การศึกษาความเกี่ยวพันโดยการวิเคราะห์สนิปสามชนิดที่มีตัวแหน่งในยีน MGP กับการเกิดนิ่วไต

Analysis of Three SNPs in MGP Gene in Genetic Association Studies for Kidney Stone Formation

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บทคัดย่อ

นิ่วไตเป็นปัญหาสำคัญที่พบทั่วโลก ปัจจัยทั้งทางด้านพันธุกรรมและสิ่งแวดล้อมมีส่วนเกี่ยวข้องกับการเกิดนิ่วไต การศึกษาวิเคราะห์มีการใช้ในการสืบค้นสาเหตุของโรค พบว่ามีการศึกษานี้เป็นส่วนหนึ่งของการวิเคราะห์ความแปรผันทางพันธุกรรมของยีน MGP ซึ่งมีพันธุกรรมและสิ่งแวดล้อมที่เกี่ยวข้องกับการเกิดนิ่วไต โดยใช้เทคนิคการวิเคราะห์สร้างโปรตีนแมททริกกลา และมีรายงานสนิป rs4236 ที่มีตัวแหน่งในส่วนแปลรหัสของยีน MGP ที่เกี่ยวข้องกับการเกิดนิ่วไต โดยมีรายงานสนิปทั้งสามไม่มีความแตกต่างกันระหว่างกลุ่มผู้ป่วยและกลุ่มควบคุม (association study) ปัจจุบันมีการศึกษาศึกษาความแปรผันทางพันธุกรรมของยีน MGP ได้แก่ rs1800802, rs4236, rs1049897 โดยวิธีพีซีอาร์-อาร์เอฟแอลพี พบว่าไม่มีความแตกต่างกันระหว่างกลุ่มผู้ป่วยและกลุ่มควบคุม (P>0.05)

ค่าสำคัญ: นี้โดย ความแปรผันทางพันธุกรรมชนิดสนิป โปรตีนแมททริกกลา ที่ซิยา-อาเระ-มณฑล

ABSTRACT

Kidney stone formation is a common problem worldwide. A combination of genetic predisposition and environmental factors has been shown to be related with the pathogenesis of the
stone formation. This study is a part of ongoing project aimed to identify genetic factors contributing to the stone formation. Association study was performed by analysis of genetic variation of \textit{MGP} gene in affected group and normal group to determine the association of the variation and renal stone formation. Previous study found that a single nucleotide polymorphism (SNP), rs4236, located on the coding region of \textit{MGP} gene, encoding for matrix Gla protein, have been shown to be associated with kidney stone and influences genetic susceptibility to kidney stone. Therefore, association studies by genotyping of three SNPs, rs1800802, rs4236 and rs1049897, located in the \textit{MGP} gene using genomic DNA of 112 patients and 112 normal controls were performed in this study. The result of genotyping obtained by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) found that genotyping of three SNPs from normal controls were not significantly different from the affected cases \((P>0.05)\).

\textbf{Keywords} : kidney stone, SNP, matrix Gla protein, PCR-RFLP
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\textbf{INTRODUCTION}

Kidney stone or kidney calculi is a common clinical disorder which caused great health and economic problem to the patient’s families and the country health-care system. In northeastern (NE) of Thailand is an endemic area. The phenotype of this disease is unique in NE Thai population. From previous work, the etiology of kidney stone in the NE Thai population is unlikely to occur from the predominant presence of stone promoters. It is more likely that the alteration or abnormality of inhibitor is involved in the kidney stone formation in this population. Recently, it has been shown that a matrix Gla protein (\textit{MGP}) gene polymorphism was associated with kidney stones (Gao \textit{et al}, 2007). Therefore, single nucleotide polymorphisms (SNPs) of \textit{MGP} gene, which encoded for matrix Gla protein (\textit{MGP}), were selected in this study in order to determine the association of these SNPs in the patients (cases) and the age-matched controls in NE Thai population.

