Analysis of Single Nucleotide Polymorphisms in the 3' Region of the Estrogen Receptor 1 Gene in Normal and Cryptorchid Miniature Dachshunds and Chihuahuas

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Abstract. This study was performed to examine the distribution of single nucleotide polymorphisms (SNPs) and estimated haplotypes in the canine estrogen receptor (ER) α gene (ESR1) and the association of them with different phenotypes of cryptorchidism (CO) in Miniature Dachshunds and Chihuahuas. Forty CO and 68 normal dogs were used, and CO was classified into unilateral (UCO; n=33) and bilateral CO (BCO; n=5) or into abdominal (ACO; n=16) and inguinal CO (ICO; n=22). Thirteen DNA fragments located in the 70-kb region at the 3’ end of ESR1 were amplified by PCR and sequenced to examine 13 SNPs (#1–#13) reported in a canine SNP database. Ten SNPs (#1–#4, #7, #8, #10–#13) were not polymorphic, and 5 new SNPs (#14–#18) were discovered. A common haplotype block in normal, CO and UCO phenotypes was identified for an approximately 20-kb region encompassing 4 SNPs (#14–#17). Allele, genotype and haplotype frequencies in CO without classification by phenotype and also in UCO, ACO and ICO phenotypes were not statistically different from the normal group. Significant differences in genotype frequencies and homozygosity for the estimated GTTG haplotype within the block were observed in BCO compared with the normal group, although the number of BCO animals was small. Our results demonstrate that the examined SNPs and haplotypes in the 3’ end of canine ESR1 are not associated with unilateral, abdominal and inguinal CO phenotypes and CO per se in Miniature Dachshunds and Chihuahuas. Further studies are necessary to suggest a clear association between the ESR1 SNPs and bilateral CO in dogs.

Key words: Cryptorchidism, Dogs, ESR1, Haplotype, Single nucleotide polymorphisms (SNP)

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firmed free of other congenital reproductive disorders except CO. When the cryptorchid dogs were categorized according to the location of the retention of the testis, abdominal (ACO; n=16, 42.1%) and inguinal (ICO; n=22, 57.9%) positions were identified. The samples consisted of unilateral (UCO; n=33, 86.8%) and bilateral (BCO; n=5, 13.2%) cases when categorized on the basis of number of undescended testes per animal. Of the UCO cases, 22 (75.9%) and 7 (24.1%) dogs had right and left retained testes, respectively. Information was missing in 2 cases each for the categories of ACO vs. ICO and UCO vs. BCO, while the side of the retained testis was unknown in 4 cases of UCO. Samples were collected from dogs presented for castration at the Veterinary Teaching Clinical Center of the Osaka Prefecture University and from other animal clinics outside the University. The ages of the animals ranged from 5 months to 16 years.

The collected testicular samples were minced into 5-mm pieces and stored at −90 C until extraction of genomic DNA. Extraction of DNA was performed by using a DNeasy Blood & Tissue Kit (QIAGEN Sciences, Germantown, MD, USA).

**Primer development**

One hundred and eighty-one SNPs of ESR1 at chromosome 1 have been reported in the canine SNPs database (CanFam 2.0, http://www.broad.mit.edu/node/459). Out of 181, 13 specific SNPs (SNP #1–#13) were selected from the 70-kb region of the 3’ terminal region (Table 1 and Fig. 1). The Primer Select (DNASTAR, Madison, WI, USA) computer software was used to design the primers shown in Table 1. The polymerase chain reaction (PCR) product sizes ranged from 200 to 400 base pairs.

**PCR**

PCR was performed using a MiniCycler (MJ Research, Waltham, MA, USA) to amplify the regions of ESR1 including specific SNPs under the following conditions. The reaction mixture consisted of 5 μl of genome DNA, 2.5 μl of 10x Ex Taq Buffer, 2 μl of dNTP mixture, 0.25 μl of each primer (100 pmol/μl) and 0.25 μl of TaKaRa Ex Taq (5 U/μl). The total volume was adjusted to 25 μl with sterile ultra pure water. The samples were amplified using a thermal cycler as follows. An initial denaturing step at 94 C for 2 min was followed by 30 cycles of 94 C for 30 sec, 60 C for 30 sec and 72 C for 2 min.

PCR products were separated by electrophoresis in a 2% agarose gel and stained with ethidium bromide. Target products were assessed by comparing results with direct sequencing. PCR-RFLP was assessed by comparing results with direct sequencing. PCR-RFLP was used only for SNP #5 and #6 in MDH (n=38) and CHH (n=25). For SNP genotyping by PCR-RFLP, 17 μl of purified DNA, 2 μl of buffer and 1 μl of the restriction enzyme were incubated for 18-20 h. The restriction enzymes used for SNP #5 and #6 were BsmAI (5,000 U/ml, New England BioLabs, Beverly, MA, USA) and Mbol (10,000 U/ml, Takara Bio, Otsu, Shiga, Japan), respectively.

