Characterization of the Cheetah Serum Amyloid A1 Gene: Critical Role and Functional Polymorphism of a Cis-Acting Element

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Abstract

Amyloid A (AA) amyloidosis is one of the principal causes of morbidity and mortality in captive cheetahs (*Acinonyx jubatus*), which are in danger of extinction. For practical conservation of this species, therefore, it is critical to elucidate the etiology of AA amyloidosis, especially to understand the mechanisms of transcriptional regulation of serum amyloid A (SAA), a precursor protein of the AA protein. In this study, the structure and nucleotide sequence of the cheetah *SAA1* gene including the 5′-flanking promoter/enhancer region was determined. Putative nuclear factor kappa-B (NF-κB) and CCAAT/enhancer binding protein β (C/EBPβ) cis-acting elements, which play key roles in SAA1 transcriptional induction in response to inflammation, were identified in the 5′-flanking region of the cheetah *SAA1* gene. Fortuitously, a single nucleotide polymorphism was identified in the captive cheetah cohort in the putative NF-κB cis-acting element and had a remarkable effect on SAA1 transcriptional induction. These results provide a foundation not only for clarifying the etiology of AA amyloidosis in the cheetah but also for contriving a strategy for conservation of this species.
had been implicated in the AA amyloidosis (Glenner 1980; Hoffman et al. 1984) and the pathogenesis of atherosclerosis (Van Lenten et al. 1995).

This dramatic change in the blood SAA1 level is primarily regulated at the transcriptional level (McAdam and Sipe 1976; Morrow et al. 1981; Lowell et al. 1986). Previous studies using primary hepatocytes and cultured liver cells revealed that interleukin-1 (IL-1), IL-6, and tumor necrosis factor, which are produced during an acute-phase response, regulate human SAA gene expression through induction of a series of other downstream transcription factors. Among the factors, C/EBP and NF-κB appeared to play key roles in SAA mRNA expression in humans (Edbrooke et al. 1989; Betts et al. 1993), as well as in rats (Li and Liao 1992), and rabbits (Ray et al. 1999). These factors function by binding to C/EBP and NF-κB cis-acting elements within the proximal promoter region of the gene (Woo et al. 1987; Edbrooke et al. 1989; Betts et al. 1993; Ray A and Ray BK 1993; Ray BK and Ray A 1993).

The cheetah (Acinonyx jubatus) is a member of the cat family and well known as the fastest of all land animals. Cheetahs are included on The World Conservation Union list of vulnerable species. Although efforts have been made in wildlife sanctuary parks and zoos worldwide to prevent extinction of the species, propagation of the cheetah is a formidable task. The principal problem is inbreeding depression, which is ascribable to reduced population size during ice age. The depression is manifested as a high degree of juvenile mortality and decreased fertility due to spermatozoa abnormalities in ejaculates (O’Brien et al. 1985). What makes the matter worse, AA amyloidosis is increasingly becoming an important cause of morbidity and mortality in captive cheetahs (Papendick et al. 1997). The prevalence of AA amyloidosis in captive cheetahs increased from 20% in pre-1990 necropsies to an unusual 70% of cheetahs necropsied in 1995. It is, therefore, important to understand the etiology of AA amyloidosis in the cheetah for effective management of this vulnerable species.

In this study, the structure and nucleotide sequence of the cheetah SAA1 gene, including the promoter/enhancer region, was characterized. The molecular mechanisms for transcriptional regulation of the cheetah SAA1 gene were also partially clarified.

**Materials and Methods**

**Cheetah Specimens**

Fourteen postmortem cheetahs (A. jubatus) collected from various zoos in Japan were examined. Five cheetahs (ID number: C69, C76, C77, C81, and C86) were imported from Africa and raised in Japan until they deceased. Both relationship and genetic background of these individuals were unknown. Three of these cheetahs (C69, C76, and C77) had no discernible AA amyloid deposition, whereas C81 had minor and C86 had relatively severe AA amyloid deposition. Other 9 cheetahs were bred in Japan (Figure 1). Seven of them were from 1 relatively large pedigree. All these individuals had relatively severe AA amyloid deposition. C87 and C82 were from different parental pairs and had minor and no discernible AA amyloid deposition, respectively. A male cheetah (C68), which died at the age of 4 years, was extensively necropsized. Severe AA amyloidosis in this cheetah was systemic and affected organs included the kidney, liver, heart, spleen, intestine, stomach, and lymph nodes. Genomic DNA was isolated from the postmortem liver of cheetah C68. Total RNA was also isolated from the postmortem liver of cheetah C68.

