Association study of the \textit{MMP1} gene polymorphism with knee osteoarthritis in Thais by high resolution melting (HRM) analysis

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\textbf{ABSTRACT}

Osteoarthritis (OA) is the most common chronic joint disease in aging population. It is characterized by degeneration and loss of cartilage at the joint surface, accompanied by subchondral bone changes. Since an important characteristic of OA is the loss of collagen network, matrix metalloproteinases (MMPs) with collagenase activity are believed to participate as a possible cause of OA. This work aimed to investigate the association between a deletion SNP in \textit{MMP1} promoter (rs1799750; 2G/1G) and knee OA susceptibility in Thai population. The study subjects consisted of 108 knee OA patients and 119 healthy controls. SNP genotyping was carried out by real-time PCR based high resolution melting (HRM) analysis, and the melting profiles from all DNA samples were analysed with Precision Melt Analysis\textsuperscript{TM} software. The allele frequencies (2G and 1G) and genotype distributions (2G/2G, 2G/1G and 1G/1G) were compared between patients and controls using the StatCalc program. Results reveal that the 2G allele is the major allele in Thai population. No statistical association between alleles or genotypes and disease occurrence was seen, both with and without
gender stratification. In conclusion, a SNP in the promoter of *MMP1* was not associated with knee OA susceptibility in a Thai population.

**Keywords:** Osteoarthritis; *MMP1*; SNP; rs1799750; HRM analysis; Thais

**INTRODUCTION**

Osteoarthritis (OA), a chronic degenerative joint disease, is the main cause of functional incapacity, physical limitation, and reduced quality of life in aging people around the world. The disease is characterized by the progressive degradation of articular cartilage together with joint space narrowing and subchondral bone changes (sclerosis and osteophytes formation), which result in joint pain, mild inflammation and restricted motion (Dieppe and Kirwan, 1994; Dieppe and Lohmander, 2005). OA is known as a multifactorial disease for several decades. Both personal risk factors such as gender, age, hormonal status, genetic character, and ethnicity, as well as local biomechanical factors such as obesity, physical activity, and joint injury play critical roles in the pathogenesis of the disease (Felson, 2004). The most commonly affected joints are small joints of the hands, knees, and hips (Hochberg *et al.*, 2012). While hand and hip OA have strong genetic background with estimated heritability of 65 and 60%, respectively, knee OA has lower heritability of 40%, and it is more likely to arise from biomechanical factors (Spector and MacGregor, 2004; Chaganti and Lane, 2011).

Unbalance between synthesis and degradation of the extracellular matrix (ECM) network in articular cartilage is one of the OA hallmarks. Although the replacement of proteoglycans is rather easy (Fell *et al.*, 1976; Jubb and Fell, 1980), a substantial damage to the adult collagen seems to be irreversible due to its very long turnover time (Maroudas *et al.*, 1992; Verzijl *et al.*, 2000). Therefore, the Zn$^{2+}$ dependent matrix metalloproteinases (MMPs) protein family has prompted much interest in OA pathophysiology (Murphy and Nagase, 2008). Among 20 MMPs, only MMP-1, -2, -8, -13 and -14 exhibit collagenase activity (Aimes and Quigley, 1995; Nagase and Woessner, 1999; Riley *et al.*, 2002). Interestingly, several MMPs are over expressed in synoviocytes and chondrocytes from OA joints (Wolfe *et al.*, 1993; Keyszer *et al.*, 1995; Borden *et al.*, 1996; Reboul *et al.*, 1996). This phenomenon has been shown to be influenced by single nucleotide polymorphisms (SNPs) in the promoter region of the genes (Rutter *et al.*, 1998; Ye, 2000). In 2009, a genetic association study in a Turkish population showed a significant association between a deletion SNP in the promoter of *MMP1* gene on chromosome 11q22.3 and knee OA susceptibility (Barlas *et al.*, 2009). This SNP is a deletion of the G nucleotide at the -1,607 position in the promoter region of the gene (GAAAG-|G|ATATG). Since the adjacent nucleotide of the SNP is also the G nucleotide, this SNP is also known as 2G/1G polymorphism. The results suggested that individuals with 1G/1G
and 2G/1G genotypes have a greater risk to develop knee OA than individuals with the 2G/2G genotype.

However, it is obvious that different population has different genetic characters, and several association studies had revealed discrepant results between different ethnic groups. Therefore, the objective of this study was to evaluate the role of polymorphism in the promoter of MMP1 as a risk factor of knee OA in Thai population.

