UNIVERSITI PUTRA MALAYSIA

GENETIC ANALYSIS OF HYPOPHOSPHATEMIC RICKETS IN MALAYSIAN PATIENTS THROUGH WHOLE EXOME SEQUENCING

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By

TAVANA NAHID

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

January 2021

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DEDICATION

This work is dedicated to

My lovely mother and father

My wonderful husband, Saeed

My precious brothers,

for their love, devotion, endless support and encouragement

And lastly, in the hope that this work may in some way be helpful for them, this is dedicated to children with hypophosphatemic rickets disease and their parents.

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

GENETIC ANALYSIS OF HYPOPHOSPHATEMIC RICKETS IN MALAYSIAN PATIENTS THROUGH WHOLE EXOME SEQUENCING

By

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January 2021

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Hypophosphatemic rickets (HR) is a rare subtype of rickets due to genetic defects in phosphate regulators. Low level of phosphate in the blood is due to low reabsorption and high excretion of phosphate by the kidneys. HR often manifests in childhood with skeletal deformities of rickets including bowing of legs, short stature and dental abnormalities. Several genes have been identified to cause HR. The most common gene is the phosphate-regulating endopeptidase homolog, X-linked (*PHEX*) in which mutations in this gene cause X-linked dominant hypophosphatemic rickets (XLHR). Other less common genes include fibroblast growth factor-23 (*FGF23*), dentin matrix protein-1 (*DMP1*), ectonucleotide pyrophosphatase/phosphodiesterase 1 (*ENPP1*), chloride channel 5 (*CLCN5*), and solute carrier family 34 member 3 (sodium/phosphate cotransporter) (*SLC34A3*). HR is rare in Malaysia and a diagnosis is usually based on both clinical and biochemical results. In 2016, a study was conducted to look for mutations in the *PHEX*, *DMP1* and *FGF23* genes in four Malaysian children with HR. However, the results could not be concluded from the previous study because a causative mutation was not found in some patients. Therefore, alternative method was needed to identify the causative mutations in the patients. In the present project, a trio study was conducted with the aim to find the gene mutations responsible for HR in Malaysian patients using whole exome sequencing (WES). The candidate variants found from WES were validate by Sanger sequencing. Paternity investigation was performed by segregation of single nucleotide polymorphisms (SNPs) extracted from the WES data. High resolution melting curve profiles were established in healthy controls to identify the presence of the mutations based on the comparison of the patient melt profiles. Quantitative real time PCR was performed to compare the expression of the *FGF23* between patients and controls. Plasma levels of FGF23 were measured using an ELISA assay. In this study,

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clinical data showed that patients had lower limb bowing, osteopenia, splaying and fraying of the metaphyses of the femur and distal tibia, and radius and ulna, which were consistent with hypophosphatemic rickets phenotypes. All patient's parents did not show any phenotypic features of HR. Molecular genetic analysis revealed 37 variants in the six candidate HR genes. Since the parents of the patients were healthy, the variants were filtered based on two strategies; "*de novo* strategy" and "double-hit strategy". After filtering, four candidate variants remained in which three were *de novo PHEX* gene mutations and one homozygous mutation in the *DMP1* gene. Two of the variants found in *PHEX* have previously been reported as HR disease-causing mutation; Patient 1 (c.871C>T) had a stop-gain mutation (p.R291*) predicted to be damaging in all prediction software tools. Patient 2, had a *PHEX* (c.1970A>G) missense mutation, which led to the replacement of Tyr657 with cysteine. This mutation was also predicted to be damaging. The two remaining variants were novel and identified in the *PHEX* and *DMP1* genes. The novel *PHEX* variant in patient 3 was an in-frame deletion (c.1946_1954delGCCTGCGGG, p.G649-651Rdel). Another novel variant was identified in patient 4, which was a homozygous splice donor variant of *DMP1* (c.54+1G>A) and was inherited from the carrier parents. The presence of all four variants were confirmed by Sanger sequencing. The paternity analysis for mendelian transmission of SNPs confirmed the biological relationship between the probands and their parents. Therefore, the *PHEX* mutations were confirmed to be *do novo*. High resolution melting analysis revealed that the mutations found in the patients were absent in fifty DNA samples from healthy controls. From the *FGF23* gene expression data, the fold change between the patients and controls demonstrated that overexpression of *FGF23* was observed only in one patient and the expression difference between controls and patients was not significant (t (5)=0.39, p=0.71). This is likely due to bone tissue samples being unavailable and white blood cells not being the appropriate tissue for the qPCR experiment. Plasma FGF23 concentrations were higher than 30pg/mL in three of the patients, however, the differences between controls and patients were not significant (Mann-Whitney test, p=0.57). In conclusion, genetic studies for HR in Malaysia showed that sporadic XLHR cases occur frequently in these study cases, and that the *PHEX* gene is likely the most common cause of HR in Malaysia.