**Cloning and Sequencing of the Cheetah SAA1 Gene**

We isolated liver amyloid protein from C68 and separated it with 16.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After Coomassie brilliant blue staining, the AA protein bands were excised and sequenced. The amino acid sequence of AA amyloid protein determined was identical to that reported in a previous study (Johnson et al. 1997), except for 3 additional amino acids in C-terminal. The cheetah AA amyloid protein had high homology to that of other animal species such as cat, cow, and mouse (Liu et al. 2007), particularly to that of cat (Felis catus; AAD24489). There were only 2 amino acid differences in the 93 amino acids of the cheetah compared with cat AA protein (Johnson et al. 1997). The 75th and 88th residues in the 93 amino acid sequences were serine and alanine in cat. Based on this result, we inferred that the SAA nucleotide sequences would be conserved between the 2 species and designed primers (sense-1, antisense-1, antisense-2, and sense-3) based on the nucleotide sequence.
First, we performed polymerase chain reaction (PCR) amplification using genomic DNA from C68 as template with the sense-1 and antisense-1 primers. Direct sequencing of the PCR product with these primers revealed the nucleotide sequence of intron 3. Additional primers of sense-2 and antisense-3 (Figure 2 and Table 1) were designed based on the determined intron 3 sequence. Then, PCR amplification was performed using 2 pairs of primers (sense-2 and antisense-2) and (sense-3 and antisense-3), followed by direct sequencing of the PCR product. As a result, we obtained the genomic DNA fragments covering the entire coding sequence, which corresponded to the region from a portion of exon 2 to the upstream part of exon 4.

**Figure 2.** Nucleotide sequence of the cheetah SAA1 gene. Nucleotide sequence from upstream of the putative TATA box (underlined) to downstream of the putative polyadenylation signal sequence (underlined) is shown. Exon and intron sequences are denoted with upper and lower case letters, respectively. The position +1, corresponding to the transcription start site of exon 1 in the cheetah SAA1 gene, was inferred from a comparison between the cheetah and human SAA1 genes (also see Figure 3; Moriguchi et al. 2001). The coding sequence is underlined and aligned with the predicted amino acid sequence. The putative N-terminal signal peptide and 18 residue peptide sequences missing in the isolated AA amyloid fibrils are marked by asterisks and solid circles, respectively. Positions of the oligonucleotide primers used for isolation and sequencing were indicated by arrows.
Table 1. Oligonucleotide primers used for isolation and sequencing of the cheetah SAA1 gene

