Summary

Hip dysplasia (HD) is a polygenic disease characterized by joint laxity and lack of congruence leading to osteoarthritis (OA). Under many pathogenic conditions including arthritis, MMP-1 synthesis is augmented. The MMP-1 gene promoter contains cytokine response elements, transcription factors AP-1 and SAF-1 that are increased in OA. The objective of the present study was to identify and characterize new polymorphisms of the canine MMP-1 promoter gene and evaluate their association with OA secondary to HD in different dog breeds. Based on the NCBI_006587 reference sequence, two novel polymorphisms, Indel CTGCCCT (bp31986794) and C>T (bpSNP 31986815) substitution were selected due their position in a consensus sequence belonging to a SAF-1 response element. Data was uploaded in the GenBank database (accession number GQ475524 and GQ475525). 125 dogs of different breeds were sampled and hip status was evaluated through ventrodorsal extended-hip radiographs. A chi-squared analysis was performed to test the association of the variables: breed, sex and genotype for each polymorphism with OA status (healthy and affected). No significant association (p > 0.05) was found between any variable and OA secondary to HD. This study does not exclude MMP-1 as a gene responsible of OA secondary to HD in the breeds sampled because only the promoter sequence was evaluated.

Key words: (canis lupus familiaris), (hip dysplasia), (osteoarthritis), (MMP-1 gene), (polymorphisms).
Introduction

Hip dysplasia (HD) in dogs is a developmental orthopaedic disease in which abnormal development of a hip joint leads to functional laxity. This results in cartilage degradation, osteophyte formation, subchondral sclerosis and ultimately osteoarthritis (OA)\(^6\). It has been shown that synovial inflammation in OA triggers the synthesis of biological stimuli such as cytokines and growth factors which subsequently affect the chondrocytes leading to cartilage destruction\(^12\). Candidate genes associated with the molecular pathogenesis of the disease have been those related with inflammation such as cytokines and structural components of extracellular matrix\(^3,4,5\). In a study of Labrador Retriever and Greyhound crossbreeds, putative quantitative trait locus (QTL) contributing to macroscopic evidence of hip OA was identified in CFA05\(^9\) close to where MMP-1 is located. MMP-1 belongs to a family of zinc-dependent endopeptidases that collectively can degrade all components of extracellular matrix and is able to cleave native collagen triple helix allowing the chains to unwind and making them susceptible to other MMPs for proteolytic degradation. Due to its extensive proteolytic capability, expression of MMP-1 protein remains very low and highly regulated. However under many pathogenic conditions, including arthritis, MMP-1 synthesis is seen to be increased\(^1,2,7,8,10,11,12,13\). MMP-1 synthesis is mediated primarily by transcriptional induction for this gene, and the promoter of the gene

Resumen

La displasia de cadera (DC) es una enfermedad poligénica caracterizada por laxitud articular y pérdida de congruencia que conduce a la osteoartritis (OA). En muchas condiciones patológicas, incluyendo a la artritis puede observarse un aumento de la síntesis de MMP-1. El promotor del gen MMP-1 contiene elementos de respuesta a citocinas, los factores de transcripción AP-1 y SAF-1 que se hallan aumentados en OA. El objetivo del presente estudio fue la identificación y análisis de polimorfismos en el promotor del gen MMP-1 canino y su asociación a la OA secundaria a DC. Basados en la secuencia de referencia NCBI_006587, se detectaron dos nuevos polimorfismos, Indel CTGCCCT (bp31986794) y una sustitución C>T (bp31986815) que fueron seleccionadas para su análisis debido a su posición en la secuencia de consenso perteneciente al elemento de respuesta SAF-1. Los polimorfismos detectados se ingresaron a la base del GenBank (números de acceso GQ475524 y GQ475525). Se tomaron muestras de 125 perros de diferentes razas y se evaluó el estado de la cadera por medio de radiografías en posición ventro-dorsal. Se realizó un análisis estadístico por medio de un chi cuadrado para probar la asociación entre las variables raza, sexo y genotipo con el estado para OA (sanos y afectados). No se hallo asociación (p>0.05) entre ninguna variable y la condición de OA secundaria a DC. El estudio no excluye al gen MMP-1 como candidato responsable de OA secundaria a DC en las razas estudiadas ya que sólo se ha evaluado la región promotora del mismo.