**Direct sequencing**

Genotyping was mainly performed with direct sequencing. Purified target PCR products were added with forward and reverse primers, and the mixture was sent to Bio Matrix Research, Nagar-eyama, Chiba, Japan, for sequencing. The samples were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and resolved on an auto sequencer (DNA analyzer, Applied Biosystems). When two peaks were detected at a single base, they were defined as a heterologous nucleotide sequence.

**Statistical analysis**

Allele, genotype frequency at each SNP and haplotype frequencies were compared among the phenotype groups, i.e., 1) between the normal and CO groups, 2) between the normal group and UCO and BCO groups and 3) between the normal group and ACO and ICO groups, by chi-square test using the online resource “Statistics to use” [26]. Allele and genotype distribution were analyzed at each SNP (SNP #5, #6, #9 and #14–#18), and the linkage disequi-
librium (LC) status of SNPs and association between estimated haplotypes were also analyzed. A P<0.05 was considered statistically significant. The odds ratio was calculated using the online calculator available at http://www.hutchon.net/ConfidOR.htm.

The test for the Hardy-Weinberg equilibrium (HWE) and estimation of haplotypes and pair-wise |D'| values were performed using the software SNPStats software (Biostatistics and Bioinformatics Unit, Catalan Institute of Oncology, http://bioinfo.iconcologia.net/snpstats/). The data of the different groups were processed for the genotype and haplotype analyses, only if the genotyping results were consistent with the HWE.

Results

SNP analysis

Out of 13 single nucleotides (#1–#13) reported in the canine SNP database (Dog SNPs-CanFam 2.0, http://www.broad.mit.edu/node/459), 3 nucleotides (#5, #6 and #9) were found to be polymorphic in MDH and CHH. The other reported single nucleotides (10 nucleotides) were not polymorphic in the above two breeds. Five SNPs (#14–#18 in Fig. 1), which have not been reported in the above database, were newly found to be polymorphic in the present study. The polymorphism was observed in both the normal and cryptorchid groups in all of the above SNPs.

When the results of PCR-RFLP and direct sequencing were compared at SNP #5, the accuracies of the results of the former in MDH and CHH were 76.3 and 84%, respectively. At SNP #6, 84.2 and 76% of the results obtained from PCR-RFLP were accurate in MDH and CHH, respectively. Direct sequencing was selected as the most appropriate method for further sequencing.

The allele (Table 2) and genotype (Table 3) frequencies in the CO (i.e., without classification by phenotype) and UCO groups were not statistically different from the normal group at any of the SNPs examined. Furthermore, the above frequencies in the ACO and ICO groups were not statistically different compared with the normal group at any of the SNPs (data not shown). However, the genotype frequency of the major allele homozygote in the BCO group was significantly higher at one SNP (#16) compared with the normal group (Table 3). The genotype frequency of the heterozygote vs. combined major and minor allele homozygotes in the BCO group was significantly lower at 4 SNPs (#9, #15–#17).

Haplotype analysis

A common haplotype block was identified in an approximately 20-kb region encompassing SNPs 14–17 (Fig.2). All the pairwise |D'| values in the considered block were greater than 0.85 in all of the groups, i.e., normal, CO, UCO, BCO, ACO and ICO. Certain SNP pairs outside the above haplotype block also showed a pairwise |D'| value greater than 0.80 and were considered in the analysis.

The frequencies of the haplotypes and their homozygotes were not statistically different in the CO, UCO, ACO and ICO groups compared with the normal group. However, the frequency of the homozygotes for the estimated GTG haplotype within the block was significantly higher (P<0.02) in BCO (80%; 4/5) than in the normal group (20%; 1/5).

Table 2. Comparisons of allele frequencies at SNPs in the 3’ end of ESR1 in cryptorchid dogs with normal dogs

