

การศึกษาความสัมพันธ์โดยการวิเคราะห์สnpสามชนิดที่มีตำแหน่งในยีน *MGP*  
กับการเกิดนิ่วไต

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

**พงษ์เพชร เบนจพลวัฒนา**<sup>1</sup> นัญวรรณ รุ่งโรจน์<sup>2</sup> ชูชัย เนตรภูวกุล<sup>3</sup> นิ่งหทัย สวัสดิ์<sup>3</sup>

นรินทร์ยา สุตตาชาติ<sup>3,4</sup> วรรณมา ทองนพคุณ<sup>2</sup> วราภรณ์ อัครปฐมวงศ์<sup>1</sup> และเพทชาย เย็นจิตโสมนัส<sup>3,4</sup>

**Pongpet Benjaponwattana**<sup>1</sup>, Nanyawan Rungroj<sup>2</sup>, Choochai Nettuwakul<sup>3</sup>, Nungthai Sawasdee<sup>3</sup>

Nirinya Sudtachat<sup>3,4</sup>, Wanna Thongnoppakhun<sup>2</sup>, Varaporn Akkarapatumwong<sup>1</sup> and Pa-thai Yenchitsomanus<sup>3,4</sup>

**บทคัดย่อ**

นิ่วไตเป็นปัญหาสำคัญที่พบทั่วโลก ปัจจัยทั้งทางด้านพันธุกรรมและสิ่งแวดล้อมมีส่วนเกี่ยวข้องกับการเกิดนิ่วไต การศึกษานี้เป็นส่วนหนึ่งของงานวิจัยที่กำลังดำเนินการอยู่เพื่อที่จะค้นหาปัจจัยทางพันธุกรรมโดยใช้การวิเคราะห์ความแปรผันทางพันธุกรรมของยีน *MGP* เปรียบเทียบระหว่างกลุ่มผู้ป่วยและกลุ่มปกติเพื่อหาความสัมพันธ์กับการเกิดนิ่วไต (association study) ยีน *MGP* มีหน้าที่ในการสร้างโปรตีนแมทริกกลาและเคยมีรายงานว่าสnp rs4236 ที่มีตำแหน่งในส่วนแปลรหัสของยีนนี้สัมพันธ์กับการเกิดนิ่วไตอย่างมีนัยสำคัญ ผลการวิเคราะห์ จีโนไทป์ของสnpสามชนิดที่มีตำแหน่งอยู่ในยีน *MGP* ได้แก่ rs1800802 rs4236 rs1049897 โดยวิธีพีซีอาร์-อาร์เอฟแอลพีจากตัวอย่างดีเอ็นเอของกลุ่มผู้ป่วยและกลุ่มปกติจำนวนกลุ่มละ 112 ราย พบว่าจีโนไทป์ของสnpทั้งสามไม่มีความแตกต่างกันอย่างมีนัยสำคัญระหว่างกลุ่มผู้ป่วยและกลุ่มคนปกติ ( $P>0.05$ )

**คำสำคัญ** : นิ่วไต ความแปรผันทางพันธุกรรมชนิดสnp โปรตีนแมทริกกลา พีซีอาร์-อาร์เอฟแอลพี

**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

<sup>1</sup> สถาบันชีววิทยาศาสตร์โมเลกุล มหาวิทยาลัยมหิดล วิทยาเขตศาลายา จ. นครปฐม 73170

Institute of Molecular Biosciences, Mahidol University, Salaya Campus, Nakhon Pathom 73170

<sup>2</sup> หน่วยอนุพันธุศาสตร์ สถานส่งเสริมการวิจัย คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล กรุงเทพฯ

Division of Molecular Genetics, Department of Research and Development, Faculty of Medicine Siriraj Hospital,

Mahidol University, Bangkok

<sup>3</sup> หน่วยอนุชีววิทยาการแพทย์ สถานส่งเสริมการวิจัย คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล กรุงเทพฯ

Division of Medical Molecular Biology, Department of Research and Development, Faculty of Medicine Siriraj Hospital,

Mahidol University, Bangkok

<sup>4</sup> หน่วยปฏิบัติการเทคโนโลยีชีวภาพทางการแพทย์ ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ สำนักพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ

Medical Biotechnology Unit, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency

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 *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 *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 *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 ( $P>0.05$ ).

**Keywords:** kidney stone, SNP, matrix Gla protein, PCR-RFLP

E-mail : g5036574@student.mahidol.ac.th, pongpet\_b@hotmail.com

## 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 (*MGP*) gene polymorphism was associated with kidney stones (Gao *et al*, 2007). Therefore, single nucleotide polymorphisms (SNPs) of *MGP* gene, which encoded for matrix Gla protein (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.

