

## Predicting the Impact of *PHEX*, *FGF23* and *DMP1* Gene Variants Found in Malaysian Malay Patients with Hypophosphataemic Rickets Through *In Silico* Analysis of Protein Function and mRNA Secondary Structure

Nurul Nadirah Razali<sup>1</sup>, Tzer Hwu Ting<sup>2</sup>, King Hwa Ling<sup>1,3</sup>, Marina L. Kennerson<sup>4,5,6</sup>, Karuppiah Thilakavathy<sup>1,3\*</sup>

<sup>1</sup>Department of Biomedical Science, Faculty of Medicine & Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

<sup>2</sup>Department of Paediatrics, Faculty of Medicine & Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

<sup>3</sup>Genetics and Regenerative Medicine Research Center, Faculty of Medicine & Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

<sup>4</sup>Northcott Neuroscience Laboratory, ANZAC Research Institute, University of Sydney Concord NSW 2139, Australia.

<sup>5</sup>Sydney Medical School, University of Sydney, Sydney, Australia.

<sup>6</sup>Molecular Medicine Laboratory, Concord Hospital, Concord, Australia.

\*Corresponding author:

Associate Professor Dr. Thilakavathy Karuppiah,  
Department of Biomedical Sciences,  
Faculty of Medicine and Health Sciences,  
Universiti Putra Malaysia,  
43400 UPM Serdang,  
Selangor,  
Malaysia.

Email: [thilathy@upm.edu.my](mailto:thilathy@upm.edu.my)

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### ABSTRACT

Hypophosphataemic Rickets (HR) is a rare bone disorder characterised by chronic hypophosphataemia caused by defective phosphate reabsorption in the renal tubules. Variants in phosphate-regulating endopeptidase homolog, X-linked (*PHEX*), fibroblast growth factor-23 (*FGF23*) and dentin matrix protein-1 (*DMP1*) genes contribute to X-linked dominant, autosomal dominant and autosomal recessive forms of HR, respectively. In this study, four Malaysian patients' DNA samples were subjected to polymerase chain reaction and Sanger sequencing to identify the types and locations of the variants. Then, *in silico* study was conducted based on the variants found to predict the effects of amino acid substitution on protein functions using SIFT and PolyPhen-2 software and RNAfold was used to construct the mRNA secondary structure. Mutational analyses had revealed two variants in *PHEX*; c.10G>C (E4Q), c.1970A>G (Y657C), one mutation in *FGF23*; c.716C>T (T239M) and three variants on *DMP1*; c.309A>T (S69C), c.1322C>T (S406S), c.1334G>A (E410E). The variants in these Malay patients were previously reported in different ethnic HR patients. Protein prediction programs suggested that the *PHEX* Y657C and *DMP1* S69C variants may affect protein function. All variants were predicted to alter the secondary mRNA structure. These findings suggest that these missense and silent variants may lead to changes in protein function and mRNA secondary structure that are associated with the manifestation of HR phenotype.

### INTRODUCTION

Hypophosphataemic rickets (HR) is a subtype of rickets due to hypophosphataemia or low serum phosphate level. The low serum phosphate in the body is related to the low reabsorption and high

excretion of phosphate by renal tubules [1, 2]. Parathyroid hormone (PTH), fibroblast growth factor 23 (*FGF23*) and 1,25 dihydroxyvitamin D [1, 25(OH)<sub>2</sub>D] are the major regulators for phosphate homeostasis in the human body [3, 4].

Familial HR is a rare disease with a low rate of occurrence; 1 in 20,000 live births worldwide [5]. Numerous studies have been carried out on HR in North American, European, African American, Saudi Arabian and subcontinent Indian, however, in Malaysia, previously only one study has been reported on the pattern of inheritance of a family with two HR individuals [6].

Individuals with familial HR tend to have growth retardation, short stature, lower limb deformities, dental abscesses, early tooth loss, and bone pain [5,7,8]. X-linked hypophosphataemia (XLH, MIM307800), autosomal dominant HR (ADHR, MIM193100) and autosomal recessive HR 1 (ARHR, MIM241520) are the hereditary forms of HR that are mainly caused by mutations in *PHEX*, *FGF23* and *DMP1*, respectively. *PHEX* accounts for the most in HR disorder, approximately 80% in all reported cases of familial HR, followed by *FGF23* and *DMP1* [9]. To date, there are more than 300 mutations reported on *PHEX* [10].

The phosphate regulating endopeptidase homolog, X-linked (*PHEX*), fibroblast growth factor 23 (*FGF23*) and dentin matrix protein-1 (*DMP1*) genes are known to be mutated in familial HR. *PHEX* helps in bone formation as it encodes a protein expressed in osteoblasts, osteocytes, and odontoblasts [11, 12]. *DMP1* shares the same functions as *PHEX*, where it encodes for a protein that is highly expressed in the osteoblasts and osteocytes [1]. It is responsible for bone and dentin mineralisation [13] and plays a role in downregulating *FGF23* [2]. Whilst *FGF23* has a role in bone formation and mineralisation [14], the overexpression of *FGF23* causes down regulation of membrane expression of sodium-phosphate co-transporter in the kidney and suppresses 1,25 (OH)<sub>2</sub>D production that subsequently interferes the process of phosphate reabsorption in the gut [3]. Gain or loss of function mutations in these genes can lead to a decrease in blood phosphate levels via elevation of *FGF23*, thereby contributing to the hypophosphataemia [15]. *FGF23* levels will increase due to loss of function mutations in *PHEX* [16] and *DMP1* [2] and gain of function mutations in *FGF23* [14].