Palabras clave: (canis lupus familiaris), (displasia de cadera), (osteoartritis), (gen MMP-1), (polimorfismos).
has been well characterized\textsuperscript{1,2,11,12}. Multiple cytokine – responsive elements were identified in the proximal promoter region of canine \textit{MMP-1} gene using a series of progressively deleted reporter constructs\textsuperscript{13}. These included DNA binding elements of activator protein 1 (AP-1), and serum amyloid A activating factor 1 (SAF-1). Mutation of AP-1 and SAF-1 elements resulted in marked reduction in the cytokine osteoarthritic cartilage tissue. Evidence of abundant expression of the inflammation-responsive transcription factor SAF-1 in moderate to severely damaged OA cartilage tissues was presented. In contrast, cells in normal cartilage matrix contain very low levels of SAF-1 protein. SAF-1 is identified as a major regulator of increased \textit{MMP-1} synthesis\textsuperscript{12}. Genetic polymorphisms can alter the binding sites of some regulatory factors and influence the \textit{MMP-1} level expression.

The objective of the present study was to identify and characterize new polymorphisms of the canine \textit{MMP-1} promoter gene and evaluate their association with OA secondary to HD in different dog breeds.

\textbf{Materials and methods}

Animals: 125 dogs from different breeds (Table 1) were sampled. The dogs belong to private owners and the Army Forces. Hip status was evaluated through ventrodorsal extended-hip radiographs at the Veterinary Hospital “Ernesto Cánepa” Veterinary School, Buenos Aires University. The animals were classified as either healthy or affected. Animal handling was according to the Committee of Care and Use of Experimental Animals belonging to the Veterinary School, Buenos Aires University.

DNA sampling and analysis: whole blood samples were taken with EDTA and genomic DNA was isolated by I Illustra blood genomicPrep Mini Spin Kit (GE Healthcare). PCR was performed to amplify the fragment corresponding to the 5’ proximal region of \textit{MMP-1} gene. Primers were designed according to the sequence of canine \textit{MMP-1} gene (NCBI\_006587 sequence reference) using the online primer design procedure Primer 3.0 (Primer 3.0, Whitehead Institute for Biomedical Research, www.cbr.nrc.ca/cgi-bin/primer3_www.cgi). All PCR reactions were carried out in a total volume of 25 µl with 50 ng of canine genomic DNA. PCR conditions are described in Table 2. PCR products of 525 base pair (bp) from 14 dogs of different breeds, both healthy and affected, were individually sequenced to screen polymorphisms. Sequencing was performed on an ABI 3130XL automated sequencer. PCR products that gave unclear results by direct sequencing were ligated into pGEM-T Easy vector (Promega). Each recombinant DNA was then transformed into DH5-\textit{α} \textit{Escherichia coli} component cells. Two clones of each genotype were selected and sequenced from both directions. All the sequences were comparatively analyzed for polymorphisms using the BioEdit sequence analysis software (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html).

Polymorphisms selection: polymorphisms identified were annotated to a genomic position by alignment of the sequence with the \textit{MMP-1} canine sequence (NCBI reference sequence NC\_006587). Data was uploaded in the GenBank database (accession number GQ475524 and GQ475525). An Indel CTGCCCT (bp 31986794) and C>T (bp 31986815) substitution were analyzed due their position in a consensus sequence belonging to a SAF-1 response element (Figure 1). PCR conditions, primers and the endonuclease used to genotype these two polymorphisms are described in Table 2. For the Indel an allele specific PCR was designed, the amplicons of 80 and 87 bp were run by electrophoresis in 12 % polyacrylamide gel and then stained with ethidium bromide. No restriction enzymes were found with a recognition site spanning the C>T substitution, therefore a forward mismatched primer was designed in order to create a restriction site with the web-based program dCAPS Finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.html). The C>T polymorphism was confirmed by digestion with the \textit{Hha} I endonuclease (FastDigest \textsuperscript{®} Fermentas). Digestion was conducted for 16
Table 1. Frequency of alleles in an Indel (80/87; 31986794) and \textit{Hha} I (C>T; 31986815) polymorphisms in Labrador Retriever (LR), Belgian Malinois (BM), German Shepherd Dog (GSD), Golden Retriever (GR), Giant Schnauzer (GS) and Rottweiler (R) in groups of affected and healthy dogs.

<table>
<thead>
<tr>
<th>Breed</th>
<th>LR</th>
<th>BM</th>
<th>GSD</th>
<th>GR</th>
<th>GS</th>
<th>R</th>
<th>Affected</th>
<th>Healthy</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>66</td>
<td>15</td>
<td>17</td>
<td>12</td>
<td>8</td>
<td>7</td>
<td>35</td>
<td>90</td>
</tr>
<tr>
<td>Indel</td>
<td>80</td>
<td>0.45</td>
<td>0.47</td>
<td>0.97</td>
<td>1</td>
<td>0.5</td>
<td>0.86</td>
<td>0.65</td>
</tr>
<tr>
<td>Hha I C</td>
<td>0.78</td>
<td>0.70</td>
<td>1</td>
<td>0.92</td>
<td>0.69</td>
<td>0.93</td>
<td>0.86</td>
<td>0.80</td>
</tr>
<tr>
<td>Hha I T</td>
<td>0.22</td>
<td>0.30</td>
<td>0</td>
<td>0.08</td>
<td>0.31</td>
<td>0.07</td>
<td>0.14</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Table 2. Primer sequences and PCRs conditions for polymorphisms analyzed in \textit{MMP-1} gene