MGP is a 12 kDa protein of 103 residues. It is encoded from *MGP* gene consisting of 4 exons and 3 introns (Cancela *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. 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 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 *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)  $\pm$  SD of  $48.26 \pm 11.69$  for cases,  $47.99 \pm 12.82$  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 *Avall* 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 *Avall* 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.

SNP	Primer name	Nucleotide sequence (5' to 3')	T <sub>m</sub> (°C)	PCR product (bp)
rs1800802	rs1800802_F	CAGAGTAGATAATATCTGGAAGGAAGGAC	64.6	288
	rs1800802_R	TCACCATAACACAAAAGTTACTACCG	61.3	
rs4236	rs4236_F	GGGGCATGGAGAGAAAGTCT	62.4	444
	rs4236_R	TCTCCTTTGACCCTCACTGC	62.4	
rs1049897	rs1049897_F	TACAGGGCTCCCTTCATTTG	60.4	401
	rs1049897_R	GTGCATTGGTATGGTCATGG	60.4	

### 3.2) Amplification of DNA fragments for SNPs analysis.

PCR reaction was carried out in 25  $\mu$ l containing 200 ng of genomic DNA, 10 pmole of each primer, 2 mM dNTPs, 2.5 mM MgCl<sub>2</sub> and 1 unit of GoTaq<sup>®</sup> 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  $\mu$ l) containing 1  $\mu$ l of PCR products and 2 units of restriction enzyme (New England Biolabs) in 1X buffer provided by manufacturer. *Ava*ll was used to determine the genotyping of rs4236 and rs1800802, and *Hin*fl was used for rs1049897. The reaction mixture was incubated at 37 °C for at least 4 hours. After digestion, 5  $\mu$ l of the digested reaction was analyzed by agarose gel (2.5%) electrophoresis in parallel with 1  $\mu$ l of undigested PCR product as a control.

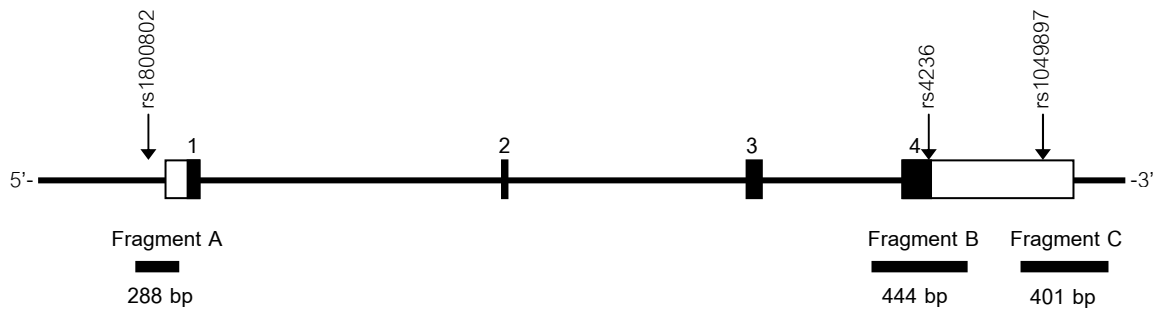
## 4. Statistical analysis

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.