<table>
<thead>
<tr>
<th>Primers</th>
<th>Oligonucleotide primer sequence</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sense-1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5'-GGGCTTGGGACATGTTG-3'</td>
<td>2338-2354 (exon 3)</td>
</tr>
<tr>
<td>Antisense-1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5'-CTTCGAGCTCCTCTG-3'</td>
<td>2961-2941 (exon 4)</td>
</tr>
<tr>
<td>Sense-2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5'-ATGGGCAATTGCTCCTCAGC-3'</td>
<td>2825-2844 (intron 3)</td>
</tr>
<tr>
<td>Antisense-2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5'-TCAGTACCTTGCAAGCCAGG-3'</td>
<td>3045-3026 (exon 4)</td>
</tr>
<tr>
<td>Sense-3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5'-GAAAGAGTATTCGTTGCTGGCGAGGCTG-3'</td>
<td>606-639 (exon 2)</td>
</tr>
<tr>
<td>Antisense-3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5'-CCAGCACTGTTCCAGACCCAGG-3'</td>
<td>2542-2503 (intron 3)</td>
</tr>
<tr>
<td>Walking primer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R1</td>
<td>5'-GACACGGGACACCTTACATCGAC-3'</td>
<td>652-625 (intron 2)</td>
</tr>
<tr>
<td>R2</td>
<td>5'-GCTTACCTGAGCAGCCTGGCAAG-3'</td>
<td>545-519 (junction of intron 2 and exon 2)</td>
</tr>
<tr>
<td>F1</td>
<td>5'-CTTCTCTGCAATCCAGCAGG-3'</td>
<td>2868-2893 (junction of intron 3 and exon 4)</td>
</tr>
<tr>
<td>F2</td>
<td>5'-CACGGGAGGACTGCAAGGCTG-3'</td>
<td>2941-2967 (exon 4)</td>
</tr>
<tr>
<td>RT-PCR primer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F3</td>
<td>5'-TCTGCTCCACTGGGCTCAG-3'</td>
<td>14-33 (exon 1)</td>
</tr>
<tr>
<td>R3</td>
<td>5'-GCTAGCCCTGAGTCTCTTGC-3'</td>
<td>2967-2947 (exon 4)</td>
</tr>
<tr>
<td>Genotyping primer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5'-TCGACGGTCGCTGAGGACAGGACAGAC-3'</td>
<td>(−244)−(−262) (promoter)</td>
</tr>
<tr>
<td>R4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5'-CCGCTCGAGTTGGAGGAGGCAGGCTGGGACCAAG-3'</td>
<td>22-3 (exon 1)</td>
</tr>
</tbody>
</table>

<sup>a</sup> The oligonucleotide primer sequence was designed based on the nucleotide sequence of *Felis catus* SAA mRNA (AF136718).

<sup>b</sup> Oligonucleotide sequence underlined in F4 and R4 primers was cheetah-specific sequence, whereas the 5'-terminal sequence contained restriction site.

Genomic sequences covering the region upstream of exon 2 and downstream of exon 4 were obtained by genome walking using the Universal GenomeWalker kit (BD Bioscience Clontech, Palo Alto, CA), according to the manufacturer’s instructions. For isolation of the upstream region of the cheetah SAA1 gene, SAA1-specific outer and nested primers (R1 and R2) were used. For isolation of the downstream region of exon 4 and downstream sequence, cheetah SAA1-specific outer and nested primers (F1 and F2) were used. PCR products were sequenced directly using nested primers. To obtain the noncoding exon 1 and exon 2, reverse transcriptase–polymerase chain reaction (RT-PCR) was performed using total RNA from the liver of cheetah C68 with 2 cheetah SAA1-specific primers (F3 and R3) (Table 1).

Genotyping of Cheetahs for a Single Nucleotide Polymorphism in the Putative NF-κB Responsive Element

Chromosomal DNA fragments containing the putative NF-κB responsive element of the cheetah SAA1 gene were amplified by PCR from genomic DNA followed by direct sequencing with 2 primers (F4 and R4) (Table 1).

Promoter Activity Assay

The chromosomal DNA fragment containing 795 bp of 5'-flanking sequence and 24 bp of untranslated exon 1 of the cheetah SAA1 gene was amplified by PCR with the following primers, which contained MluI or XbaI sites at their 5' and 3' ends, respectively: 5'-primer (5'-TCAGCGTGCTCCTACGAGGCAGGACGCTGTA-3') and 3'-primer (5'-CCGCGCTCACTGGAGGACAGGCTGACG-3'). Promoter reporter plasmid pGL3-SAA1-795 was constructed by inserting the DNA fragment into the pGL3-Basic plasmid (Promega, Madison, WI). Four 5' deletion constructs (pGL3-SAA1-526, pGL3-SAA1-263, pGL3-SAA1-175, and pGL3-SAA1-84) were generated in a similar fashion by using the 3'-primer described above paired with different 5'-primers. Two deletion constructs for the putative C/EBPβ and NF-κB elements (pGL3-SAA1-noCE and pGL3-SAA1-no-NF) were made by inverse PCR with the pGL3-SAA1-795 plasmid as a template and primers directed outward from the elements and introduced EozRI sites at their 5' ends. PCR products were digested with EozRI, self-ligated, and cloned. The sequence of all recombinant plasmids was verified.