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Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

ANALISIS GENETIK MELALUI PENJUJUKAN KESELURUHAN EKSOM BAGI PESAKIT RIKET HIPOFOSFATEMIK DI MALAYSIA

Oleh

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Riket hipofosfatemik (HR) adalah subjenis riket yang jarang berlaku kerana kecacatan genetik pada pengatur fosfat. Tahap rendah fosfat dalam darah adalah disebabkan oleh penyerapan semula yang rendah dan perkumuhan fosfat yang tinggi oleh buah pinggang. HR sering muncul pada kanak-kanak yang mengalami kecacatan tulang riket termasuk kaki yang bengkok, perawakan pendek dan ketidaknormalan kepada gigi. Beberapa gen telah dikenalpasti dalam menyebabkan HR. Gen yang paling biasa terlibat adalah *phosphate-regulating endopeptidase homolog, X-linked (PHEX)* di mana mutasi pada gen ini menyebabkan *X-linked dominant hypophosphatemic rickets (XLHR)*. Gen lain termasuklah *fibroblast growth factor-23 (FGF23)*, *dentin matrix protein-1 (DMP1)*, *ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1), chloride channel 5 (CLCN5)*, dan *solute carrier family 34 member 3 (sodium/phosphate cotransporter) (SLC34A3).* HR jarang berlaku di Malaysia dan diagnosis biasanya dilakukan berdasarkan hasil klinikal dan biokimia. Pada tahun 2016, satu kajian dilakukan untuk mencari mutasi pada gen *PHEX*, *DMP1* dan *FGF23* pada empat kanak-kanak Malaysia yang mempunyai HR. Namun, hasilnya tidak dapat disimpulkan dari kajian sebelumnya kerana penyebab mutasi tidak dijumpai pada beberapa pesakit. Oleh itu, kaedah alternatif diperlukan untuk mengenal pasti penyebab mutasi pada pesakit. Dalam projek ini, satu kajian trio dilakukan dengan tujuan untuk mencari mutasi gen yang bertanggungjawab terhadap HR pada pesakit Malaysia menggunakan whole exome sequencing (WES). Varian calon yang dijumpai dari WES disahkan oleh penjujukan Sanger. Penyelidikan terhadap bapa dilakukan dengan pemisahan *single nucleotide polymorphisms (SNP)* yang diekstrak dari data WES. *High resolution melting curve profiles* dibuat bagi kumpulan yang sihat untuk mengenal pasti adanya mutasi berdasarkan perbandingan profil lebur pesakit. *Quantitative real time PCR*