<table>
<thead>
<tr>
<th>SNP</th>
<th>Allele</th>
<th>Position</th>
<th>CO vs. normal</th>
<th>UCO vs. normal</th>
<th>BCO vs. normal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>P value</td>
<td>OR (95% CI)</td>
<td>P value</td>
</tr>
<tr>
<td>SNP #5</td>
<td>A/C</td>
<td>45367641</td>
<td>0.60</td>
<td>1.17 (0.65–2.10)</td>
<td>0.80</td>
</tr>
<tr>
<td>SNP #6</td>
<td>A/G</td>
<td>45367861</td>
<td>0.64</td>
<td>1.16 (0.62–2.18)</td>
<td>0.90</td>
</tr>
<tr>
<td>SNP #9</td>
<td>C/G</td>
<td>45385854</td>
<td>0.85</td>
<td>1.08 (0.49–2.39)</td>
<td>0.72</td>
</tr>
<tr>
<td>SNP#14</td>
<td>G/T</td>
<td>45386006</td>
<td>0.31</td>
<td>1.39 (0.74–2.61)</td>
<td>0.52</td>
</tr>
<tr>
<td>SNP #15</td>
<td>T/C</td>
<td>45407473</td>
<td>0.81</td>
<td>1.07 (0.61–1.89)</td>
<td>0.64</td>
</tr>
<tr>
<td>SNP #16</td>
<td>T/G</td>
<td>45407775</td>
<td>0.68</td>
<td>1.12 (0.64–1.97)</td>
<td>0.70</td>
</tr>
<tr>
<td>SNP #17</td>
<td>G/A</td>
<td>45407781</td>
<td>0.84</td>
<td>1.06 (0.59–1.91)</td>
<td>0.72</td>
</tr>
<tr>
<td>SNP #18</td>
<td>G/A</td>
<td>45407811</td>
<td>0.59</td>
<td>0.43 (0.13–1.41)</td>
<td>0.93</td>
</tr>
</tbody>
</table>

CO, cryptorchid (n=40); BCO, bilateral cryptorchid (n=5); OR, Odds ratio; UCO, unilateral cryptorchid (n=33); Normal, normal dogs (n=68).

* Positions were decided according to the NCBI map viewer and canine SNP database.
Table 3. Comparisons of genotype frequencies at SNPs in the 3' end of ESR1 in cryptorchid dogs with normal dogs

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype</th>
<th>CO vs. normal</th>
<th>UCO vs. normal</th>
<th>BCO vs. normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNP #5</td>
<td>AA vs. AC+CC</td>
<td>0.25 1.58 (0.72–3.47)</td>
<td>0.68 1.19 (0.51–2.75)</td>
<td>0.09 5.71 (0.61–53.9)</td>
</tr>
<tr>
<td>SNP #6</td>
<td>AA vs. AG+GG</td>
<td>0.31 1.50 (0.68–3.31)</td>
<td>0.67 1.20 (0.52–2.76)</td>
<td>0.20 4.00 (0.42–37.7)</td>
</tr>
<tr>
<td>SNP #9</td>
<td>CC vs. AC+CC</td>
<td>0.55 1.39 (0.47–4.08)</td>
<td>0.30 1.76 (0.59–5.25)</td>
<td>0.38 0.30 (0.02–4.64)</td>
</tr>
<tr>
<td>SNP #14</td>
<td>GG vs. GT+TT</td>
<td>0.31 1.50 (0.68–3.31)</td>
<td>0.67 1.20 (0.52–2.76)</td>
<td>0.20 4.00 (0.42–37.7)</td>
</tr>
<tr>
<td>SNP #15</td>
<td>TT vs. TC+CC</td>
<td>0.56 1.27 (0.57–2.82)</td>
<td>0.74 0.86 (0.36–2.06)</td>
<td>0.06 0.17 (0.03–1.09)</td>
</tr>
<tr>
<td>SNP #16</td>
<td>TT vs. TG+GG</td>
<td>0.42 1.39 (0.62–3.14)</td>
<td>0.84 0.91 (0.37–2.34)</td>
<td>0.03 8.36 (0.88–79.3)</td>
</tr>
<tr>
<td>SNP #17</td>
<td>GG vs. GA+AA</td>
<td>0.52 1.29 (0.59–2.84)</td>
<td>0.86 0.93 (0.40–2.17)</td>
<td>0.09 5.71 (0.61–53.9)</td>
</tr>
<tr>
<td>SNP #18</td>
<td>GG vs. GA+AA</td>
<td>0.54 1.48 (0.42–5.19)</td>
<td>0.60 1.43 (0.37–5.44)</td>
<td>0.41 2.58 (0.25–27.0)</td>
</tr>
<tr>
<td>SNP #19</td>
<td>GG vs. GA+AA</td>
<td>0.59 1.34 (0.47–3.86)</td>
<td>0.91 1.06 (0.36–3.11)</td>
<td>0.28 3.63 (0.34–38.3)</td>
</tr>
</tbody>
</table>

CO, cryptorchid (n=40); BCO, bilateral cryptorchid (n=5); OR, Odds ratio; UCO, unilateral cryptorchid (n=33); Normal: normal dogs (n=68).

Fig. 2. Pairwise linkage disequilibrium maps. A haplotype block comprising SNPs #14–#17 was identified in all groups. The pairwise |D'| values were greater than 0.85 among all SNP pairs considered for the haplotype block. Since SNP #9 and #18 are not polymorphic in BCO, the above SNPs are not included in the map of BCO. ACO, abdominal cryptorchid (n=16); BCO, bilateral cryptorchid (n=5); CO, all cryptorchid dogs (n=40); ICO, inguinal cryptorchid (n=22); Normal (n=68); UCO, unilateral cryptorchid (n=33).
normal group (27.9%; 19/68).

