Gene 637 (2017) 100–107

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

The *ENPP1 K121Q* polymorphism modulates developing of bone disorders in type 2 diabetes: A cross sectional study

Nahid Neamati[a], Seyed Reza Hosseini[b], Mahmood Hajiahmadi[c], Sohrab Halalkhor[a], Hajighorban Nooreddini[d], Haleh Akhavan Niaki[e], Bahare Korani[a], Hadi Parsian[b,e,*]

[a] Department of Clinical Biochemistry, Faculty of Medicine, Babol University of Medical Sciences, Babol, Iran
[b] Social Determinant of Health Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, Iran
[c] Department of Biostatistics, Faculty of Medicine, Babol University of Medical Sciences, Babol, Iran
[d] Department of Radiology, Faculty of Medicine, Babol University of Medical Sciences, Babol, Iran
[e] Cellular and Molecular Biology Research Centre, Babol University of Medical Sciences, Babol, Iran

ARTICLE INFO

Keywords:
ENPP1 gene
K121Q polymorphism
rs1044498
Osteopenia
Osteoporosis
Type 2 diabetes

ABSTRACT

Background: Osteoporosis and osteopenia are common diseases in every population. Type 2 diabetes mellitus (T2DM) can lead to the development of various complications, such as bone disorders especially among elderly individuals. Studies suggested that ectonucleotide pyrophosphatase/phosphodiesterase1 (ENPP1) is contributed in insulin resistance and also the inhibition of bone mineralization. In this study, association of *K121Q* (rs1044498) polymorphism of the *ENPP1* gene with T2DM and bone disorders is evaluated.

Methods: Four-hundred-and-ninety females who were classified based on bone mineral density (BMD) at lumbar spine and femur were included in this study. In addition, participants were classified according to their diabetes status. *K121Q* polymorphism was evaluated by the PCR-PFLF technique. One-way ANOVA was used for comparison of various analyzed factors in diseases subgroups and *K121Q* genotypes. Association of *K121Q* polymorphism with diabetes and bone disorders was evaluated by logistic regression.

Results: Significant association was observed between *K121Q* polymorphism with osteoporosis and osteopenia ($p = 0.041$, $p = 0.029$, respectively), but a similar pattern was not observed in T2DM status ($p = 0.723$). Moreover, in diabetic patients, *K121Q* polymorphism showed a better prediction potential for the development of bone disorders in comparison to non-diabetic subjects ($p = 0.018$; OR = 4.63, $p = 0.540$; OR = 1.31). There were no significant differences between *K121Q* genotypes with FBS, Ca, P, vitamin D, PTH and BMD status.

Conclusions: The present study implies that *K121Q* polymorphism of *ENPP1* gene is able to modulate the development of bone disorders in T2DM. Therefore in diabetic patients screening of this polymorphism is suggested for the monitoring of these persons.

1. Introduction

Osteoporotic fractures usually occur due to decrease in bone mineral density (BMD) and destruction of bone structure (Genant et al., 1999). This disease is an important problem for societies because its prevalence is dramatically increasing in recent years (Cummings and Melton, 2002). Around the world, there are more than two hundred million people with this disease (Kannus, 2003). In Iran 50% of men and 70% of women, over 50 years, suffer from bone problems (osteoporosis or osteopenia) (Rahnavard et al., 2009).

Bone strength is affected by many risk factors such as age, gender, body mass index (BMI), menopausal and nutritional status, hormonal disorders, use of alcohol and some drugs, smoking, physical activity, genetic factors and so on. Studies have shown that > 50% (50–80%) of BMD variations are affected by genetic factors. Nowadays, evaluation of BMD is one of the main methods for estimation of bone strength and is used for identifying and predicting the future fractures risks (Hui et al., 1988, Lau et al., 2001, Dontas and Yiannakopoulos, 2007).

