Sox9 Duplications Are a Relevant Cause of Sry-Negative XX Sex Reversal Dogs

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Abstract

Sexual development in mammals is based on a complicated and delicate network of genes and hormones that have to collaborate in a precise manner. The dark side of this pathway is represented by pathological conditions, wherein sexual development does not occur properly either in the XX and the XY background. Among them a conundrum is represented by the XX individuals with at least a partial testis differentiation even in absence of SRY. This particular condition is present in various mammals including the dog. Seven dogs characterized by XX karyotype, absence of SRY gene, and testicular tissue development were analysed by Array-CGH. In two cases the array-CGH analysis detected an interstitial heterozygous duplication of chromosome 9. The duplication contained the SOX9 coding region. In this work we provide for the first time a causative mutation for the XXSR condition in the dog. Moreover this report supports the idea that the dog represents a good animal model for the study of XXSR condition caused by abnormalities in the SOX9 locus.

Introduction

Gonadal differentiation in mammals is initiated, controlled, and regulated by the coordinated action of several genes and hormones.

During the last two decades many genes involved in this process have been identified [1], and in recent times, epigenetic factors have also come into play [2]. The scepter of power remains firmly in the hands of SRY, the sex determination key gene [3] located on the Y chromosome that is necessary and sufficient to induce the primordial undifferentiated gonad to develop into a testis [4]. In the absence of SRY, that is in the XX embryos a different set of genes is activated, and the undifferentiated gonad becomes an ovary [5]. SRY role takes place in a short period of time, and ceases after the activation of SOX9. This gene is a main actor in testis differentiation and in several other embryogenetic fields [6]. Normally this process follows well-defined tracks: the XY embryos develop the testis and a male phenotype, while the XX embryos develop ovaries and a female phenotype. However, this complex process can result in the appearance of developmental errors on account of the discordance between the chromosomal, gonadic, and phenotypic sex.

One of the most interesting issues is represented by the XX sex-reversal cases. In humans most of them do have the SRY gene that is transposed to the tip of Xp due a recurrent Non Allelic Homologous Recombination between of PRKX and PRKY in a particular Y haplotypic background [7]. However, in both humans and other mammals SRY-negative XX males have been observed displaying testicular tissue, with or without ovarian tissue. It has been observed, at least in pig, that in these cases SOX9 gene is surprisingly activated in the absence of SRY [8]. Subjects with XX sex reversal have been observed in different species: human, pig, goat, llama, dog, and horse [9–14]. In the dog this pathology appears with a relatively high frequency compared to the other species and has been described in various breeds [15]. XX sex-reversal in dogs can show a very different structure of gonads, ranging from bilateral testis to one ovo-testis and one ovary. With regard to the causes of occurrence of this anomaly in different species, to date, only three genetic causes have been identified: FOXL2 in goat [16] and SOX9 [17] and RSPO1 [18] in humans. SOX9 alterations in XXSR cases include duplications, triplications, and reciprocal translocations [17,19–21]. Surprisingly, despite the many cases investigated in XXSR dogs, till date no causative mutations have been reported, but only a linkage for a genomic region has been detected in a single specific pedigree [22].

In this article, we report the molecular analysis of seven XX sex-reversal dogs and we clearly show, for the first time in literature, that two of them carry SOX9 gene duplication.

Materials and Methods

Case Description

Seven dogs from different breeds have been considered in this study: Four of these have already been described, while three are still unreported. Case C2, C9, C10, and C44 [23–25] have been
previously characterized to show a presence of testicular tissue with a XX karyotype in the absence of the SRY gene. The other three cases, C61, C64, and C65, have been characterized in this study.

Histological Examination

All clinical activities and surgical experiments on the dogs were carried out at the Veterinary Hospital of the University of Milan by veterinary surgeons. During the research no animals were sacrificed. The anesthetic and surgical protocol fulfilled the Federation of European Laboratory Animal Science Association’s recommendations and European Union legislation (Council Directive 86/609/EEC). Blood (1.5 ml) and gonad samples were collected for a routine medical procedure and stored for further analysis. Consistent with Italian regulation (D.L. 116/1992), the owners signed a voluntary consent, for their animals before undergoing surgery. This consent includes the possibility that the removed tissue may be used in scientific researches without economic interest.