Discussion

In the present study, we discovered 5 new SNPs in the 3’ end of the ESR1 in Miniature Dachshunds and Chihuahuas. These SNPs are not registered in the canine SNP data base, CanFam 2.0 (http://www.broad.mit.edu/node/459). We analyzed samples from the above two small breeds, which are currently the most popular breeds in Japan, while the SNP data in CanFam 2.0 consists of data collected from large- or medium-size breeds such as the Beagle, Boxer, Grey Wolf, Labrador Retriever, Rottweiler, Shepherd and Standard poodle. It remains to be determined whether the 5 SNPs in the 3’ end of the ESR1 found in this study are specific to these two small breeds or not.

The absence of significant differences in allele, genotype and estimated haplotype frequencies in CO per se and in 3 CO phenotypes, i.e., unilateral, abdominal and inguinal CO, shows that the tested parameters in the 3’ end of the ESR1 are not associated with the above CO groups in Miniature Dachshunds and Chihuahuas. When the polymorphic nucleotides in the 3’ end of the canine ESR1 are considered, ESR1 is not a causative gene for the unilateral, abdominal and inguinal CO and is also not a causative gene for the CO without classification by phenotype. Even though significant differences were observed in genotype frequencies and homozygotes for the GTTG haplotype in bilateral CO compared with the normal group, a conclusion based on the above results may not be appropriate since the sample number (n=5) is too small. The bilateral CO samples consisted of 3 samples from Miniature dachshunds and 2 samples from Chihuahuas, but complete pedigree data was not available to rule out familial relationships among the bilateral CO dogs. Further studies with an increased number of subjects of bilateral cases are required to understand the association of SNPs in ESR1 with bilateral CO, which is less prevalent compared with unilateral CO in dogs as previously reported [2, 8, 27] and as observed in this study. In dogs, no causative or associated genes for CO have been identified according to the reports available to date. Previously, Wende et al. [28] compared the coding region of calcitonin gene-related peptide gene (CGRP) between cryptorchid and normal dogs, but no mutations or polymorphisms were identified.

In humans, ESR1 has been suggested to be an associative gene for cryptorchidism [13, 14]. However, the findings have not been consistent among independent multiracial groups. A previous study showed that the allele and genotype frequencies were significantly different at certain SNPs between control males and CO patients in a 50-kb region of the 3’ end of human ESR1 [13]. Furthermore, the same study identified the AGATA haplotype as a susceptibility factor for CO in the Japanese population. In another recent study carried out in Europe using two Caucasian populations [14], the “A” allele of SNP 12 (the tag SNP for the AGATA haplotype) was a protective factor for CO, and this was the opposite effect compared with the Japanese population. In an American study in which multiracial populations including Caucasians, African Americans and Asian Americans [15] were used, the AGATA haplotype was not found within the estimated haplotypes. That study reported that SNP 12 in ESR1 is not associated with CO in the populations that were examined.

Based upon the SNPs discovered in this study, we identified a common haplotype block of 20-kb encompassing four new SNPs from #14 to #17 in the 3’ end of canine ESR1. In humans, SNPs and haplotypes in the 3’ end of ESR1 have been found to be related not only with CO, but also with the occurrence of other diseases such as male genital abnormalities and breast cancer. Watanabe et al. [29] reported that the AGATA haplotype is associated with micropenis and hypospadias. Furthermore, certain SNPs located in the 3’ end of ESR1 have been found to be related with the occurrence of breast cancer [30, 31]. Hence, it is worth examining the association of the five new SNPs and haplotypes arising from the newly identified block with other diseases such as male genital abnormalities and mammary tumors in dogs.

In summary, the present study represents the first time an SNP analysis has been conducted for the 3’ end of the ESR1 in Miniature Dachshunds and Chihuahuas, and five new SNPs were discovered. A common haplotype block encompassing four newly discovered SNPs (#14–#17) was identified. No significant differences in allele, genotype or haplotype frequencies were observed in the CO group without classification by phenotype or in the unilateral, abdominal and inguinal CO groups compared with normal dogs. Therefore, the polymorphic nucleotides and estimated haplotypes at the 3’ end of ESR1 are not associated with the risk for CO or for the above phenotypes. Significant differences in genotype frequencies and homozygosity for the estimated GTTG haplotype within the block were observed in bilateral CO compared with normal group, although the number of animals was small. Further studies are necessary to suggest a clear association between the ESR1 SNPs and bilateral CO in dogs.

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References