It is interesting to note that all the patients diagnosed with HR in several hospitals in Malaysia were of Malay ethnicity. Finding the gene mutations could give a better understanding of the disease pathogenesis in this population. We have screened the *PHEX*, *FGF23* and *DMP1* genes in HR patients of Malay ethnicity and identified six known variants. *In silico* analyses predicted the effect of the particular *PHEX*, *DMP1* and *FGF23* variants on protein functions and mRNA secondary structures.

## METHODS

### Patients

Children and adolescents aged less than 18 years diagnosed with hypophosphataemic rickets were recruited for this study. Records of clinical features and laboratory parameters of each patient since the diagnosis of HR were obtained from the clinic medical records. Criteria for inclusion in the study were manifestation of HR clinical features (short stature and bone deformities), reduced serum phosphate levels, raised alkaline phosphatase, normal serum calcium levels and reduced maximum rate of renal tubular reabsorption of phosphate normalised to the glomerular filtration rate (TmP/GFR) [17]. Patients with raised serum creatinine and urinary calcium levels, urine glycosuria and metabolic acidosis on venous blood gas were excluded to eliminate the possibility of renal failure, Fanconi syndrome or other renal tubulopathies.

Informed consent was obtained from the parents or guardians of the patients prior to sample collection in accordance with the approved Ethics Committee guidelines; Universiti Putra Malaysia..Ethics..Committee (UPM/TNCPI/RMC/1.4.18.1(JKE UPM)/F2) and Ministry of Health Medical Research and Ethics Committee (NMRR-13-609-16031).

### DNA analysis

Genomic DNA was extracted from the buffy coat of patient blood samples using the QIAamp® DNA Blood Mini Kit (Qiagen, USA) according to the protocol recommended by the manufacturer. Exons and exon-intron boundaries were amplified with sets of previously described primer pairs specific to *PHEX*, *FGF23* and *DMP1* [18, 19].

PCR reactions were performed in 20 µL reactions, containing 1X Colorless GoTaq® Reaction Buffer, 0.8-1.5 mM MgCl<sub>2</sub> solution, 0.2 mM dNTP Mix, 2.5 U GoTaq® DNA Polymerase (Promega, USA), nuclease free water (Vivantis, Inc. USA), 0.3 µM primers and 100ng of template DNA. Thermal cycling was initiated with 10 min at 95°C, followed by 40 cycles of 95°C (45 s), annealing temperature (45 s) and extension at 72°C (45 s), with a final extension step at 72°C for 10 min using a PCR Mastercycler Gradient machine (Eppendorf, Germany). PCR amplicons were size fractionated on 2% (w/v) agarose gels in 1X TAE buffer. PCR product purification was performed using the MEGAquick-spin™ Total – Fragment DNA purification kit (Intron Biotechnology, Inc., Korea). The purified PCR products were sent for direct sequencing service (First Base Laboratories, Singapore). Sequencing traces were viewed and analysed using DNA Baser software (v4.16.0).

### *In silico* analysis

All missense variants were analysed by using *in silico* study; SIFT (<http://sift.jcvi.org/>) and PolyPhen (<http://genetics.bwh.harvard.edu/pph2/>) for prediction of the effects of amino acids substitution on the protein functions. RNAfold (<http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>) software was used to predict the secondary structure of mRNA. The predicted function by SIFT and PolyPhen-2 were interpreted following the parameters of the program. The range of SIFT and PolyPhen-2 scores is from 0 to 1, to categorize tolerated or deleterious. For SIFT, score < 0.05 is considered as deleterious and score > 0.05 is tolerated. PolyPhen-2 scores benign from 0 to 0.5, possibly damaging from > 0.5 to 0.9 and probably damaging from > 0.9 to 1.0 [20, 21].

## RESULTS

### Clinical findings on hypophosphataemic rickets patients

Four children (Patients 1 to 4) diagnosed with hypophosphataemic rickets were recruited for this study. The clinical features and laboratory data of these patients were shown in Table 1. All were Malay female patients with early onset of disease with noticeable bowing of legs at age 1-3 years. All had growth retardation manifesting as short stature. The serum phosphate levels of all four patients and TmP/GFR reading from three patients were low, and serum alkaline phosphatase levels were raised. All had normal vitamin D status indicating the absence of vitamin D deficiency as a contributing cause of rickets in these patients. None had a positive family history of hypophosphataemic rickets or skeletal deformity. Radiological findings were consistent with rickets in all study patients.

**Table 1.** Clinical data at diagnosis of hypophosphataemic rickets patients.

	Patient 1	Patient 2	Patient 3	Patient 4
Age at study	9y3m	14y9m	7y10m	4y5m
Age at diagnosis	6y6m	6y1m	3y	3y3m
Gender	Female	Female	Female	Female
Ethnicity	Malay	Malay	Malay	Malay
Clinical features at diagnosis	Short stature, bowing of legs, frontal bossing	Short stature, bowing of legs, swelling of wrists, rachitic rosary	Short stature, mild bowing of legs, swelling of wrists, frontal bossing	Short stature, bowing of legs, swelling of wrists and ankles
Age of onset of bowing of legs	1-2y	2-3y	2y	18m
Family history of skeletal deformity	No	No	No	No
<b>Laboratory parameters at diagnosis</b>				
Serum phosphate (normal 1.29-1.94 mmol/L for 2-18yo)	0.78	0.82	0.63	0.64
Alkaline phosphatase (normal 58-400 U/L for 2-18yo)	436	644	620	726
Serum calcium (normal 2.1-2.55 mmol/L)	2.16	2.35	2.22	2.34
Parathyroid hormone (normal 1.6-6.9 pmol/L)	Not available	7.5	4.9	3.3
Serum creatinine (normal 20-72 µmol/L)	39	28	32	24
25-hydroxyvitamin D (normal >50 nmol/L)	56.5	51.6	55.0	90.9
TmP/GFR (normal 1.15-2.44 mmol/L for 2-15yo)	Not available	0.68	0.49	0.51
Urine calcium/creatinine (normal <0.7)	Not available	0.15	0.22	0.5
Radiological findings at diagnosis	Osteopenia, splaying and fraying of femoral and tibial metaphyses	Splaying and fraying of femoral and tibial metaphyses	Mild splaying and fraying of radial, ulnar and tibial metaphyses	Cupping, splaying and fraying of tibial metaphyses