In addition to osteoporosis, diabetes mellitus is another serious disease. Unfortunately, the number of people that suffer from diabetes

Abbreviations: AHAP, Amirkola health and aging project; BMD, Bone mineral density; BMI, Body mass index; Ca, Calcium; DEXA, Dual energy X-ray absorptiometry; *ENPP1*, Ectonucleotide pyrophosphatase/phosphodiesterase1; FBS, Fasting blood sugar; HWE, Hardy-Weinberg Equilibrium; P, Phosphorus; PCR, Polymerase chain reaction; PTH, Parathyroid hormone; RFLP, Restriction fragment length polymorphism; T2DM, Type 2 diabetes mellitus; vit D, Vitamin D

* Corresponding author at: Clinical Biochemistry, Social Determinant of Health Research Center, Health Research Institute, Babol University of Medical Sciences, Ganjafrooz Ave, Babol, Iran.
E-mail address: hadiparsian@mubabol.ac.ir (H. Parsian).

http://dx.doi.org/10.1016/j.gene.2017.09.042
Received 10 June 2017; Received in revised form 17 September 2017; Accepted 19 September 2017
Available online 21 September 2017

is increasing in all countries. Currently, 382 million people worldwide have diabetes and this number is expected to rise up to 592 million until 2035 (Guariguata et al., 2014).

Diabetes as a metabolic disease can lead to complications such as microvascular diseases, retinopathy, neuropathy, nephropathy, and even functional disorders (Association, A. D, 2010). In addition, some researchers have shown that diabetic individuals are more susceptible to osteoporosis than non-diabetic ones. Diabetes could be a predisposing factor to various kinds of fractures. It has been proposed that the incidence rate of fractures in wrist, leg, and hip in elderly diabetics is much more than non-diabetics (Karimifar et al., 2012, Jiajue et al., 2014, Jiao et al., 2015).

Despite the impact of recognized risk factors that are involved in developing the diabetes and osteoporosis, substantiated evidence suggests that certain genetic factors could be effective in occurrence and severity of their complications. It is suggested that ectonucleotide pyrophosphatase/phosphodiesterase 1 (*ENPP1*) is one of the candidate genes that is involved and contributed to the pathogenesis of diabetes and also osteoporosis (Gaulton et al., 2008, Cheung et al., 2010, Ermakov et al., 2010).

It has been reported that extracellular matrix mineralization is an important process in bone formation, resorption and also its maintenance (Harmey et al., 2004; Forriol and Shapiro, 2005). Any defect in this process could be associated with changes in bone strength and structure. Inorganic phosphorus (Pi) is an essential element for bone mineralization and is tightly regulated by three enzymes: ENPP1, alkaline phosphatase, liver/bone/kidney (ALPL) and ANKH inorganic pyrophosphate transport regulator (ANKH) (Ermakov et al., 2010). ENPP1 that is one of the subtypes of ENPP family, serves as a mineralization modulator by hydrolyzing tri-nucleotide phosphate and generating pyrophosphate (PPi) (Harmey et al., 2004). Another enzyme, called ALPL, is able to hydrolyze PPi to Pi and provide required inorganic phosphate for bone mineralization (Hessle et al., 2002). ANKH serves as a transporter of PPi from intracellular to extracellular space and vice versa (Ho et al., 2000). PPi by itself is an inhibitor of bone mineralization. Interestingly, it serves as ALPL substrate for production of Pi and can contribute in bone mineralization. In addition to the above mentioned function, another property that is attributed to the *ENPP1* gene is that encoding a transmembrane glycoprotein that interacts with insulin receptor and inhibits its tyrosine kinase activity (Maddux et al., 1993). Therefore, it can involve in insulin signaling. Thus *ENPP1* may be a suitable candidate for research in the diabetes field due to insulin resistance.

A linkage between hip, spine and wrist BMD with *ENPP1* gene polymorphisms has been reported and some studies suggested *ENPP1* as a susceptible gene for BMD variations (Cheung et al., 2010; Ermakov et al., 2010). To our best knowledge, there is no available data on the association between diabetes and osteoporosis (or osteopenia) regarding to *ENPP1*, rs1044498 (*K121Q*) polymorphism. *K121Q* (rs1044498) polymorphism of *ENPP1* gene is a functional missense mutation where adenine is replaced by cytosine and results in transcoding of glutamine amino acid instead of lysine at codon 121 of mRNA transcript. There are some controversial data that suggested an association between rs1044498 polymorphism with insulin resistance in type 2 diabetes mellitus (T2DM) patients (Keshavarz et al., 2006, Saberi et al., 2011, Jing et al., 2012, Prakash et al., 2013, Badaruddoza et al., 2014). Summary of some investigations about the association between ENPP1 polymorphisms and its related phenotypes are presented in Table 1.