HepG2 cells were seeded in 6-well plates with Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 50 U/ml penicillin–streptomycin. When the cells were 90–95% confluent, they were transfected with 4–8 μg of plasmid DNA and 0.08 μg of pRL-TK plasmid (Promega, Madison, WI) according to the manufacturer’s instructions. To reproduce an inflammatory condition, 48 h after transfection, cells were stimulated with recombinant human IL-6 and IL-1β (Peprotech EC Ltd., London, United Kingdom) at final concentrations of 10 ng/ml and 0.1 ng/ml, respectively, for 3 h (Hagihara et al. 2005). Cells were then lysed in passive lysis buffer and assayed with the Dual-Luciferase Reporter Assay System (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Assays were conducted in triplicate, and the experiments were repeated at least 3 times. Data are expressed as mean ± standard deviation of 3 independent experiments. Statistical
significance was assessed with the Student's t-test. P values, 0.05 were considered significant.

Results
Structure and Nucleotide Sequence of the Cheetah SAA1 Gene

The complete nucleotide sequence (3300 bp) spanning the entire coding region of the cloned cheetah SAA1 gene is presented in Figure 2. Comparison of amino acid sequence deduced from the nucleotide sequence to the previously determined amino acid sequence of the cheetah SAA protein allowed assignment of 3 coding exons (exons 2–4). Human SAA1 and mouse Saa1 genes have a short non-coding exon 1. The cheetah gene also had a sequence with high homology to the noncoding exon 1 of these 2 species (Figures 2 and 3). Thus, the overall structure of the cheetah SAA gene was highly similar to that of the human SAA1 gene and mouse Saa1 (data not shown).

The isolated AA amyloid protein was determined as a protein with 93 amino acid residues, whereas the precursor SAA protein deduced from the obtained nucleotide sequence consisted of 129 amino acid residues (Figure 2). This precursor protein contained N-terminal hydrophobic sequence (18 amino acids), which was not present in the AA amyloid protein isolated from the cheetah liver (marked by asterisk in the 5’ region in Figure 2). This N-terminal sequence is presumably a signal peptide for secretion, which is inherently removed during secretion from hepatocytes. The reading frame for translation in putative exon 4 was extended for 18 amino acid residues (marked by solid circles) beyond a codon for the C-terminal glycine residue of the isolated AA amyloid protein. This sequence is likely to be lost during AA amyloid fibril formation. Removal of signal and C-terminal peptides was demonstrated for AA amyloid of other mammalian species.

Characterization of Cis-Acting Elements in the Upstream Region of the Cheetah SAA1 Gene

The 5’-flanking sequence of approximately 800 bp of the cheetah SAA1 gene was isolated (Figure 3). When inserted in the luciferase reporter plasmid (pGL3-SAA1-795) and used in the promoter assay under artificial inflammatory conditions, this sequence gave 8 times higher relative luciferase activity than that of the mock pGL3 plasmid under the same conditions, suggesting the presence of promoter/enhancer elements in this 795-bp sequence (Figure 4). To define more precisely the location of functionally important cis-elements in this region, we prepared a series of 5’-end deletion reporter constructs. Two deletion reporter constructs, pGL3-SAA1-526 and pGL3-SAA1-263, had almost identical luciferase activities to the original pGL3-SAA1-795 (Figure 4). Further deletion of the 5’ end from the putative promoter/enhancer region, represented by pGL3-SAA1-175, resulted in a dramatic reduction in luciferase activity compared with the intact pGL3-SAA1-795. These results narrowed the location of cis-elements in the 5’-flanking 263-bp sequence, which confer promoter/enhancer activity under inflammatory conditions.
Figure 4. Assignment of the cis-elements in the promoter/enhancer region of the cheetah SAA1 gene. HepG2 cells were transfected with 4 μg the intact or a series of 5’ deletion or internal deletion reporter constructs. Cytokine stimulation was performed with IL-6 (10 ng/ml) and IL-1β (0.1 ng/ml) for 3 h. Cells were then lysed, and the luciferase activities were determined. The relative luciferase activity is presented as a ratio to that of the mock pGL3-Basic plasmid. Data presented here represent the mean values of 3 independent experiments.