MATERIALS AND METHODS

Study population

In total, 227 Thai participants were included in this study. The patient group consisted of 108 individuals, 20 men and 88 women, with a median age of 70 years (51–91 years). They all had grades 3-4 of radiographic signs of OA according to the Kellgren–Lawrence grading system (Kellgren and Lawrence, 1957) and undergone total knee replacement (TKR) surgery at Thammasat Commemoration Hospital. The control group consisted of 119 healthy individuals, 26 men and 93 women, with a median age of 55 years (50–60 years). They had annual check-up and routine screening at Chulalongkorn Memorial Hospital. All were diagnosed by physicians for no symptoms or signs of OA, other joint disorders and family history of those diseases. The protocol for taking the blood sample from knee OA patients has been approved by the Clinical Research Ethics Committee of the Faculty of Medicine, Thammasat University (protocol No. MTU-OT-4-CR010-010/53), whereas the protocol for healthy controls has been approved by the Clinical Research Ethics Committee of Faculty of Medicine, Chulalongkorn University (protocol No. 533/54). All research participants provided written informed consents.

Sample collection and DNA extraction

Peripheral blood from all study subjects was collected by standard venipuncture and kept at 4 °C in ethylenediaminetetraacetic acid (EDTA)-containing tubes until use. Genomic DNA was extracted from the whole blood using the innuPREP Blood DNA Master Kit (Analytikjena, Seerasen, Eisfeld, Germany) according to the manufacturer’s instructions. The DNA quantity and quality were assessed by the spectrophotometer at the wavelength of 260 and 280 nm. DNA was then diluted to the working concentration of 50 ng/µl and used as a template in SNP genotyping.

High Resolution Melting SNP genotyping

Genotyping of the rs1799750 (2G/1G) polymorphism was carried out by HRM analysis on the CFX96™ real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). The forward primer 5’-GCCACTTAGATGAGGAAATTGTAG-3’ and the reverse primer 5’-CGTCAAGACTGATATCTTACTCAT-3’ were used to amplify the interested region. The
resulting amplicon of the 1G genotype is 110 bp, whereas the 2G genotype is 111 bp. The real-time PCR was conducted in a total volume of 10 µl containing 50 ng of genomic DNA, 5 µl of EvaGreen Super mix (Bio-Rad Laboratories, Hercules, CA), 0.2 µM of each primer and 3.2 µl of sterile dH₂O. The thermal cycling protocol started with an initial denaturation at 98 ºC for 30 sec; then 40 cycles of 98 ºC for 2 sec, 54 ºC for 10 sec followed by a plate read. Melt profiles were generated by heating PCR products to 98 ºC for 30 sec, cooling it down to 55 ºC for 30 sec, and then slowly increasing the temperature by 0.2 ºC from 55 ºC to 80 ºC. The melt profile was tracked in the form of fluorescence signal from a 10 sec plate read at each temperature. The analysis of melt profiles from all samples was carried out by Precision Melt Analysis™ software (Bio-Rad Laboratories, Hercules, CA). Briefly, the software plots data for each temperature increment from all samples on the x-axis and fluorescence signal intensity on the y-axis. Both before and after the melt phase values are then rescaled (normalized) so that fluorescence intensity of each melt profile ranges from 0-1 (baseline noise to maximum signal). For each point of temperature, the average fluorescence value of the most common genotype in the run (baseline genotype) is calculated and afterward subtracted from normalized relative fluorescence unit (RFU) value of each sample to plot a difference curve. Different genotypes were discriminated by the automatic grouping function of the software based on their melting patterns in difference curve plotting. Each sample was determined in duplicate. Accuracy of HRM genotypic procedure was confirmed by direct DNA sequencing from several randomly selected samples.

**Statistical analysis**

For comparison of participants’ demographic data, Microsoft Excel 2007 was used. In addition, *unpaired* Student's *t*-test was used for the continuous traits, while Chi-square test was used for the categorical traits. The allele frequencies (2G and 1G) and genotype distributions (2G/2G, 2G/1G and 1G/1G) in patient and control groups were assessed for Hardy–Weinberg Equilibrium (HWE) using the previously reported program (Rodriguez *et al.*, 2009; http://www.oege.org/software/hardy-weinberg.html). The correlation between rs1799750 and knee OA susceptibility was determined using the StatCalc Program (AcaStat Software, Leesburg, VA). The odds ratio (OR) with 95% confidence intervals (CI) was analysed for the genotype-disease risk association. *P* value < 0.05 was considered statistically significant. The results were also analyzed in gender stratified groups to investigate the SNP effect in males and females.

**RESULTS**

In this study, the HRM was successfully analysed for rs1799750 (2G/1G) SNP genotyping in 227 subjects. Characteristics of the study population were
shown in our previous report (Poonpet et al., 2013). In summary, both patient and control groups have about the same average height, weight, body mass index (BMI), as well as the sex ratio. However, the average age of the patient group was significantly greater than that of the control group (Table 1).