Note: TmP/GFR - maximum rate of renal tubular reabsorption of phosphate normalised to the glomerular filtration rate

**Variants screening on PHEX, FGF23 and DMP1 genes in hypophosphataemic rickets patients**

Based on the DNA sequencing screening, total six variants have been found in this study (Table 2). Two variants were identified in Patient 1; c.716C>T in *FGF23* exon 3 and c.309A>T in *DMP1* exon 6. Meanwhile, Patient 2 and Patient 3 have three variants in all three studied genes; P02 has variants of c. 10G>C in *PHEX* exon 1, c.716C>T in *FGF23* exon 3 and c.309A>T in *DMP1* exon 6, whereas P03 has variants of c.1970A>G in *PHEX* exon 20, c.716C>T in *FGF23* exon 3 and c.309A>T in *DMP1* exon 6. As for P04, variants were only found in exon 6 of *DMP1*; c.1322C>T and c.1334G>A.

**Table 2.** Description of variants on PHEX, FGF23 and DMP1 discovered in four Malaysian hypophosphataemic rickets patients.

Gene	Patient 1		Patient 2		Patient 3		Patient 4			
	FGF23	DMP1	PHEX	FGF23	DMP1	PHEX	FGF23	DMP1	DMP1	
Exon	3	6	1	3	6	20	3	6	6	
Location	c.716C>T	c.309A>T	c.10G>C	c.716C>T	c.309A>T	c.1970A>G	c.716C>T	c.309A>T	c.1322C>T	c.1334G>A
Amino acid change	T239M	S69C	E4Q	T239M	S69C	Y657C	T239M	S69C	S406S	E410E
Mutation type	m	m	m	m	m	m	m	m	s	s

Note: m missense, s silent

Exon 1 of *PHEX* is 118 bp in size, and at location of c.10, the heterozygous substitution of G to C (rs147859619) lead to amino acid changes, from glutamic acid to glutamine at codon 4 (E4Q). Whereas exon 20 of *PHEX* has 105 bp, and a substitution of A to G at location c.1970 causing the tyrosine to be replaced by cysteine at codon 657 (Y657C). Exon 3 of *FGF23* comprises of 441 bp and C>T at location c.716 (rs7955866) causes amino acid changes from threonine to methionine at codon 239 (T239M). Exon 6 is the longest exon of *DMP1* with 1360 bp from A>T at location c.309 (rs10019009) alters the amino acid from serine to cysteine at codon 69 (S69C). Meanwhile from C>T at c.1322 (rs2615498) and G>A at c.1334 (rs2615497) led to no changes of amino acid serine at codon 406 and glutamic acid at codon 410, respectively.

**In silico analysis on protein function based on amino acid substitutions by using SIFT and PolyPhen-2 software**

Analyses in predicting the impact of amino acid substitutions on protein function were conducted via prediction tools; SIFT and PolyPhen-2 (Table 3). SIFT studies the type of amino acid changes and its position by aligning the related protein sequence with the query. Ng & Henikoff [22] explained the multistep procedure of SIFT which begins with searching for similar sequences, and then the closely related sequences that share similar function will be selected and aligned. From the alignment, the normalised probabilities of all possible substitutions occurring at each position of the alignment will be calculated. From the calculation, if the probability is less than a chosen cutoff, it is predicted as deleterious, while it will become tolerated if the value is greater or equal to the cutoff. PolyPhen-2 operates by using eight sequence-based and three structure-based predictive features which have been chosen automatically by an iterative greedy algorithm [23]. Position-Specific Independent Counts (PSIC) score is then calculated based on the difference between wild type (ancestral, normal) and mutant (derived, disease causing) amino acid, hence classifying the results into benign, possibly damaging, probably damaging [24].