After surgical excision of the gonads, they were fixed in 10% neutral buffered formalin for at least three days. For histological examination, several slices of gonads were processed histotechno-

Figure 1. Histological examination of the new cases reported. Case C61: Histologic section of the right (A) and left (B) gonad showing seminiferous tubules with diffuse atrophy of the seminal line. Case C64: Right Ovotestis (C): The gonads were surrounded by ovarian bursa and shown some follicular structures and corpora lutea (white arrow). In the medulla hypoplastic seminiferous tubules were present (black arrow). Case C65 (D): Dog ovotestis. In the gonad, follicular structures including oocytes (arrow) coexist with testicular tubuli lined by Setoli cells (asterisc) (Courtesy of Valeria Grieco, University of Milan). doi:10.1371/journal.pone.0101244.g001
### Table 1. List of CNVs identified with array-CGH in the seven cases with the indication of their code, type, location and size (CanFam2 assembly).

| Case code | CFA | CNV code | Log ratio | Size Kb | Last unaffected bp | First affected bp | Last affected bp | First unaffected bp | Already Described (Y/N) | Genes |
|-----------|-----|----------|-----------|---------|-------------------|------------------|-----------------|---------------------|------------------------|-------|
| C2        | 9   | DEL      | -0.7      | 459     | 20,436,097        | 20,465,561       | 20,924,123      | Y                   |                       |       |
|           | 9   | DUP      | 0.5       | 541     | 21,021,894        | 21,562,129       | 21,574,304      | Y                   |                       |       |
| C9        | 9   | DEL      | -0.8      | 459     | 20,439,097        | 20,465,561       | 20,924,123      | Y                   |                       |       |
|           | 9   | DUP      | 0.5       | 541     | 21,021,894        | 21,562,129       | 21,574,304      | Y                   |                       |       |
| C10       | 9   | DUP      | 3         | 0.5     | 577               | 10,414,955       | 11,016,965      | 12,062,144         | N                      | SOX9  |
|           | 9   | DUP      | 4         | 0.3     | 414               | 19,864,938       | 20,022,338      | 20,447,061         | Y                      |       |
|           | 9   | DEL      | -0.75     | 458     | 20,447,061        | 20,465,561       | 20,924,123      | Y                   |                       |       |
|           | 9   | DUP      | 0.5       | 541     | 21,021,894        | 21,574,304       | 21,589,624      | Y                   |                       |       |
| C44       | 9   | DUP      | 3         | 0.57    | 577               | 10,414,955       | 11,016,965      | 12,062,144         | N                      | SOX9  |
|           | 9   | DEL      | -0.9      | 1300    | 19,766,692        | 19,819,256       | 21,119,179      | 21,292,889         | Y                      |       |
| C61       | 9   | DEL      | 6         | 0.8     | 809               | 20,097,414       | 20,115,306      | 20,924,123         | Y                      |       |
|           | 9   | DUP      | 2         | 0.5     | 541               | 21,021,894       | 21,562,129      | 21,574,304         | Y                      |       |
| C64       | 9   | DEL      | -0.8      | 809     | 20,097,414        | 20,115,306       | 20,924,123      | Y                   |                       |       |
|           | 9   | DUP      | 2         | 0.5     | 541               | 21,021,894       | 21,562,129      | 21,574,304         | Y                      |       |
| C65       | 9   | DEL      | -0.8      | 809     | 20,097,414        | 20,115,306       | 20,924,123      | Y                   |                       |       |
|           | 9   | DUP      | 2         | 0.5     | 541               | 21,021,894       | 21,562,129      | 21,574,304         | Y                      |       |

CNVs were checked for occurrence in the Database of Genomic Copy Number Variants in the dog genome (http://dogs.genouest.org/LUPA.dir/CNV.html) and in several papers [29–33].
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logically according to standard laboratory procedures, cut at 5 μm, and stained with hematoxylin and eosin [26].

Cell Cultures and Genetic Analyses

Peripheral blood lymphocyte cultures were performed following the standard procedures [27]. SRY gene analysis was performed as reported [23]. Briefly, the entire SRY coding region (GenBank AF107021) was amplified by polymerase chain reaction (PCR) using the following primers: (5'-3'):

- SRY-Dog-F: ctttccaacttccctccgta
- SRY-Dog-R: ggacgtttcgttagccagag

The PCR product was 813 bp long. PCR was performed using AmpliTaq Gold DNA Polymerase (Applied Biosystems) according to the manufacturer’s instructions.