\textit{MGP} is a 12 kDa protein of 103 residues. It is encoded from \textit{MGP} gene consisting of 4 exons and 3 introns (Cancela \textit{et al}, 1990) and located at 12p13.1-p12.3 of human chromosome with 4527 bp in size. Messenger RNA (mRNA) of this gene is 661 bp in size. \textit{MGP} is bone related protein and a potent calcification inhibitor in arterial vessels. It is highly expressed in the kidney, bone, lung and heart. After \textit{MGP} is synthesized, it undergoes a post-translational modification in the endoplasmic reticulum (ER) where specific glutamic acid (Glu) residues are converted to $\gamma$-carboxyglutamic acid (Gla) by a vitamin K-dependent $\gamma$-carboxylase enzyme (Price \textit{et al}, 1983). A Gla containing domain has high affinity for calcium, phosphate ions and hydroxyapatite crystal (Price, 1989) and it was found in the macromolecular proteins of the calcium containing kidney stone matrix (Lian, 1977).
OBJECTIVE

Analysis of three single nucleotide polymorphisms (SNPs) located in MGP gene for determination the association of MGP gene and kidney stone formation.

MATERIALS AND METHODS

1. Subjects

The total of 224 subjects, 112 from affected cases and 112 from normal controls, were used in this study. They borned and lived in Khon-Kaen province or neighboring area. All subjects were diagnosed and examined by nephrologists from Division of Nephrology, Department of Medicine, Faculty of Medicine Siriraj Hospital. The affected cases were identified from the presence of opaque kidney stone by the X-ray of kidney-ureter-bladder (KUB) or the scar from operation to remove kidney stone. The normal control did not have the history of pass stone, dysuria, hematuria and turbid urine, and negative result in KUB or scar from operation to remove the stone. Their family members did not have history of stone in kidney or urinary tract. The affected cases and normal control groups are age-matched with mean age (year) ± SD of 48.26 ± 11.69 for cases, 47.99 ± 12.82 for normal controls. Blood samples of the all subjects were used to extract genomic DNA by standard protocol using phenol-chloroform extraction.

2. SNP selection

Three SNPs located in MGP gene were selected from SNPs database (dbSNPs) of the International HapMap Project and the National Center for Biotechnology Information (NCBI) based on allele frequency of HCB (Han Chinese in Beijing, China) population. They are rs1800802, rs4236, and rs1049897 located in the promoter, exon4 and 3' untranslated region of MGP with allele frequencies of T(0.578)/C(0.422), A(0.911)/G(0.089) and A(0.898)/T(0.078), respectively.

3. Analysis of SNPs by PCR-RFLP

3.1) Primer design for PCR-RFLP

Two pairs of primer were designed to amplify fragments containing two SNPs, rs4236 and rs1049897 which can be analyzed by AvaII and HinfI, respectively. A pair of primer used for analysis of rs1800802 was designed by using dCAPS Finder 2.0 web-based program to generate AvaII restriction site at the 3' end. The sequence of primers used in this study was shown in Table 1. These primers were designed based on accession number M55270.
Table 1 Nucleotide sequence of primers used to generate amplified DNA fragments for SNP analysis.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Primer name</th>
<th>Nucleotide sequence (5’ to 3’)</th>
<th>Tm (°C)</th>
<th>PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1800802</td>
<td>rs1800802_F</td>
<td>CAGAGTAGATAATATCTGGAAAGGAAGGAC</td>
<td>64.6</td>
<td>288</td>
</tr>
<tr>
<td></td>
<td>rs1800802_R</td>
<td>TCACCATAACACAAAGTTACTACCG</td>
<td>61.3</td>
<td></td>
</tr>
<tr>
<td>rs4236</td>
<td>rs4236_F</td>
<td>GGGGCATGGGAGAAAGTCT</td>
<td>62.4</td>
<td>444</td>
</tr>
<tr>
<td></td>
<td>rs4236_R</td>
<td>TCTCCTTTGACCCTCAGTCGG</td>
<td>62.4</td>
<td></td>
</tr>
<tr>
<td>rs1049897</td>
<td>rs1049897_F</td>
<td>TACAGGGCTCCCTTCATTG</td>
<td>60.4</td>
<td>401</td>
</tr>
<tr>
<td></td>
<td>rs1049897_R</td>
<td>GTGCAATTGGTGATGGTACCATG</td>
<td>60.4</td>
<td></td>
</tr>
</tbody>
</table>

3.2) Amplification of DNA fragments for SNPs analysis.