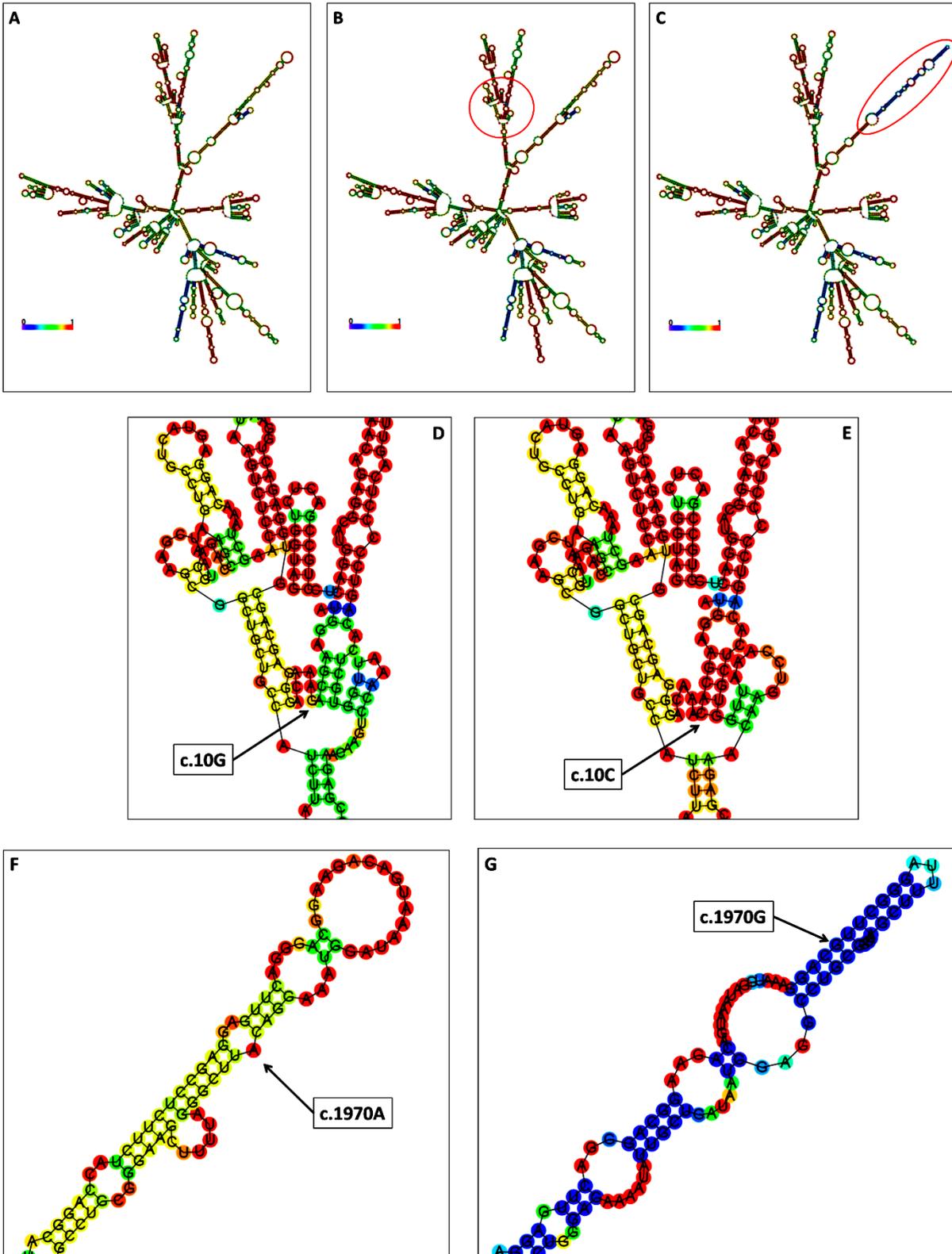
**Table 3.** Prediction of SIFT and PolyPhen-2 on the impact of missense nucleotide substitutions on protein function.

Gene	Amino acid changes	SIFT	SIFT score	PolyPhen-2	PolyPhen-2 score
<i>PHEX</i>	E4Q	Tolerated	0.29	Probably damaging	0.981
	Y657C	Damaging	0.01	Probably damaging	1.000
<i>FGF23</i>	T239M	Tolerated	0.29	Benign	0.001
<i>DMP1</i>	S69C	Damaging	0.02	Probably damaging	1.000

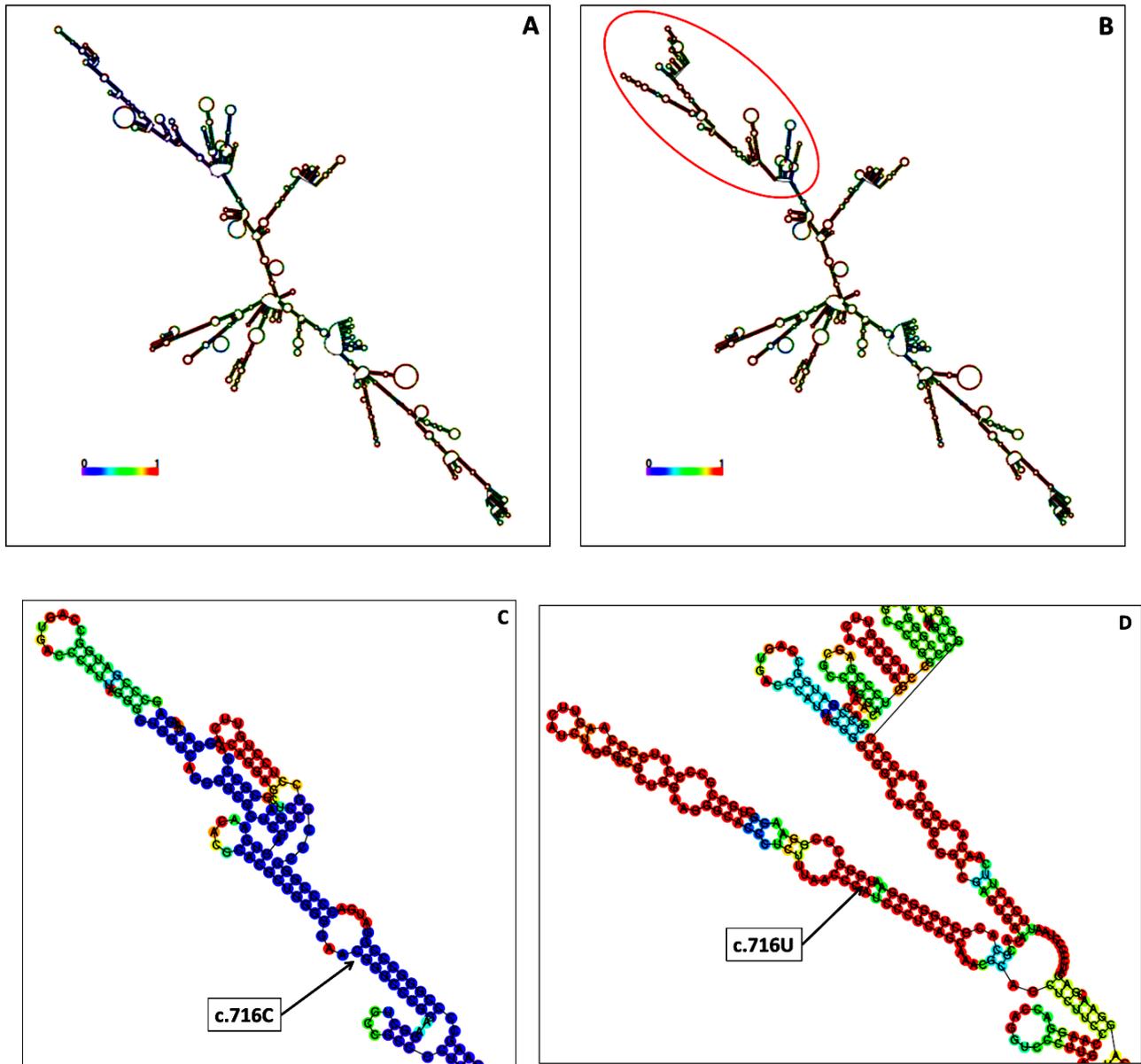
Based on the computational analysis, both software predicted with high confidence that the *PHEX* Y657C and *DMP1* S69C variants affect protein function or structure (SIFT score <0.05, PolyPhen-2 score 1.0), whilst *FGF23* T239M variants was predicted as not affecting the protein function (SIFT score >0.05, PolyPhen-2 score 0.001). *PHEX* E4Q has mixed prediction of tolerated by SIFT (score >0.05) and probably damaging by PolyPhen-2 (score 0.981).