<table>
<thead>
<tr>
<th>SNP Primers</th>
<th>Indel</th>
<th>C/T</th>
<th>Promoter</th>
</tr>
</thead>
<tbody>
<tr>
<td>F: 5’ AGCGCACAGCACACCTACCAG3’</td>
<td>F: 5’ GCCTTGATTTGAGGAGCCCGG 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R: 5’ GGCTATGAAGTGCTCCAGGCT 3’</td>
<td>R: 5’ CAGAAATGGTGACGCCATGCAG 3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>“annealing” temperature</th>
<th>62º C</th>
<th>60º C</th>
<th>63 ºC</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO (%)*</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Cl2 Mg (mM)</td>
<td>2 mM</td>
<td>2,5 mM</td>
<td>2 mM</td>
</tr>
<tr>
<td>PCR product (bp)</td>
<td>87</td>
<td>235</td>
<td>525</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Restriction Endonuclease</th>
<th>Hha I</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Allele (pb)</th>
<th>+: 87</th>
<th>T: 235</th>
<th>C: 19 + 216</th>
</tr>
</thead>
</table>

* DMSO: Dimethyl sulfoxide

Figure 1. Putative binding site for SAF-1 transcription factor in the promoter region of canine \textit{MMP-1} gene. In bold the polymorphisms analyzed in the present study
hours according to the manufacturer directions. The DNA fragments from the digested PCR products of 235 bp (allele T) and 216 + 19 bp (allele C) were separated on 3.5 % agarose gel and visualized by ethidium bromide staining.

Statistical Analysis: A chi-squared analysis was performed to determine the association of the variables breed, sex and genotype for each polymorphism with OA status using the program R version 2.10.1. Copyright (C) 2009 the R Foundation for Statistical Computing. ISBN 3-900051-07-0.

Results and discussion

Allele frequencies among different breeds and distribution between healthy and affected dogs can be seen in Table 1. No significant association (p>0.05) was found between any variable and OA secondary to HD in the dog.

An extensive body of literature supports an association of MMPs (including MMP-1) with a number of human diseases such as cancer, rheumatoid arthritis and other autoimmune disorders, cardiovascular and fibrotic diseases, suggesting that their inhibition or stimulation may have a therapeutic role. In arthritic disease a correlation between the increase of MMP-1 and collagen degradation has been demonstrated, suggesting that cleavage of cartilage collagen is directly related to the activity of this enzyme and can be blocked by a broad range of MMP-1 inhibitors in a dose dependent manner.

A study was done to compare gene expression in normal intake skin and wound margin biopsies in horses; an increased level of MMP-1 was seen within wound biopsies. MMP-1 participates in remodeling during wound healing in the horse and the differences in expression in body and limb lesions may contribute to the excessive proliferative response seen in the limb (keloid).

In dogs, immunohistochemical analysis indicated higher levels of MMP-1, SAF-1 and AP-1 proteins in osteoarthritic but not in normal cartilage tissue. These results show that induction and activation of AP-1 and SAF-1 transcription factors are involved in the regulation of MMP-1 expression in the chondrocytes which could be used as therapeutic targets to combat the pathogenesis of OA.

We report the partial sequence of the MMP-1 promoter region in six dog breeds that are commonly affected with OA secondary to HD. Two polymorphisms in the consensus region of SAF-1 principal transcription factor in up regulated the MMP-1 gene were analyzed but none of them could explain the difference in the illness status. The breeds in which the polymorphisms were identified are not necessarily closely related. This indicates that both polymorphisms may be older mutations. Because of the structure of dog breeds, diseases seen within breeds are identical by descent, therefore in which case other breeds should be tested in order to confirm the role of these polymorphisms. This study does not exclude MMP-1 as a gene responsible for OA secondary to HD in the breeds sampled, because only the promoter sequence was evaluated; others like the 3’ untranslated region that contains the sequence AUUUA which destabilizes the mRNA should be analyzed.

Conclusion

The polymorphisms Indel CTGCCCT (bp 31986794) and C>T (bp 31986815) substitution analyzed were not associated with the OA affected status in the breeds included in this study, however MMP-1 could not be excluded as a candidate gene in OA associated to HD.

Acknowledgements

The study was supported by grants from Buenos Aires University, Argentina, UBACYT 2008-2010. Project V405.

References