PCR reaction was carried out in 25 μl containing 200 ng of genomic DNA, 10 pmole of each primer, 2 mM dNTPs, 2.5 mM MgCl₂ and 1 unit of GoTaq® Flexi DNA polymerase (Promega) in buffer provided by the manufacturer. The PCR profile for fragment carrying rs4236 and rs1800802 was performed as follows: 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 59 °C for 30 sec and 72 °C for 2 min, and then final extension at 72 °C for 5 min. The PCR profile for rs1049897 was performed at 94 °C for 2 min, followed by 35 cycles consisting of 94 °C for 30 sec, 61 °C for 30 sec and 72 °C for 2 min, and then final extension step at 72 °C for 5 min. After amplification, the reaction was analyzed by 1.5% agarose gel electrophoresis. DNA band was visualized using the Gel Documentation system after staining with ethidium bromide solution.

3.3) Restriction fragment length polymorphism analysis

After amplification, the PCR products were digested in a reaction (total volume of 20 μl) containing 1 μl of PCR products and 2 units of restriction enzyme (New England Biolabs) in 1X buffer provided by manufacturer. AvaII was used to determine the genotyping of rs4236 and rs1800802, and HinfI was used for rs1049897. The reaction mixture was incubated at 37 °C for at least 4 hours. After digestion, 5 μl of the digested reaction was analyzed by agarose gel (2.5%) electrophoresis in parallel with 1 μl of undigested PCR product as a control.

4. Statistical analysis

The statistical tests for deviation from Hardy-Weinberg equilibrium (HWE) of genotypes and association between SNP frequencies and disease were performed by using DeFinetti web-based program. The data with P<0.05 was considered as statistical significant.
RESULTS AND DISCUSSION

1. Amplification of three fragments carrying three SNPs

Genomic DNAs of 112 affected cases and 112 normal controls were used as template to generate three amplified fragments, named fragments A, B and C, containing rs1800802, rs4236 and rs1049897, respectively (Figure 1). The results showed that three amplified fragments were obtained from all DNA template used in this study. The size of the amplified fragments obtained is 288, 444 and 401 bp as expected (Table 1). An example of agarose gel electrophoreses used for analysis of the amplified products is presented in Figure 2.

![Figure 1](image1.png)

**Figure 1** Schematic representation of human MGP gene. Open boxes indicate untranslated regions. Filled boxes indicate exons 1 to 4. The positions of SNPs used in this study are indicated. Three amplified fragments named A, B and C containing the SNPs are shown under the map of the MGP gene.

![Figure 2](image2.png)

**Figure 2** The 2.5% ethidium bromide stained agarose gel showed expected size of three fragments. M : 100 bp+1.5 kb DNA ladder marker. (a) The amplified products, fragment A, containing rs1800802 is 288 bp. (b) The amplified products, fragment B, containing rs4236 is 444 bp. (c) The amplified products, fragment C, containing rs1049897 is 401 bp.
2. Analysis of SNPs by PCR-RFLP

After amplification of three fragments carrying three SNPs, rs1800802, rs4236 and rs1049897, the fragment was digested with restriction enzyme. An example of SNPs genotyping by RFLP is presented in Figure 3. The genotyping of all samples was shown in Table 2.

For genotyping of rs1800802, fragment A (288 bp) was digested with Avall. Two digested bands (262 bp and 26 bp) were obtained indicating that the fragment A carrying allele C whereas the one carrying allele T cannot digested with Avall (Figure 3a). For genotyping of rs4236, allele A generate two digested fragments (235 bp and 209 bp) after digestion of fragment B (444 bp) with Avall (Figure 3b). For analysis of rs1049897, the amplified fragment of 401 bp was digested with HinfI. After HinfI digestion of amplified fragment rs1049897, the fragment carrying allele A gave the two digested bands (342 bp and 59 bp) but the fragment carrying allele T cannot digested with HinfI (Figure 3c).

![Figure 3](image)

**Figure 3** The 2.5% ethidium bromide-stained agarose gel showed digested PCR products (D) in parallel with the undigested products (U). M : 100 bp+1.5 kb DNA ladder marker. (a) Analysis of rs1800802 by Avall shows heterozygous (T/C) of this SNP. Two bands of 288 bp (undigested fragment) and 266 bp (digested fragment) were observed. (b) Analysis of rs4236 by Avall shows heterozygous (A/G) of this SNP. The digested fragments of 235 bp and 209 bp were observed. (c) Analysis of rs1049897 by HinfI shows heterozygous (A/T) of this SNP. The digested fragment (342 bp) and undigested fragment (401 bp) were observed.