Array-CGH Analyses

Array-CGH was performed using a custom Agilent Canine Genome CGH Microarray 180 K (Agilent Technologies, Santa Clara, California, USA) and processed as reported [28]. Briefly, 500 ng of purified DNA of a subject and a control, were double-digested with RsaI and AluI for two hours at 37°C. After 20 minutes at 65°C, each digested sample was labeled by the Agilent random primers; labeling was performed for two hours using Cy5-dUTP for the subject DNA and Cy3-dUTP for the control DNA. The labeled products were columns purified and prepared according to the Agilent protocol. After probe denaturation and pre-annealing with 5 μl of Cot-1 DNA, hybridization was performed at 65°C, with rotation for 40 hours. After two washing steps, the arrays were analyzed with the Agilent scanner and the Feature Extraction software (v10.7.3.1). A graphical overview was obtained using the CGH analytics software (v7.0.4.0). The DNA extracted from a normal female (boxer breed) was used as the control in all cases. All experimental data were submitted to GEO repository with the following Series accession number: GSE57137.

Quantitative real Time PCR

Sox9 duplications detected by array CGH were confirmed by Real-Time-qPCR with SYBR Green detection (Brilliant II SYBR Green QPCR master mix, Agilent Technologies), using one non-polymorphic marker located within the duplicated region. The primers were designed by using the Primer3 Software online (http://frodo.wi.mit.edu/primer3/), with the following criteria: Amplicon size 80–200 bp, GC content of 20–80%, and melting temperature (Tm) of 59–61°C. The primer sequences are available on request. Real-time detection was performed using the Stratagene Mx3000P. The Real-Time-qPCRs were performed in triplicate for each reaction.

The comparative CT method (ΔΔCT method) was used to discriminate between two and three allele copies of the DNA target sequence (Sox9) in the two dogs (resulted duplicated by a-CGH) relative to five normal control dogs DNA samples. The data have been normalized against two different reference sequences (Abs17, Bgr2).

Results

All the three new cases, C61, C64, and C65, showed a normal 78,XX karyotype in all the observed metaphases, and PCR analyses confirmed the absence of the SRY gene (not shown).

Moreover, the histological analyses revealed the presence of testicular tissue in all the three cases, indicating that the male pathway was active during the fetal period in the absence of SRY.

The testes of Case 61 are composed of testicular parenchyma with absence of the germline. In Cases 64 and 65 the right and left gonads are ovotestes (Figure 1a, b, c, d).

The results of array-CGH in the seven cases analyzed are listed in Table 1.

In cases C10 and C44, the array-CGH analysis detected an interstitial heterozygous duplication of chromosome 9, of 577 Kb (from 11,016,965 to 11,593,933; all data are referred to CanFam2 genome assembly) (Figure 2). The duplication contained the SOX9 gene. This was confirmed by reverse transcriptase (RT)-PCR (Figure 3) and it was never described as copy number variations (CNVs) in different dog breeds [29–33]. The duplicated region was flanked by small, 168 bp, directly oriented repeats of 97.6% sequence identity, suggesting that non-allelic homologous recombination (NAHR) might have mediated these duplications. Furthermore, array-CGH identified several CNVs (data not available).
shown) in our cases; all of them were described as polymorphic in previous dog aCGH reports [29–33]. The complex CNV region on CFA9:19,761,852–21,600,512 has been observed, and reported with slightly different boundaries depending on the array platform used, in multiple studies [29–33]. Several CNV patterns have been described: gains or losses across the whole region, gains or losses of only a part of the region or alternate gains and losses within a single individual. The desert region between 20,115,306 and 21,119,179 is orthologous (61.9% of bases, 84.0% of span; http://genome.ucsc.edu/index.html) to the human region chr17:68,723,331–69,717,418 (genome assembly Hg19), located 500–600 Kb upstream of SOX9, which is suggested to be the human regulatory region critical for gonadal SOX9 expression [20]. It is particularly interesting because, taking into account that the Dog Genome Assembly is a working progress and contains many assembly errors (Rossi E. personal communication), the actual distance between SOX9 and this region within the dog genome could be the same of the human one. A more stable and defined dog assembly will demonstrate the actual distance between the two regions and will help to clarify the related effects. As shown in Table 1, in our cases the CNV from CFA9:19,761,852–21,600,512 has different patterns: complex in cases 2, 9, 10, 61, 64, 65 and simple as a deletion in case 44.

**Discussion**

Genes in the SOX family play a critical role in the sex determination process. *SRY* is the master gene of this process [34] while *SOX9* represents the genetic factor that, activated by *SRY*, starts and regulates testis development. Although *SRY* is mammal-specific (with very few exceptions) *SOX9* plays an important role in bird also [35], *SOX3* and *SOX8* genes are also involved in the sex determination process [36–37]. Chromosomal duplications as well as triplications involving the *SOX9* locus on HSA17q24.3, have been reported to be the causative mutations of the XX sex-reversal condition [38], however, all these duplications/triplications, except one, do not involve the *SOX9* coding region (CDS), but all are located 5′ to this gene. Indeed only the first reported *SOX9*
determination includes the SOX9 CDS [39]. This duplication, characterized by Variable-Number Tandem Repeat (VNTR) analyses, is at least 11.7 Mb long and is starts at 9.4 Mb 5' and ends at 2.2 Mb 3' of SOX9 CDS.