**Table 1** Demographic data of knee OA patients and normal controls

<table>
<thead>
<tr>
<th>Variables</th>
<th>Controls</th>
<th>Knee OA patients</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=119</td>
<td>n=108</td>
<td></td>
</tr>
<tr>
<td>Female/ Male</td>
<td>93/26</td>
<td>88/20</td>
<td>0.53</td>
</tr>
<tr>
<td>Age (years)</td>
<td>54.5±2.7</td>
<td>69.9±8.4</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>63.5±12.1</td>
<td>63.6±10.9</td>
<td>0.95</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>157.2±6.3</td>
<td>155.6±7.1</td>
<td>0.06</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.7±4.3</td>
<td>26.3±4.5</td>
<td>0.27</td>
</tr>
</tbody>
</table>

Note: *statistically significant

The unique melting profiles were not exclusively dependent on their melting temperature (72.2 ºC for the 2G/2G genotype; 72.0 ºC for the 2G/1G and the 1G/1G genotypes) but also a vertical shift in relative fluorescence units on the y-axis, following reference (baseline) signal subtraction at each data collection temperature. The shape of difference curve from different genotypes was generated with the Precision Melt Analysis™ software. As shown in Fig. 1, the fluorescent signal from all samples were normalized (Fig. 1a), and the most common melting pattern in a run (mostly came from the 2G/1G genotype) was used as the baseline (red lines) for plotting difference curves (Fig. 1b). The 1G/1G genotypes (blue lines) displayed lower difference relative fluorescence units (RFU) than the 2G/2G genotypes (green lines) when compared with the 2G/1G baseline. The melting pattern of all PCR product samples were differentiated and clustered, with the aid of auto-grouping function of the program into three groups according to similar melting profiles as illustrated in Fig. 1c. The average percent confidence for genotype calling was 98.58% in cases and 98.70% in controls. The corresponding sequencing electropherograms are shown in Fig. 2.

The comparison of allele and genotype frequencies of the SNP between case and control are shown in Table 2. The distributions of all genotypes in patient and control groups were in Hardy–Weinberg equilibrium (P>0.05). The overall allele frequencies for the 2G and the 1G alleles were 57.8% and 42.2%, respectively. The 2G/1G genotype was the most frequent genotype in both OA patients.
Figure 1 The rs1799750 SNP genotypes of the study population were investigated by HRM analysis using Precision Melt Analysis software. a, melt profile of the 2G/2G (green lines), 2G/1G (red lines) and 1G/1G genotypes (blue lines) after normalization; b, difference curve plotting from data in Fig. 1Aa using the 2G/1G genotype as the baseline. c, Melt profiles were discriminated by the auto-grouping function of the program into 3 genotypes. RFU, relative fluorescence unit.

(44.4%) and control group (45.4%). There was no significant difference between patient and control groups regarding genotype distributions or allele frequencies in pooled or gender stratified groups.

DISCUSSION

High-resolution melting (HRM) analysis is commonly used to investigate genetic variations in various diseases based on the difference in melting profiles of PCR products with different DNA sequences such as SNP. This cost-effective method allows rapid SNP genotyping by real-time PCR system without the need for expensive labeled probes or allele-specific primers (Reed et al., 2007; Erali et al., 2008).

In this work, we determined the relationship between a SNP (rs1799750 in MMP1) and knee OA susceptibility in Thai population using HRM analysis. The 2G allele is the ancestral allele and considered as the major allele in Asian (67%), American (56%) and European (51%) populations, whereas its frequency is lower than the 1G allele in African population (45 vs 55%) (The International HapMap Consortium, 2003). This promoter polymorphism has been shown to influence the
transcriptional regulation of the MMP-1 protein. The presence of the 2G allele creates the consensus sequence for the Ets family of transcription factors (AAGGAT). Thus, the 2G allele possesses greater transcriptional activity than the 1G allele (Rutter et al., 1998; Ye, 2000; Price et al., 2001).

![Electropherograms](image)

**Figure 2** Electropherograms of the corresponding sequencing analysis. From top to bottom, the first electropherogram shows the 2G/2G sample, the second shows the 2G/1G sample and the third shows the 1G/1G sample, the regions that show nucleotide variation were highlighted by a dashed box. Mixed electropherograms due to a single strand G deletion was observed in the 2G/1G sample.