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dilakukan untuk membandingkan ekspresi *FGF23* antara pesakit dan kumpulan kawalan. Tahap plasma FGF23 diukur menggunakan ujian ELISA. Dalam kajian ini, data klinikal menunjukkan pesakit mempunyai kaki yang bengkok, osteopenia, metafisis femur dan distal tibia serta radius dan ulna yang terpisah, dan ini selaras dengan fenotip hipofosfatemik riket. Semua ibu bapa pesakit tidak menunjukkan ciri fenotip HR. Analisis genetik molekul mendedahkan 37 varian pada enam gen HR calon. Oleh kerana ibu bapa pesakit adalah sihat, varian disaring berdasarkan dua strategi iaitu; "*de novo* strategy" dan "double-hit strategy". Setelah menyaring, empat varian calon tetap ada di mana tiga adalah mutasi gen *PHEX de novo* dan satu mutasi homozigot dalam gen *DMP1*. Dua varian yang terdapat dalam *PHEX* sebelum ini dilaporkan sebagai mutasi penyebab penyakit HR; Pesakit 1 (c.871C>T) mengalami mutasi stop-gain (p.R291*) yang diramalkan akan merosakkan di semua alat perisian ramalan. Pesakit 2, mempunyai mutasi missense *PHEX* (c.1970A>G), yang menyebabkan penggantian Tyr657 dengan cytosine. Mutasi ini juga diramalkan akan merosakkan. Dua varian yang berbaki adalah novel dan dikenal pasti dalam gen *PHEX* dan *DMP1*. Varian *PHEX* novel pada pesakit 3 adalah penghapusan inframerah (c.1946_1954delGCCTGCGGG, p.G649-651Rdel). Varian novel lain dikenal pasti pada pesakit 4, yang merupakan varian penderma sambatan homozigot *DMP1* (c.54+1G>A) dan diwarisi dari ibu bapa pembawa. Kehadiran keempat-empat varian tersebut disahkan oleh penjujukan Sanger. Analisis terhadap bapa untuk penghantaran SNP mendelian mengesahkan hubungan biologi antara proband dan ibu bapa mereka. Oleh itu, mutasi *PHEX* disahkan sebagai *de novo*. Analisis *high resolution melting* menunjukkan bahawa mutasi yang terdapat pada pesakit tidak ada dalam lima puluh sampel DNA dari kawalan yang sihat. Dari data ekspresi gen *FGF23*, perubahan lipatan antara pesakit dan kawalan menunjukkan bahawa ekspresi berlebihan *FGF23* hanya diperhatikan pada satu pesakit dan perbezaan ekspresi antara kawalan dan pesakit tidak signifikan (t (5)=0.39, p=0.71). Ini mungkin kerana tiada sampel tisu tulang dan sel darah putih adalah tidak sesuai untuk eksperimen qPCR. Kepekatan plasma FGF23 lebih tinggi daripada 30pg/mL pada tiga pesakit, namun, perbezaan antara kawalan dan pesakit tidak signifikan (Mann-Whitney test, p=0.57). Kesimpulannya, kajian genetik untuk HR di Malaysia menunjukkan bahawa kes XLHR sporadis sering berlaku dalam kes kajian ini, dan gen *PHEX* mungkin merupakan penyebab HR yang paling biasa di Malaysia.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

HR	Hypophosphatemic rickets
PTH	Parathyroid hormone
1,25 $(OH)_2D$	1,25-dihydroxyvitamin D
FGF23	Fibroblast growth factor 23 protein
PHEX	Phosphate regulating endopeptidase homolog, X-linked gene
FGF23	Fibroblast growth factor 23 gene
DMP1	Dentin matrix protein-1 gene
ENPP1	Ectonucleotide pyrophosphatase/phosphodiesterase 1 gene
CLCN5	Chloride channel 5 gene
SLC34A3	Solute carrier family 34 member 3 gene
SLC34A1	Solute carrier family 34 member 1 gene
CYP27B1	Cytochrome P450 family 27 subfamily B member 1 gene
XLHR	X-linked dominant hypophosphatemic rickets
LOF	Loss of function
PHEX	Phosphate regulating endopeptidase homolog, X-linked protein
ALP	Alkaline phosphatase
ADHR	Autosomal dominant hypophosphatemic rickets
ARHR	Autosomal recessive hypophosphatemic rickets
DMP1	Dentin matrix protein-1 protein
FAM20C	Family with sequence similarity 20, member c gene
Fam20c	Family with sequence similarity 20, member c gene in mice
KL	Klotho gene
HHRH	Hereditary hypophosphatemic rickets with hypercalciuria
DNA	Deoxyribonucleic acid
mRNA	Messenger ribonucleic acid
NGS	Next generation sequencing
SNP	Single nucleotide polymorphism
HRM	High resolution melting
NCBI	National Center for Biotechnology Information
dbSNP	Single nucleotide polymorphism database
HGMD	Human gene mutation database
gnomAD	Genome aggregation database
ACMG	American college of medical genetics and genomics
qPCR	Quantitative real time polymerase chain reaction
cDNA	Complementary DNA
ELISA	Enzyme-linked immunosorbent assay
mmol/L	Millimoles per liter
U/L	Units per liter
pmol/L	Picomoles per liter
%	Percentage
℃	Degree Celsius
x g	Times gravity
min	Minute