3. Statistical analysis

The data of SNPs genotyping of all samples was collected and tested for deviation from HWE and for association with the disease phenotype by DeFinetti web-based program. All genotyped SNPs were in HWE ($P>0.05$) according to Pearson’s goodness-of-fit chi-squared test (Table 2) but
none of their allele and genotype frequencies showed significant differences between case and control groups (Table 3).

**Table 2** Genotyping of all three SNPs in this study

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Allele frequency, n (%)</th>
<th>Genotype frequency, n (%)</th>
<th>HWE testing</th>
<th>Controls</th>
<th>Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pearson chi-square, $P$ value$^a$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>Cases</td>
<td>Controls</td>
<td>Cases</td>
<td>Controls</td>
</tr>
<tr>
<td>SNP1*</td>
<td>T</td>
<td>C</td>
<td>T</td>
<td>C</td>
<td>TT</td>
</tr>
<tr>
<td></td>
<td>178 (79.5)</td>
<td>(20.5)</td>
<td>170 (75.9)</td>
<td>(24.1)</td>
<td>70 (62.5)</td>
</tr>
<tr>
<td>SNP2*</td>
<td>A</td>
<td>G</td>
<td>A</td>
<td>G</td>
<td>AA</td>
</tr>
<tr>
<td></td>
<td>169 (75.4)</td>
<td>(24.6)</td>
<td>173 (77.2)</td>
<td>(22.8)</td>
<td>62 (55.4)</td>
</tr>
<tr>
<td>SNP3*</td>
<td>A</td>
<td>T</td>
<td>A</td>
<td>T</td>
<td>AA</td>
</tr>
<tr>
<td></td>
<td>193 (86.2)</td>
<td>(13.8)</td>
<td>189 (84.4)</td>
<td>(15.6)</td>
<td>82 (73.2)</td>
</tr>
</tbody>
</table>

$^*$ SNP1 = rs1800802, SNP2 = rs4236, SNP3 = rs1049897

$^a$ = Analyzed by DeFinetti web-based program

**Table 3** Association between single SNP and kidney stone risk

<table>
<thead>
<tr>
<th>SNP</th>
<th>Test for association</th>
<th>Allele frequency difference</th>
<th>Heterozygous</th>
<th>Homozygous</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1800802</td>
<td>Odds ratio (95% CI)</td>
<td>0.81 (0.52-1.27)</td>
<td>0.60 (0.16-2.22)</td>
<td>0.53 (0.15-1.9)</td>
</tr>
<tr>
<td></td>
<td>$P$</td>
<td>0.36</td>
<td>0.44</td>
<td>0.32</td>
</tr>
<tr>
<td>rs4236</td>
<td>Odds ratio (95% CI)</td>
<td>1.10 (0.71-1.71)</td>
<td>0.91 (0.25-3.38)</td>
<td>1.07 (0.29-3.86)</td>
</tr>
<tr>
<td></td>
<td>$P$</td>
<td>0.66</td>
<td>0.89</td>
<td>0.92</td>
</tr>
<tr>
<td>rs1049897</td>
<td>Odds ratio (95% CI)</td>
<td>0.87 (0.51-1.46)</td>
<td>1.14 (0.07-19.02)</td>
<td>0.95 (0.06-15.47)</td>
</tr>
<tr>
<td></td>
<td>$P$</td>
<td>0.59</td>
<td>0.93</td>
<td>0.97</td>
</tr>
</tbody>
</table>

The limitation in this study is the less number of SNPs for genotyping. This study used only three SNPs that were obtained from SNPs database (dbSNPs) of the International HapMap Project and the National Center for Biotechnology Information, NCBI. SNPs selection focused on the data from HCB (Han Chinese in Beijing, China) population as their genetic background is closely related to
Thai population. Each three SNPs were selected by following criterion. The criterion are that SNPs should be in coding region as a non-synonymous coding variants altered amino acid sequence > regulatory element > intron, spread throughout the gene and minor allele frequency more than 5% that are more likely to capture variation in the region. Based on the available information and the selected criterion, only three SNPs of \( MGP \) gene were obtained and analyzed in this study.

**CONCLUSION**

Association study was performed to determine the association of \( MGP \) gene and kidney stone formation in northeastern Thai population by analysis of three SNPs. The results indicate that the genotyping of \( MGP \) gene in affected cases is not significant different from control groups \((P>0.05)\).

**REFERENCES**


**ACKNOWLEDGMENTS**

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