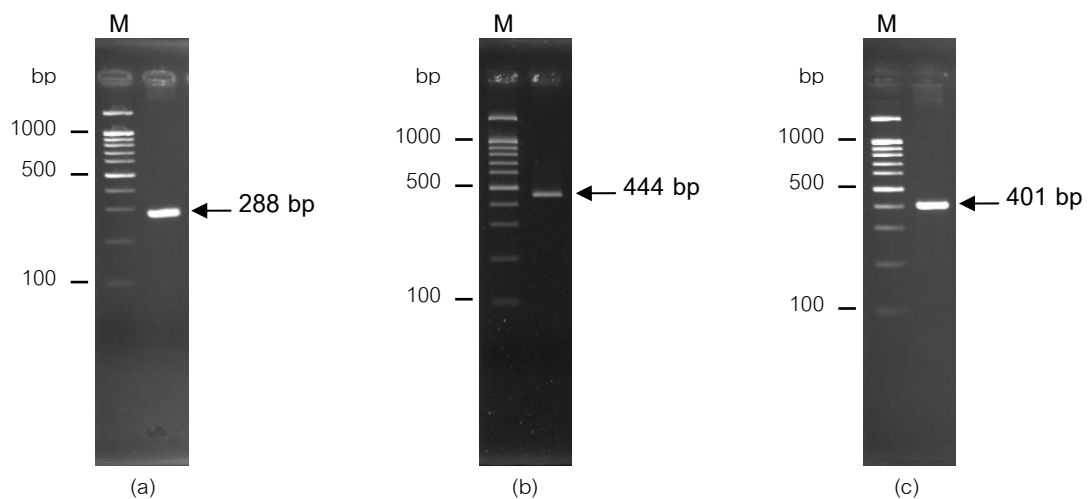
## RESULTS AND DISCUSSION

### 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** 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.

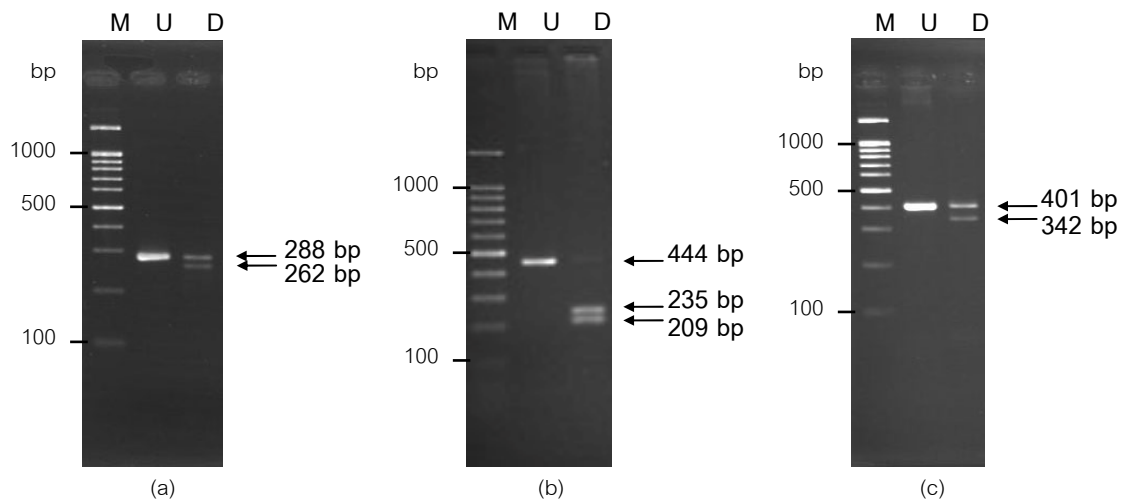


**Figure 2** The 2.5% ethidium bromide stained agarose gel showed expected size of three fragments. M : 100 bp+1.5 kb DNA ladder marker. (a) The amplified products, fragment A, containing rs1800802 is 288 bp. (b) The amplified products, fragment B, containing rs4236 is 444 bp. (c) The amplified products, 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 *Hinfl*. After *Hinfl* 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 *Hinfl* (Figure 3c).



**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 *Hinfl* 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

SNPs	Allele frequency, n (%)				Genotype frequency, n (%)						HWE testing	
	Controls		Cases		Controls			Cases			Pearson chi-square, <i>P</i> value <sup>a</sup>	
	T	C	T	C	TT	TC	CC	TT	TC	CC	Controls	Cases
SNP1*	178 (79.5)	46 (20.5)	170 (75.9)	54 (24.1)	70 (62.5)	38 (33.9)	4 (3.6)	65 (58.0)	40 (35.7)	7 (6.3)	0.67	0.80
SNP2*	169 (75.4)	55 (24.6)	173 (77.2)	51 (22.8)	62 (55.4)	45 (40.2)	5 (4.5)	66 (58.9)	41 (36.6)	5 (4.5)	0.37	0.67
SNP3*	193 (86.2)	31 (13.8)	189 (84.4)	35 (15.6)	82 (73.2)	29 (25.9)	1 (0.9)	78 (69.6)	33 (29.5)	1 (0.9)	0.36	0.21

\* SNP1 = rs1800802, SNP2 = rs4236, SNP3 = rs1049897

<sup>a</sup> = Analyzed by DeFinetti web-based program

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

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

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

- Cancela, L., Hsieh, CL., Francke, U., Price, PA. (1990). Molecular structure, chromosome assignment, and promoter organization of the human matrix Gla protein gene. *J Biol Chem* 265, 15040-15048.
- Gao, B., Yasui, T., Itoh, Y., Tozawa, K., Hayashi, Y., Kohri, K. (2007). A polymorphism of matrix Gla protein gene is associated with kidney stones. *J Urol* 177, 2361-2365.
- Price, PA., Urist, MR., Otawara, Y. (1983). Matrix Gla protein, A new gamma-carboxyglutamic acid-containing protein which is associated with the organic matrix of bone. *Biochem Biophys Res Commun* 117, 765-771.
- Price, PA. (1989). Gla-containing proteins of bone. *Connect Tissue Res* 21, 51.
- Lian, JB., Prien EL. Jr., Glimcher MJ., Gallop OM. (1977) The presence of protein-bound gamma-carboxyglutamic acid in calcium-containing renal calculi. *J Clin Invest* 59, 1151.

## ACKNOWLEDGMENTS

This work is supported by Thailand Research Fund Senior Research Scholar to Prof. Pa-thai Yenchitsomanus and Mahidol University research grant.