The present study aimed to determine probable association between rs1044498 polymorphism of *ENPP1* gene with osteoporosis in T2DM patients.

2. Materials and methods

2.1. Study design

Among the 1616 individuals that participated in Amirkola health and aging project (AHAP) there were 733 old women (> 60 years old) (Hosseini et al., 2013). After excluding the person who have taken medications related to BMD (such as alendronate or Ibandronate, …), 490 females were recruited and were evaluated for BMD at lumbar spine (L1-L4) and femur using a dual energy X-ray absorptiometry (DEXA) densitometer (DMS Lexxos DR, French). Based on WHO criteria, participants were stratified in three separate groups according to their BMD: osteoporosis, osteopenia and normal groups. If a person had BMD 2.5 standard deviations (or more) below than average value for young healthy adults, i.e. T-score ≤ -2.5 SD, considered as osteoporosis patient; T-score higher than -1.0 SD as normal and BMD measures between $-2.5 <$ T-score ≤ -1 considered as osteopenia. In our study, 279 females suffered from osteoporosis, 154 females had osteopenia and 57 subjects had no sign of any bone defect.

The status of body weight, height and serum levels of biochemical and hormonal biomarkers such as fasting blood sugar (FBS), calcium (Ca), phosphorus (P), parathyroid hormone (PTH), and vitamin D (vit. D) were extracted from the AHAP database. Body mass index (BMI) was calculated by dividing weight (Kg) to height (m^2).

In addition to the classifications of participants according to their BMD results, we also classified our included persons according to their diabetes status. Diabetes status was determined based on physician report sheet and measurement of fasting blood glucose level in two separate sampling (Association, A. D, 2014). Our study was approved by our university's ethics committee.

2.2. DNA extraction, PCR and RFLP

Genomic DNA has been extracted from leukocytes in whole blood using QIAamp DNA blood mini kit (Qiagen, Korea) according to manufacture protocol. Quality of the extracted DNA was assessed by running on 1% agarose gel and its concentration measured by nanodrop (Thermoscientific, USA). DNA extraction has been done in base AHAP study and resulted DNAs have been stored at -80 °C.

To amplify the rs1044498 region from the *ENPP1* gene, primers designed by Gene Runner software (version 5.1.06 beta) and confirmed via NCBI (primer blast) and online oligo-analyzer. The forward primer was 5′-GTAGTGGCAGATTCTGTGAGTGAC-3′ and the reverse one was 5′-CCGCTAAGACGCTGGAAGATACC-3′.

Amplification was performed in 25 μl total reaction mixture volume contained forward and reverse primer (10 pmol), tris-HCL buffer (pH 8.8) (1 mM), dNTPmix (0.2 mM), $MgCl_2$ (1.5 mM), taq DNA polymerase (1 unit) and DNA template (20 ng). The amplification was performed according to the following program: initial denaturation at 94 °C for 5 minutes, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 1 min and final extension at 72 °C for 10 min. In order to ensure the amplification of the ENPP1 rs1044498 region, its electrophoresis was performed by running on 2% agarose gel for 30 min and ethidium bromide staining. The amplified DNA was visualized on a trans-illuminator under ultraviolet light by gel documentation and analysis system (UVdoc, England).

In order to assess the *K121Q* polymorphism, 10 μl of PCR product was digested by 3.3 Unit of *Ava*II restriction enzyme (Thermoscientific, USA) at 37 °C for 7 h. After digestion, the presence of *K121Q* polymorphism was determined by running on polyacrylamide gel electrophoresis for 30 min and ethidium bromide staining. Samples with KK genotype had one fragment (i.e. one band), samples with QQ genotype had two separate fragments and samples with KQ genotype had three separate fragments.

The lengths of the PCR product and resulting fragments after digestion were assessed according to the 50 bp DNA size markers. PCR