Nucleotide sequences of the 263-bp putative promoter/enhancer region of the cheetah SAA1 gene are 70% homologous to that of the corresponding region of the human SAA1 gene (Figure 3). Within this region of the human gene are 2 cis-acting elements for C/EBPβ and NF-κB, both of which play critical roles in transcriptional induction of SAA1 (Edbrooke et al. 1991; Betts et al. 1993; Kumon et al. 2002). Nucleotide sequences of NF-κB and C/EBPβ target sites were highly conserved between these 2 species. We then explored the possibility that the C/EBPβ and NF-κB responsive element-like sequences in the cheetah SAA1 gene are functionally important. The relative luciferase activity of the reporter construct lacking the putative C/EBPβ-binding site and upstream sequence but that retained the putative NF-κB responsive element (pGL3-SAA1-175) was reduced to less than 50% of that of intact pGL3-SAA1-795 (Figure 4). When the deletion included the putative NF-κB responsive element (pGL3-SAA1-84), the relative luciferase activity was largely eliminated. Similarly, the relative luciferase activity was reduced to baseline when the C/EBPβ-binding site alone (pGL3-SAA1-no-CE) was internally deleted, suggesting that the NF-κB element alone cannot confer promoter activity. In contrast, the construct without the NF-κB sequence alone (pGL3-SAA1-no-NF) still gave 4 times higher relative luciferase activity than that of the mock pGL3 plasmid, suggesting the retention of partial promoter activity in the C/EBPβ element even in the absence of the NF-κB element. The different response between pGL3-SAA1-175 and pGL3-SAA1-no-CE constructs is puzzling. It could be accounted by the presence of a suppressor element for NF-κB in the 5’-upstream region of the C/EBPβ element, which was demonstrated in a previous study for human SAA1 gene (Edbrooke et al. 1991). Thus, deletion of the suppressor element in the pGL3-SAA1-175 construct might have released the promoter activity of the NF-κB element from the suppressor element. It also could not rule out the possibility that other cis-acting elements were responsible for different response, such as SAA-activating sequences (SAS) that had been implicated in IL-6-mediated transcriptional induction of the SAA1 gene in nonhepatic cells (Ray A and Ray BK 1996). These results as a whole indicated that transcriptional induction of the cheetah SAA1 gene by inflammatory cytokines IL-1 and IL-6 is dependent on the presence of both the C/EBPβ and NF-κB cis-acting elements, analogous to the human SAA1 gene.

Functional Polymorphism in the NF-κB Cis-Acting Element of the Cheetah SAA1 Gene

Although the PCR products for the 5’-flanking region of the SAA1 gene taken from cheetah C68 were directly sequenced, we noticed overlap of 2 chromatograms of almost equal signal intensity beginning from nucleotide ~97 (Figure 5A). This result strongly suggested that this cheetah was heterozygous for sequences derived from 2 alleles. Indeed, when the PCR products were cloned into a plasmid vector and several clones were sequenced, 2 distinct sequences were obtained: one with 3 consecutive G residues and the other with only 2 G residues (Figure 5B and C). More importantly, this insertion/deletion of a G residue was in the putative NF-κB-binding site. In order to evaluate the effect of this polymorphism on promoter/enhancer activity, we prepared a new luciferase reporter construct, pGL3-SAA1-795-2G, which had 2 consecutive G residues in the putative NF-κB-binding site. The relative luciferase activity of pGL3-SAA1-795-2G was significantly lower than that of the original pGL3-SAA1-795 with 3 G residues (P < 0.001; Figure 5D).

We then examined the prevalence of the 2 alleles defined by insertion/deletion of a G nucleotide (3Gs or 2Gs) in 13 additional cheetahs. Two cheetahs (C83 and C85) were homozygous for the 3Gs allele. The other 11 cheetahs were heterozygous for both alleles. We found no homozygous cheetah for the 2Gs allele. Thus, the 3Gs allele associated with high promoter/enhancer activity appeared to be prevalent as a major allele.