**Determination of mRNA secondary structure by using RNAfold**

RNAfold works by entering single sequence of DNA or RNA in plain text or FASTA format, then it will be analysed using the dynamic programming algorithm [25]. The differences between wild type and mutant mRNA secondary structure (additional of hairpin or stem loop structure) can be compared once it is predicted by RNAfold. The RNA secondary structure predictions comparing the wild type and altered *PHEX* are shown (Figure 1A-C). A different minimum free energy (MFE) was observed for the wild type and variants which is a measure of the structure stability. The MFE values for *PHEX* wild type and variants c.10G>C and c.1970A>G are -617.60 kcal/mol, -620.50 kcal/mol and -619.20 kcal/mol, respectively.



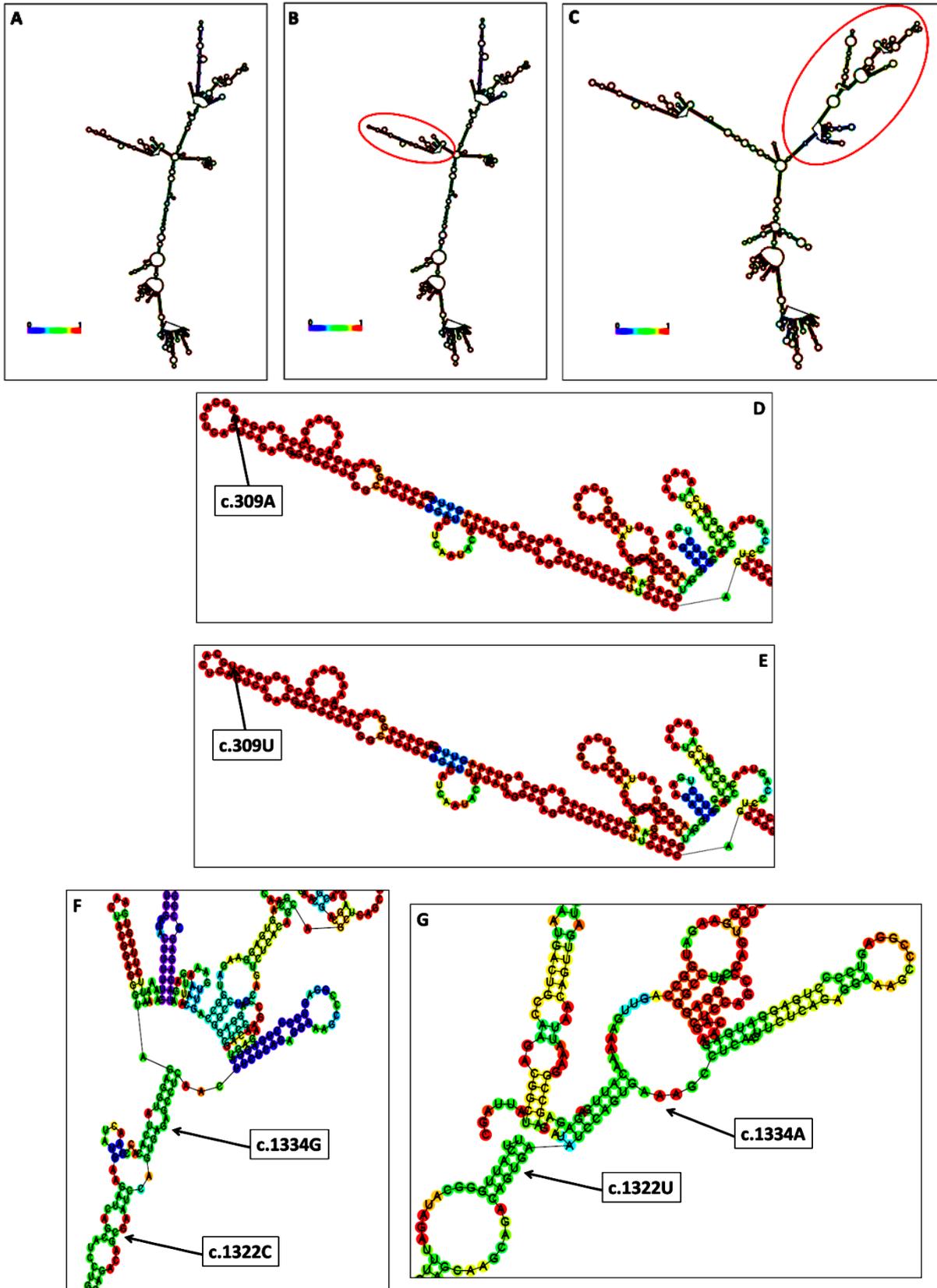
**Fig. 1.** Secondary structure of *PHEX* mRNA. The secondary mRNA structures of wild type (A) and the missense c.10G>C (B) and c.1970A>G (C). The red circle and oval circle show the region of the mRNA structure where the c.10G>C and the c.1970A>G variants localize respectively. The secondary structures differ between the wild type and gene variants. Enlargement of *PHEX* wild type (D) and variant (E) mRNA secondary showing the G to C substitution at nucleotide position c.10. The red arrow indicates the loop size near the substitution site is larger when compared to normal *PHEX*. Enlargement of wild type *PHEX* structure (red oval in Fig. 1C) showing the location of the wild type (F) and variant (G) at nucleotide position c.1970 (G).