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s	Second
ng/µL	Nanogram/microliter
XLSX	Microsoft excel open XML spreadsheet
MAF	Minor allele frequency
mM	Millimolar
µM	Micromolar
MgCl2	Magnesium chloride
bp	Base pair
TAE	Tris Acetate-EDTA
V	Volt
ACTB	ß-actin gene
RPL13A	Ribosomal protein L13a gene
pg/mL	Picograms per milliliter
TmP/GFR	Tubular maximum reabsorption of phosphate per glomerular filtration rate
SNV	Single nucleotide variant
indel	Insertion and deletion
P01	Patient 1
P02	Patient 2
P03	Patient 3
P04	Patient 4
MP01	Mother of patient 1
MP02	Mother of patient 2
MP03	Mother of patient 3
MP04	Mother of patient 4
FP01	Father of patient 1
FP02	Father of patient 2
FP03	Father of patient 3
FP04	Father of patient 4
ESP	Exome Sequencing Project
IGV	Integrative Genomics Viewer

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CHAPTER 1

INTRODUCTION

Rickets is a bone disorder due to deficiency in vitamin D, phosphorus, or calcium that occurs mainly during childhood. It happens during the process of bone formation owing to mineralization flaws. Calcium and phosphate are two essential minerals that humans need to maintain the strength and growth of the bones. Imbalance of these two minerals may result in deposition of uncalcified bone matrix, known as osteoid, during the replacement of woven bone into lamellar bone (Elder & Bishop, 2014; Jagtap et al., 2012; Wharton & Bishop, 2003). As the process of transformation of cartilage into bone is disrupted, rickets and osteomalacia occur in growing bones and non-growing bones, respectively. Therefore, rickets only occur in growing children.

Hypophosphatemic rickets (HR) is a genetic disorder causing defects in the renal handling of phosphorus, resulting in rickets. Hypophosphatemia is characterized by low level of serum phosphate that is caused by defects in the renal tubular reabsorption of phosphate which can occur in isolation or as part of renal tubular disorders (Baroncelli et al., 2012; Cho et al., 2005). Low level of phosphate in the body is due to low reabsorption and high excretion of phosphate in the renal tubules in kidney (Jagtap et al., 2012).

Individuals with HR tend to have growth retardation, short stature, dental abscesses, early tooth loss, bone pain, lower limbs deformities, and backache (Bhadada et al., 2010; Douyere et al., 2009). Blood tests show low phosphate level, normal calcium and parathyroid hormone levels with urine showing increased phosphate loss (Baroncelli et al., 2012; Cho et al., 2005). X-ray of the affected limbs show changes of rickets. Oral medication such as phosphate supplements, and calcitriol are given as treatment, which cause improvement of rickets. However, this treatment is unsuccessful for a significant number of patients (Haffner et al., 2019) and in some cases is associated with problems such as nephrocalcinosis and hyperparathyroidism (Erik A Imel et al., 2019).

Phosphate is one of the essential minerals needed by living organisms, especially in building and repairing bone and tooth structure, where majority of phosphate (85%) can be found (Penido & Alon, 2012). It is regulated by parathyroid hormone (PTH), 1,25-dihydroxyvitamin D (1,25 $(OH)_2D$ or calcitriol), and fibroblast growth factor 23 (FGF23) (Penido & Alon, 2012). Abnormalities in phosphate-related genes may interrupt the regulations of phosphate, leading to excess of phosphate excretion and hypophosphatemia.

