 1G > A results in two aberrant splicing products: one with exon 14 skipping and the other with partial exon 14 skipping due to activation of a cryptic splice donor site GC in exon 14. Nearly all splice-site pairs (99.24%) are canonical GT-AG dinucleotides. The non-canonical splice-site pairs are rare, occurring 0.69–0.9% for GC-AG and 0.05–0.09% for AT-AC [9,28]. It has been shown that non-canonical splice-sites are frequently involved in alternative splicing and are prone to annotation errors [29]. The activation or utilization of a cryptic splice donor site GC in exon 14 was not predicted by the computer programs and only confirmed by RNA analysis, indicating the complexity of RNA splicing mechanisms.

Most online splice prediction programs offer tools to predict putative 5′ splice donor and 3′ splice acceptor sites (including cryptic splice-site) with probability scores or potential alteration of splicing such as Splice-Port (http://spliceport.cbcb.umd.edu/), ESEfinder (http://krainer01.cshl.edu/tools/ESE2/), BDGP Spice Site Prediction by Neural Network (http://www.fruitfly.org/seq_tools/splice.html), Human Splicing Finder Version 3.0 (http://www.umd.be/HSF3/), and Alternative Splice Site Predictor (http://wangcomputing.com/assp/). None of these programs offer direct prediction of functional consequences of splice-site mutations such as exon skipping, exon deletion, or intron retention. End-users have to make their own prediction based on the probability scores. Some splice-site mutations can result in both exon skipping and exon deletion (due to activation of cryptic splice-site in the exon) such as c.437-3C > G and c.1586 + 1G > A. In this situation, it would be much more difficult to predict the functional consequences. CRYP-SKIP (http://cryp-skip.img.cas.cz/) is the only available online program to predict directly the probability of exon-skipping or activation of cryptic splice sites. The program has higher successful rate in prediction of exon skipping than activation of cryptic splice sites since none of the activated cryptic splice-sites described in our study is predicted by the program. The accuracy of prediction may be improved if there is a large database correlating splice-site mutations and functional consequences.

The genotype-phenotype correlation of the *PHEX* gene mutation in X-linked hypophosphatemic rickets has not been well established. No correlation is found between the severity of disease and the type or location of the mutation [30]. However, among patients with a family history of hypophosphatemic rickets, there is a trend toward more severe skeletal disease in patients with truncating mutations [30] or with a mutation in the C-terminal half of the *PHEX* gene [31]. Lower tubular reabsorption of phosphate and 1,25(OH)2D levels have also been found in patients with truncating *PHEX* mutations, indicating that *PHEX* mutation type may predict phenotype severity [32]. Among 22 splice-site mutations studied in the current study, 20 are predicted to result in truncated proteins from exon-skipping or intron retention and 16 of them are associated with moderate or severe phenotype (Table 2). The phenotype severity is unknown in the remaining 4 mutations. There are two splice-site mutations (c.436 + 6T > C and c.1586 + 6T > C) that could not completely destroy the splice-site and 40% wild-type transcripts could still be detected. Makras et al. described a family with 3 cases (mother and her two sons) who carry the c.436 + 6T > C [16]. All of them had very mild clinical presentations: the mother had hypophosphatemia and normal height (Z score, −0.6) without receiving any treatment; her two sons also achieved normal heights despite low serum phosphate levels and late initiation of treatment. The presence of 40% wild-type transcripts may explain the mild phenotype of the patients. Regarding to the c.1586 + 6T > C, we are unable to confirm whether it would result in mild phenotype due to lack of clinical information in the original publication [17]. Our current study has provided some evidence of genotype-phenotype correlation. More studies are needed.

In summary, we have performed functional analysis of 22 previously reported *PHEX* splice-site mutations by a customized minigene assay, which offers a simple and reliable method to analyze the outcome of splice-site mutations. Exon skipping is the most common outcome from *PHEX* splice-site mutations. Both canonical and non-canonical splice-site mutations can result in either severe or mild RNA splicing defects. Non-canonical splice-site mutations should not be overlooked especially for those located within 50 bp from canonical splice site.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2019.05.017.

Authors' contributions

HB, MZ, AA, BA, MA, and RA contributed to the genetic analysis. YS, MZ and BM designed the experiments and drafted the manuscript. All of the authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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