Results from some studies concerning the role of this SNP were controversial and conflicting. In one study, the 2G allele frequency was significantly higher in the group of ovarian cancer than in the control. The MMP1 expression levels in ovarian cancer patients carrying 2G alleles were increased significantly when compared with 1G homozygotes (Kanamori et al., 1999). In addition, clinical researches on colorectal tumors and lung cancer also showed similar results (Ghilardi et al., 2001; Zhu et al., 2001). On the contrary, studies in sarcoidosis (Ninomiya et al., 2004), endobronchial tuberculosis (TB) (Kuo et al., 2008), primary sclerosing cholangitis (PSC) (Wiencke et al., 2004), as well as the previous reports of knee OA (Barlas et al., 2009) have shown a significant increase in 1G/1G or 2G/1G genotypes in patients. Nevertheless, there was no significant association to knee OA neither from the 2G allele nor the 1G allele in the present study. The discrepancy in these reports could be attributed to different genetic background, population size, gene pool, age and sex ratio of the study populations. In addition, after joint injury and inflammation, the mean levels of proMMP-1 and MMP-1 activities were increased (Tchetverikov et al., 2005). The increased levels of activated MMP-1 in some studies might be, at least partially, a consequence of knee OA pathophysiologic background not related to MMP1 polymorphism. However, the MMP1 gene expression and protein activity were not determined for the patients in this work. Finally, some environmental factors or gene-environment interaction in each population such as selected physical activity, diets and lifestyles may affect the roles of investigated SNP to knee OA susceptibility.

In conclusion, the deletion SNP in MMP1 promoter (rs1799750) was not
Table 2: Genotype distribution and allele frequency of *MMP1* polymorphism between knee OA patients and normal controls

<table>
<thead>
<tr>
<th>rs1799750</th>
<th>OA</th>
<th>Control</th>
<th>OR (95% CI)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1G/1G</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole group</td>
<td>20 (18.5%)</td>
<td>25 (21.0%)</td>
<td>0.80 (0.36-1.78)</td>
<td>0.55</td>
</tr>
<tr>
<td>Female</td>
<td>15 (17.0%)</td>
<td>19 (20.4%)</td>
<td>0.81 (0.33-2.02)</td>
<td>0.63</td>
</tr>
<tr>
<td>Male</td>
<td>5 (25.0%)</td>
<td>6 (23.1%)</td>
<td>0.71 (0.10-4.78)</td>
<td>0.69</td>
</tr>
<tr>
<td>2G/1G</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole group</td>
<td>48 (44.4%)</td>
<td>54 (45.4%)</td>
<td>0.89 (0.47-1.67)</td>
<td>0.69</td>
</tr>
<tr>
<td>Female</td>
<td>40 (45.5%)</td>
<td>40 (43.0%)</td>
<td>1.03 (0.51-2.08)</td>
<td>0.93</td>
</tr>
<tr>
<td>Male</td>
<td>8 (40.0%)</td>
<td>14 (53.8%)</td>
<td>0.49 (0.10-2.43)</td>
<td>0.32</td>
</tr>
<tr>
<td>2G/2G</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole group</td>
<td>40 (37.1%)</td>
<td>40 (33.6%)</td>
<td>1**</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>33 (37.5%)</td>
<td>34 (36.6%)</td>
<td>1**</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>7 (35.0%)</td>
<td>6 (23.1%)</td>
<td>1**</td>
<td></td>
</tr>
<tr>
<td>Allele (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1G</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Whole group</td>
<td>88 (40.7%)</td>
<td>104 (43.7%)</td>
<td>0.89 (0.60-1.31)</td>
<td>0.52</td>
</tr>
<tr>
<td>Female</td>
<td>70 (39.8%)</td>
<td>78 (41.9%)</td>
<td>0.91 (0.59-1.42)</td>
<td>0.68</td>
</tr>
<tr>
<td>Male</td>
<td>18 (45.0%)</td>
<td>26 (50.0%)</td>
<td>0.82 (0.33-2.03)</td>
<td>0.63</td>
</tr>
<tr>
<td>2G</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole group</td>
<td>128 (59.3%)</td>
<td>134 (56.3%)</td>
<td>1**</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>106 (60.2%)</td>
<td>108 (58.1%)</td>
<td>1**</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>22 (55.0%)</td>
<td>26 (50.0%)</td>
<td>1**</td>
<td></td>
</tr>
</tbody>
</table>

* compared between the test genotype to the 2G/2G phenotype, and the test allele to the 2G allele
**Reference genotype/allele.

associated with the occurrence of knee OA in Thai population reported herein. This result draws attention to the importance of each population’s own genetic association study and SNP database. However, the potential limitation in this study is the small size of the study population and age difference between patients and controls. The association study in larger samples and in different ethnic groups need to be done to see whether the 2G/1G polymorphism in promoter of *MMP1* should be used as a genetic marker in a certain
population, with the future goal to improve therapeutic approach of OA by targeting the specific MMP enzymes and related molecules.

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