**Fig. 2.** Secondary structure of *FGF23* mRNA. The secondary mRNA structure of wild type (A) and the mutation c.716C>T (U) (B) The whole branch appears entirely different with additional branching and presence of smaller loops due to the presence of the substitution. Enlargement of *FGF23* wild type (C) and variant (D) mRNA secondary showing the nucleotide substitution of C to T at position c.716.

The c.10G>C variant caused increase in the loop structure size compared to the wild type structure (Fig. 1D and E). Similarly, a change in structure was observed between wild type and the c.1970A>G variant. The substitution predicted the formation of different size of loop structures on different locations of the mRNA branch (Fig. 1F and 1G). Fig. 2 shows the mRNA secondary structure prediction of the wild type and *FGF23* variant. Obvious changes in the structure of *FGF23* were seen in Fig. 2B, where it had increased number of branches compared to the wild type structure (Fig. 2A). The MFE value for wild type *FGF23* was -750.21 kcal/mol which was higher than MFE for variant -750.74 kcal/mol. Fig. 2C and 2D show the location of the substitution of c.716C>T (U). c.716C was originally located near a loop structure, however, due to the nucleotide change, the mutated position was shifted onto a hairpin structure. Additional branches and loops existed in the variant mRNA structure too.

The mRNA secondary structure of wild type and variant *DMP1* are shown in Fig. 3A, 3B and 3C. At a glance, structure of variant c.309A>T (U) and wild type *DMP1* appear to be similar, nevertheless, the MFE values for both structures were different; wild type had higher value (-464.30 kcal/mol) than variant (-467.40 kcal/mol). Meanwhile mRNA secondary structure of variant c.1322C>T (U) and c.1334A>G variant exhibited obvious changes on the right-side branch and recorded a higher MFE (-463.90 kcal/mol) when compared with the wild type structure. The dissimilarity between wild type and variant c.309A>T (U) structures was not noticeable. However, a close-up on the loop near the end of the mutated branch showed that the size of the loop in the altered structure was smaller compared to that in the wild type (Fig. 3D and 3E).



**Fig. 3.** Secondary structure of *DMP1* mRNA. The secondary mRNA structure of wild type (A), missense altered c.309A>T (U) (B) and silent variants c.1322C>T (U) and c.1334A>G (C). The secondary structures differ between the wild type and gene variants. Enlargement of *DMP1* wild type (D) and mutant (E) mRNA secondary structure indicating substitution of adenine to uracil at position c.309. The branch is quite similar to the wild type except for the loop at the end of the branch, which is smaller. Expansion of wild type *DMP1* structure (F) and variants (G) with altered structure at position c.1322 and c.1334. The arrows mark the substitutions from cysteine to uracil and guanine to adenine, respectively. The dual variants altered the whole structure of the branch.

The presence of double variants c.1322C>T (U) and c.1334A>G in *DMP1* changed the secondary structure of the mRNA extensively (Fig. 3F and 3G). Separate analysis on the mRNA secondary structure for each variant of the dual variants was carried out and it showed that the *DMP1* structure was greatly impacted by the presence of one of the variants, c.1322C>T (U) (Fig. 4A), with MFE value of -463.90 kcal/mol.

showed the variant mRNA secondary structure with the presence of c.1334A>G. This variant did not seem to change the mRNA structure. However, its MFE value differ from the wild type; 463.20 kcal/mol.