Discussion

In this study, we determined the structure and nucleotide sequence of the cheetah SAA1 gene. In humans, the SAA multigene family includes 4 genes (SAA1–SAA4) (Sellar et al. 1994). SAA3 expression is induced by lipopolysaccharide in mammary gland epithelial cells (Larson et al. 2003). SAA4 is constitutively expressed and is not particularly increased during inflammation (Liang et al. 1997). Thus, these SAA proteins were irrelevant to AA amyloidosis. Meanwhile, SAA1 and SAA2, which share 96% sequence
homology, are dramatically upregulated during inflammation. We consider that the gene identified in this study represents the cheetah SAA1 for the following reasons. The exon/intron organization of the cheetah SAA gene was highly homologous to that of the human SAA1 and SAA2 genes (data not shown). Both SAA1 and SAA2 derivatives are found in human and mouse AA deposits (Baba et al. 1992). However, the SAA1 derivative dominates over the SAA2 derivative in most cases (Liepnieks et al. 1995; Bell et al. 1996). In addition, the greater potential of SAA1 to form AA amyloid fibrils was demonstrated by in vitro studies (Yamada et al. 1994). It would, therefore, be reasonable to consider that the AA amyloid deposited in cheetahs is derived from SAA1. Meanwhile, the amino acid sequence deduced from the nucleotide sequence was identical to that of AA amyloid fibrils isolated from the liver of cheetahs with AA amyloidosis, except for the additional 18 amino acids each at N- and C-terminals in the deduced sequence, strengthening our hypothesis that the gene isolated was indeed SAA1.

We also characterized the 5′-upstream region of the cheetah SAA1 gene and identified putative promoter/enhancer elements of the gene. The results obtained in this study suggest that the cheetah SAA1 gene is under similar transcriptional regulation under inflammatory conditions as is the human SAA1 gene. As in humans, inflammatory cytokines IL-1β and IL-6 appeared to play key roles in upregulation of the cheetah SAA protein during inflammation. In humans, sustained high blood IL-6 levels are associated with another morbid condition, rheumatoid arthritis (RA). It has been reported that IL-6 blocking therapy by anti-IL-6 receptor antibody reduced the blood SAA level and ameliorated RA symptoms (Nishimoto et al. 2004). Our data suggest that an anticytokine treatment is a feasible and effective therapy to prevent AA amyloidosis in captive cheetahs, if such antibodies for cheetah IL-6 are available.

The most intriguing point in this study is the finding of a functional polymorphism in the putative NF-κB target element of the cheetah SAA1 gene. We could not rule out the possibility that the PCR product with 2Gs was a derivative from a gene, which has high homology to SAA1 (e.g., SAA2). However, this possibility is low. There was no difference between the 2 PCR products other than the 3Gs or 2Gs in approximately 800 bp. This contrasted to the presence of several nucleotide substitution and/or insertion/deletion variations between the corresponding region of human SAA1 and SAA2 genes or mouse Saa1 and Saa2 genes. It would, therefore, be reasonable to consider that the 2 PCR products were derived from 2 alleles of the same SAA1 locus. The remarkable difference in the promoter/enhancer activities of the 2 alleles highlighted again the importance of the NF-κB-binding motif in the transcriptional regulation of the cheetah SAA1 gene.

In humans, a single nucleotide polymorphism (SNP) in the 5′-flanking region of SAA1 gene (−13T/C) is documented (Moriguchi et al. 2005). The allele SAA1-13T is associated with high transcriptional activity compared with −13C and hence is a risk factor for AA amyloidosis in both Japanese and Caucasian populations. This observation is similar to the finding in this study. However, in this study, we did not examine the correlation between 3Gs allele with strong promoter/enhancer activity and susceptibility for AA amyloidosis in cheetah. In the 14 deceased cheetahs examined in this study, 7 of 8 cheetahs with severe AA amyloidosis were from the same pedigree (Figure 1). All these individuals had at least 1 copy of the 3Gs allele associated with strong promoter/enhancer activity. Meanwhile, 6 cheetahs from different pedigrees or imported from
Africa had no or minor AA amyloid deposition, although they also had 1 copy of the 3Gs allele. As widely recognized, having a risk allele (3Gs allele in the case of cheetah) does not necessarily imply that such individuals would develop AA amyloidosis. Rather, expression of SAA protein is largely affected by inflammation. Thus, it is necessary to examine more cheetahs (possibly including live captive as well as wild animals and belong to different groups) to explore the possibility that identified SNP is genetically associated with AA amyloidosis in this species.

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**References**


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