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HR is found to be due to mutations in several genes; phosphate regulating endopeptidase homolog, X-linked (*PHEX*), fibroblast growth factor-23 (*FGF23*), dentin matrix protein-1 (*DMP1*) and ectonucleotide pyrophosphatase/phosphodiesterase 1 (*ENPP1*) are the related genes that are involved in FGF23-mediated HR. FGF23-independent HR are as part of renal tubular disorders and are caused by mutation in chloride channel 5 (*CLCN5*) and solute carrier family 34 member 3 (sodium/phosphate cotransporter) (*SLC34A3*) genes. Any gain or loss of function mutations in any of these six genes contributes to the hypophosphatemia state. Majority of the cases reported are due to inherited mutations in the involved genes with different modes of inheritance. However, sporadic cases have also been reported.

In the past, the criterion for selecting clinical genetic testing was the patient's clinical signs, which tested one or a few disease-related genes by polymerase chain reaction (PCR) and Sanger sequencing. However, reliable genetic diagnosis was possible in only less than half of the patients (Rauch et al., 2006). There are many disadvantages to PCR amplification, including the fact that gene sequencing and optimization for designed primers require many reactions, and there may be limitations in coverage due to PCR conditions (Hoppman-Chaney et al., 2010). In addition, Sanger sequencing cannot provide adequate throughput for large-scale projects due to sequencing on a single amplicon. The development of next-generation sequencing technologies has helped to detect mutations in both previously identified genes as the cause of disease and novel genes, and has also accelerated the gene discovery rate (Retterer et al., 2016).

The exome is the part of the genome that encodes protein. Because exons contain most of the known disease-causing mutations, whole exome sequencing (WES) is highly efficient for research and is used to identify mutations that cause Mendelian diseases (Stenson et al., 2017). However, 100% of the genes in the genome are not targeted in WES and about 97% of the coding regions are covered. In addition, it is difficult to detect certain types of mutations, such as mutations located in GC-rich or repetitive regions, as well as large rearrangements (Burdick et al., 2020). Today, WES is widely used to identify disease-causing mutations in known and novel genes. WES is a cost-effective method for clinical applications that could be an alternative to whole genome sequencing (WGS). WES produces a smaller set of data that enables easier and faster analysis of data compared to WGS (Gilissen et al., 2011).

HR was first described in 1937 by Fuller Albright and most countries have conducted HR related studies (Albright et al., 1937). However, there is paucity of information on HR in Malaysia. Diagnosis of HR in Malaysian patients is usually made based on clinical and radiological features and abnormal biochemistry in blood and urine tests which sometimes do not show consistent

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results. Only two studies in Malaysia had been reported; One study of a family tree of XLHR (Yong & Aik, 2000), another study was conducted to look for mutations in *PHEX*, *DMP1* and *FGF23* genes in four Malaysian children with HR. However, the causative mutations for HR and the pattern of inheritance in Malaysians could not be concluded because only polymorphisms were found in some patients as well as their asymptomatic parents (Razali et al., 2019). Moreover, since genetic background of HR in Malaysian population has not been established, burosumab (a recently approved treatment by FDA for XLHR) cannot be given to HR patients without genetic analysis.

This study was designed to address the insufficient genetic information of HR in Malaysia. The presence of genetic mutations was studied among children who showed the symptoms of HR and also their parents. Therefore, the general objective of this study was to identify the pathogenic gene mutations causing HR disease in Malaysian patients through whole exome sequencing (WES).

The specific objectives of this study were:

i. To identify the presence and types of mutations and the pattern of transmission of HR among patients and their parents;
ii. To investigate mRNA and protein expression of FGF23 in HR patients

The two hypotheses of this study are:

i. Children with clinical features of HR carry mutations either in a recessive gene or represents a *de novo* mutation;
ii. *FGF23* mRNA and protein are overexpressed in patients with HR.

This study aims to find the underlying genetic defect in HR patients. The selection of variant types for further analysis in this thesis was based on phenotype status of the patients and their parents. Therefore, since parents were unaffected, three *de novo* variants and one recessive variant transmitted from the parents were identified and chosen to be further analyzed to confirmed the pathogenicity. The identification of pathogenic gene mutations is essential to our understanding of the disease mechanisms causing HR. This directly contributes to the diagnosis and genetic counselling, and allows the opportunity for development of new treatment strategies.

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