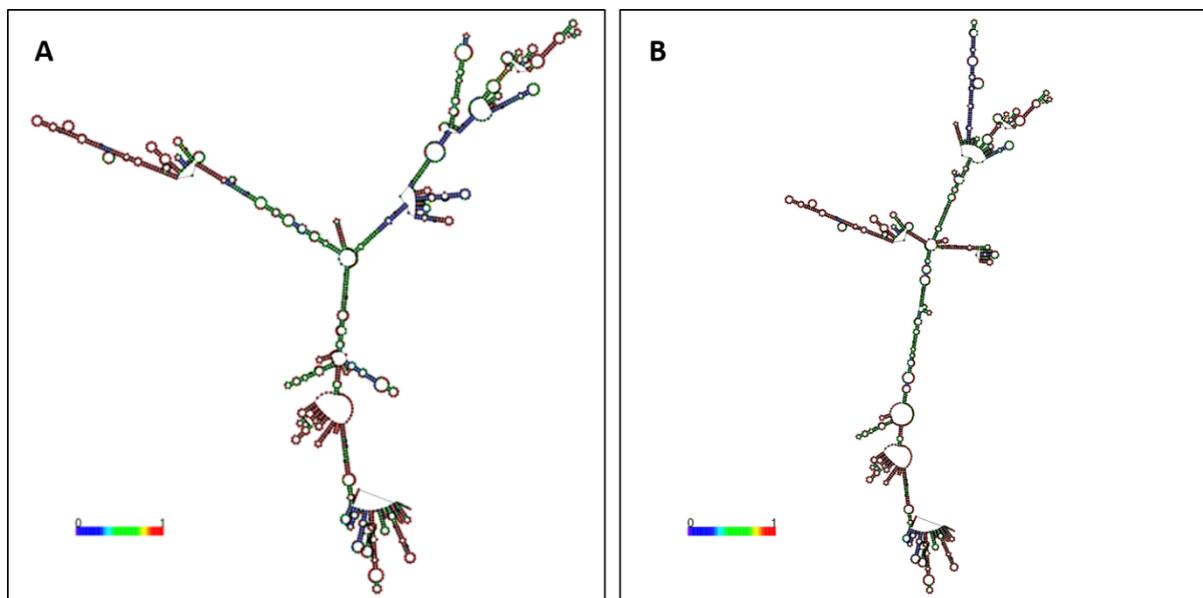


Fig. 4. Secondary structure of variant *DMP1* mRNA with silent substitution at position c.1322C>T (U) (A) and c.1334A>G (B).

## DISCUSSION

According to Li et al., [27], the *PHEX* mutation database shows different frequencies of mutation types; 25% frameshifts, 23% alternative splicing, 22% missense, 18% nonsense, 8% deletion and 4% polymorphisms. Although mutations in *PHEX* are the most common in HR cases, in this study, not all patients had a *PHEX* variant. Patient 1 has only variants on *FGF23* and *DMP1*, while Patient 4 carried *DMP1*. On the other hand, Patient 2 and Patient 3 recorded variants in all three studied genes. Nevertheless, HR is a single gene disorder [28], hence a single altered gene is enough to cause the disease. The severity of the disease is not associated with the location of variant and gender as no correlation has been found between these two factors and severity of HR in previous studies [29, 30].

In this study, the missense substitution found on *PHEX* (c.10G>C) has been reported in a previous study among Chinese patients [31]. This substitution causes E4Q amino acid change and was predicted to be non-pathogenic. However, the study by St-louis [32] revealed that E4Q was associated with bone mineralisation. This substitution is believed in obstructing the interaction of PHEX to COPII; which is responsible for training secretory vesicles. This interference subsequently affecting the incorporation of PHEX into calcification vesicles that would be important in mineralisation process. Thus, although E4Q was predicted with no change in protein function, the phenotype may result from transformed mRNA secondary structure and altered translation efficiency. The prediction tools indicated c.1970A>G substitution in *PHEX* leading to Y657C alteration as damaging. There were various additions of loops with different sizes and hairpin on the right-side branch that made the top structure of the variant appeared completely different from the wild type. Fig. 4B

This variant was also reported to occur sporadically in European HR patients [33]. It was speculated that this substitution occurred in the conserved region, consequently giving an impact on protein functions. SIFT and PolyPhen-2 prediction on this variant concur with the speculation.

*PHEX* is a zinc metalloendopeptidase and the protein has high specificity of binding with matrix extracellular glycoprotein (MEPE), a protein involved in biomineralisation. *PHEX* mutations may fail to interrupt the process of intermediate cleavage of MEPE that will generate free protease-resistant acidic serine aspartate rich MEPE associated (ASARM) peptides. ASARM peptide is a mineralisation inhibitor in bone and teeth, and causes mineralisation defects and inhibits renal and intestinal phosphate uptake [34]. Thus, interruption in phosphate regulation results in higher phosphate excretion and reach hypophosphataemia state.

Variant at c.716C>T in *FGF23* was also found in hypophosphataemic stone former due to renal phosphate leak [35]. Despite being predicted as benign and tolerable, previous *in vitro* studies by Rendina et al., [35] indicated that T239M modification influenced *FGF23* biological function. This was supported by Merlotti et al., [36] whose study showed that T239M enhanced *FGF23* protein secretion. Mutated *FGF23* causes prevention of proteolytic degradation and promotes *FGF23* production, increased *FGF23* results in renal phosphate loss leading to hypophosphataemia [14].

The three different variants in exon 6 of *DMP1* in our study were also reported by Ruppe et al., [8] in American HR patients. Missense substitution c.309A>T (S69C) was foretold by the prediction tools used in this study that it might be likely damaging the protein function. Turan et al., [37] in their study stated that

S69C was associated with bone mineralisation and concluded the importance of *DMP1* in the pathogenesis of autosomal recessive HR and in the normal regulation of phosphate homeostasis. *DMP1* is a member of small integrin-binding ligand N-linked glycoprotein (SIBLING). ASARM is one of the features that are highly conserved in SIBLING protein. Defective *DMP1* may enhance ASARM peptides in bone and teeth that lead to hypophosphataemia state [34].

RNAfold was used to predict the mRNA secondary structure and calculate the MFE values of wild type and altered *PHEX*, *FGF23* and *DMP1*. As stated by de Smit & Van Duin [38] mRNA secondary structure is a key factor in determining the translation efficiency. Thus, studying the mRNA secondary structure widens the scope of analyzing the impact of all types of substitution variants. Stability is an important factor in mRNA secondary structure, as stable complexes improve translation efficiency [39]. MFE is provided on all structures constructed by RNAfold.