The mechanism underlying the XXSR condition in the presence of SOX9 duplications is still not clear, although it is clear that a Sox9 over expression is required to induce the testis development in a XX background.

Therefore all different Sox9 locus duplications must be organized to allow this possibility. The Sox9 transgenic mouse effectively develops the XXSR phenotype. Incidentally, in this case, the gene is under the regulation of a strong promoter, which is initially expressed in both the developing gonads (XY and XX), but only in the XY gonads its expression increases greatly. This upward regulation is due to SRY activation, and later on, to an auto-loop induced by additional positive feed-forward signals (Fig9). In the XX developing gonad the auto-loop is not able by itself to up-regulate Sox9 expression; moreover, female-specific genes repress additional feed-forward signals.

The analyses of human duplications in XXSR suggest a model of action. In these subjects two CDS SOX9 doses are present (as in normal subject), but the upstream region in one allele is of action. In these subjects two CDS SOX9 doses are present (as in normal subject), but the upstream region in one allele is of action. In these subjects two CDS SOX9 doses are present (as in normal subject), but the upstream region in one allele is of action. In these subjects two CDS SOX9 doses are present (as in normal subject), but the upstream region in one allele is.
31. Nicholas TJ, Baker C, Eichler EE, Akey JM (2011) A high-resolution integrated map of copy number polymorphisms within and between breeds of the modern domesticated dog. BMC Genomics 12: 414.

32. Quilez J, Short AD, Martinez V, Kennedy LJ, Ollier W, et al. (2011) A selective sweep of >8 Mb on chromosome 26 in the Boxer genome. BMC Genomics 12: 339.

33. Berglund J, Nevalainen EM, Molin AM, Perloski M, Andre C, et al. (2012) Novel origins of copy number variation in the dog genome. Genome Biol 13: R73.

34. Sinclair AH, Berta P, Palmer MS, Hawkins R, Griffiths BL, et al. (1990) A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. Nature 346: 240–245.

35. Morais da Silva S, Hacker A, Harley V, Goodfellow P, Swain A, et al. (1996) Sox9 expression during gonadal development implies a conserved role for the gene in testis differentiation in mammals and birds. Nature Genet 14: 62–68.

36. Sutton E, Hughes J, White S, Sekillo R, Tan J, et al. (2011) Identification of SOX3 as an XX male sex reversal gene in mice and humans. J Clin Invest 121: 326–341.

37. Chaboissier MC, Kobayashi A, Vidal VI, Lutzkendorf S, van de Kant HJ, et al. (2004) Functional analysis of Sox8 and Sox9 during sex determination in the mouse. Development 131: 1891–1901.

38. Fonseca AC, Bonaldi A, Bertola DR, Kim CA, Otto PA, et al. (2013) The clinical impact of chromosomal rearrangements with breakpoints upstream of the SOX9 gene: two novel de novo balanced translocations associated with acampomelic campomelic dysplasia. BMC Med Genet 14: 50.

39. Huang B, Wang S, Ning Y, Lamb AN, Bartley J (1999) Autosomal XX sex reversal caused by duplication of SOX9. Am J Med Genet 87: 349–353.

40. Vidal VP, Chaboissier M-C, de Rooij DG, Schedl A (2001) Sox9 induces testis development in XX transgenic mice. Nature Genet 28: 216–217.

41. Montazer-Torbati F, Kocer A, Auguste A, Renaud I, Charpigny G, et al. (2010) A study of goat SRY protein expression suggests putative new roles for this gene in the developing testis of a species with long-lasting SRY expression. Dev Dyn 239: 3324–3333.

42. Georg I, Bagheri-Fam S, Knowler KC, Wieacker P, Scherer G, et al. (2010) Mutations of the SRY-responsive enhancer of SOX9 are uncommon in XY gonadal dysgenesis. Sex Dev 4: 321–325.

43. Chen YS, Racca JD, Phillips NB, Weiss MA (2013) Inherited human sex reversal due to impaired nucleocytoplasmic trafficking of SRY defines a male transcriptional threshold. Proc Natl Acad Sci U S A 110: E3567–3576.