MFE indicates the stability of a structure, where it is hypothesised that the lower the MFE, more stable is the secondary structure, thus enhancing protein translation [39]. In this study, the mRNA secondary structures of all variant genes with missense substitutions showed lower MFE than the wild type, indicating higher mRNA structural stability. Opposing the MFE hypothesis, study by Dvir et al., [40] found a significant association between stable structure (lower MFE) and less protein translation. Hence, they suggested that stable mRNA secondary structures would not necessarily yield more translation of protein, instead the stable secondary structures would have probability and tendency of impediment on the translation initiation, thus producing less protein.

In contrast, the two synonymous *DMP1* variants in exon 6 showed less stability as indicated by higher MFE and would hypothesise there is less protein translation. Komar [41] showed in studies of translation kinetics and protein folding that abnormal translation kinetics arose from a silent mutation. Abnormal translation kinetics describes the behaviour of ribosome speed that can be faster or slower through certain regions of mRNA, hence producing different protein conformation. Therefore, it is possible that defective protein translation could occur due to the presence of variants found in the three genes in this study regardless of their MFE value and mRNA structure stability.

Hairpin, loop and stem in mRNA secondary structures cause interference in protein translation [42]. Thus, comparing the mRNA secondary structure of wild type and mutant gene is necessary as small changes in the structure might give adverse effects. Paek et al., [43] reported that translational process is enhanced by the presence of loopings. Paulus et al., [44] explained the importance of the RNA stem-loop for mediating translation initiation and concluded that the presence of stem loop may enhance formation of a translation initiation domain. Any deletion or addition of a single loop may give impact on the translation and affects the protein yield eventually.

The mRNA secondary structures of the altered *PHEX*, *FGF23* and *DMP1* depicted different structures from the wild type, with the obvious addition of loopings and branchings. Although mRNA secondary structures of mutant *PHEX* c.10G>C and *DMP1* c.309A>T (U) did not seem to appear different from the wild type, the presence of different sizes of loops could be spotted near the variant sites. Apart from the presence of hairpin and loop, codon usage bias could be another promising factor in interfering the efficiency of protein translation [46, 47]. Codon usage is calculated based on the occurrence frequency of a synonymous codon in a

coding sequence, which is the actual region of DNA that is translated to form proteins. It has been proven by study conducted by Athey et al., [48], where they have shown that codon usage is affecting protein structure and function through interference of translation kinetics and co-translational protein folding.

Moreover, previous study by Huang et al., [49] analysed the relationship between the codon usage frequencies of open reading frame and their translation rate. Their findings confirmed the strong correlation of codon usage frequencies and translation efficiency, with additional identification of which codon usage may increase and decrease the translation efficiency. Higher frequency of AAC, TCT, ACC, TCC, GCC, GCT, CCA codons are believed to have tendency elevating the rate of protein synthesis, meanwhile translation could be reduced with the frequent presence of ATA, CGA, TGC, GTA, GGA, CTT, AGG, CGG, TAT codons [49]. Recent study on manipulation of codon usage by Frumkin et al., [50] had shown quite similar finding, where genes with higher occurrence of CGG codon has demonstrated reduce translation efficiency. In contrast, there was elevation of translation efficiency in the genes that are supplemented with CGU, CGC, and CGA codons. Nevertheless, in this study we did not assess the correlation of the variants with codon usage bias manipulation.

These discoveries perhaps support the reasons how protein translation was affected by the presence of synonymous and nonsynonymous variants, despite of none or slight mRNA structural changes, as shown by *PHEX* c.10G>C and *DMP1* c.1334A>G variants.

In this study, the existence of dual silent variants on *DMP1* especially by the presence of c.1322C>T (U), has changed the mRNA secondary structure greatly at the upper part; 1151-1647 bp. In fact, these are the only variants that give less structure stability due to high MFE values. Therefore, this result support the report by Supek et al., [45] that synonymous variants may no longer be silent and could impact the protein function through oncogenic potential or by altering transcript splicing.

## CONCLUSION

This study provides new knowledge for the genetics of HR in an unstudied Malaysian population. This information provides genetic knowledge of ethnicity in relation to patients described from other countries. For further validation on HR variants, further functional studies are required to understand the impact of the variants on protein function.

## ABBREVIATION

HR	Hypophosphataemic Rickets
PHEX	Phosphate-regulating endopeptidase homolog, X-linked
FGF23	Fibroblast growth factor-23
DMP1	Dentin matrix protein-1
PTH	Parathyroid hormone
1,25(OH) <sub>2</sub> D	1,25 dihydroxyvitamin D
TmP/GFR	Maximum rate of renal tubular reabsorption of phosphate normalised to the glomerular filtration rate
MFE	Minimum free energy
COPII	Coatome, a type of vesicle coat protein
MEPE	matrix extracellular glycoprotein
ASARM	acidic serine aspartate rich MEPE associated
SIBLING	small integrin-binding ligand N-linked glycoprotein

## DECLARATION OF INTEREST

The authors declare that there is no conflict of interest.

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## AUTHOR CONTRIBUTIONS

All authors have made a significant contribution to the research described in this manuscript. NNR: Study concept and design, acquisition of data, analysis and interpretation of data, and manuscript writing. KT: Study concept and design, interpretation of data, and manuscript writing. THT: Acquisition of clinical data and manuscript writing. KHL: Interpretation of *in silico* data and revision of the manuscript. MLK: Revision of the manuscript. All authors approved the final manuscript as well as the authorship list.

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