Research

Expression of the insulin-like growth factor (IGF) system and steroidogenic enzymes in canine testis tumors

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Abstract

Testis tumors occur frequently in dogs. The main types of tumors are Sertoli cell tumors, seminomas, and Leydig cell tumors. Mixed tumors and bilateral occurrence of tumors may be encountered frequently. To elucidate the possible relationship between the insulin-like growth factor (IGF) system and the development of different types of testis tumors in dogs, the expression of insulin-like growth factor-I and II (IGF-I and IGF-II), their type I receptor (IGF-IR), and their binding proteins (IGFBPs) was examined. In addition the expression of the steroidogenic enzymes p450-aromatase and 5α-reductase type I and type II, and the androgen receptor (AR) was investigated by a semiquantitative reverse-transcriptase PCR (RT-PCR). Both normal testes and testes with tumors were studied. In normal testes a clear expression of IGF-I, IGF-II, IGF-IR, IGFBP2, IGFBP4 and IGFBP5 was found. Expression of IGFBP1 and IGFBP3 was weak. There was also clear expression of the steroidogenic enzymes 5α-reductase, aromatase, and the AR. Quantification of RT-PCR products revealed significantly less expression of IGFBP1, IGF-I, and 5α-reductase type I in Sertoli cell tumors and seminomas. Leydig cell tumors and mixed tumors had a significantly higher expression of IGFBP4 and IGF-IR than normal testes. The expression of aromatase was lower in seminomas and in mixed tumors. The expression of AR, IGF-II and IGFBP2, IGFBP3, IGFBP5, and 5α-reductase type II did not differ among the different types of tumors. It was concluded that Sertoli cell tumors and seminomas have a comparable expression of the IGF system while Leydig cell tumors have a different pattern, suggesting difference in pathobiology among these types of tumors.

Background

The prevalence of testicular tumors is higher in dogs than in any other species of domestic animals and higher than in humans. Especially in older dogs the prevalence can be as high as 60% [1]. Testis tumors in dogs seldom metastasize and can be considered to be benign proliferations [2]. The three main types of testis tumors in dogs are Sertoli cell tumors, seminomas, and Leydig cell tumors and

combinations of them occur often. These tumors are seldom lethal, but can cause feminization of the dog, which in severe cases can lead to a fatal bone marrow depression [2]. Feminization is caused by hyperestrogenism and is mostly associated with Sertoli cell tumors but Leydig cell tumors and seminomas have also been associated with this syndrome. When feminization occurs in dogs with a seminoma, it is presumed that a co-existing Sertoli or Leydig cell tumor is responsible for the hyperestrogenism [2].

There is evidence that growth factors of the insulin-like growth factor regulatory system, such as insulin-like growth factor-I (IGF-I) and IGF-II, are involved in the pathobiology of neoplasia, both in terms of the risk of developing a tumor and in terms of its behavior [3].

Insulin-like growth factor-I (IGF-I) and IGF-II are peptides believed to play an important role in the regulation of cellular growth and differentiation. The IGFs are synthesized and secreted by many tissues. They can act as endocrine hormones that are being transported by the circulation to distant sites of action, but they can also act locally by paracrine or autocrine mechanisms. The biological activity of IGF-I and IGF-II is modulated by their binding proteins and receptors. Two distinct receptors and six different high-affinity binding proteins have been identified [4,5].

Both IGF-I and IGF-II are produced locally in the testis [6–9]. The IGF system plays an important role in the local regulation of testicular function [10]. IGF-1 has different roles in the two major compartments of the testis, the interstitium containing the Leydig cells and the seminiferous tubules containing Sertoli and germ cells. In Leydig cells it stimulates testosterone synthesis [11], in spermatogonia it is involved in stimulation of DNA synthesis [12], while in Sertoli cells it stimulates lactate synthesis [13] and glucose transport [14]. IGF-II has a clearly established role in embryonic and fetal development, but its postnatal function remains unclear [15] although culture experiments have shown that it can stimulate spermatogonial proliferation [12].

Two major gene classes, the proto-oncogenes and tumor suppressor genes, can trigger tumor formation after becoming mutated. It is known that components of the IGF system are upregulated by oncogenes, while tumor suppressor genes can inhibit this system [16]. Hence, mutated proto-oncogenes may induce the abundant synthesis of growth factors like the IGF's [17]. Subsequently, autocrine regulation of growth can occur in a number of tumors, implying that the tumor cells have gained the ability to grow autonomously [18]. Since mixed tumors are encountered frequently in the canine testis, one could argue that both autocrine and paracrine mechanisms may play a role in tumor induction [19]. This could raise the hypothesis that

neighboring cells following several mutations could be triggered to transform and become neoplastic even if they are of different origin, leading to formation of different types of tumors in one testis.

When investigating a model for carcinogenesis, testis tumors could be considered as hormone-related cancers whereby hormones drive cell proliferation. Genes involved in steroid hormone metabolism and transport are of interest. We investigated four genes: p450-aromatase, the androgen receptor and 5α-reductase type I and II. The p450-aromatase (P450-aro) enzyme converts testosterone to estrogens, the androgen receptor is responsible for androgen transport and 5α-reductase is responsible for the conversion of testosterone to the metabolically more active dihydroxytestosterone (DHT). Two isoforms of 5α-reductase have been identified and are referred to as type I and type II. The formation of DHT is IGF-I dependent [20].

Because tumors occur more frequently in the canine testis than in any other species we hypothesize that the IGF-system is stimulated in the testis of the aging dog. To test this hypothesis we examined the gene expression of IGF-I, IGF-II, and IGF-IR and the binding proteins IGFBP1 to IGFBP5 in Sertoli cell tumors, seminomas, Leydig cell tumors, and mixed tumors. Furthermore, we also studied the expression of the enzymes p450-aromatase and 5α-reductase (type I and II) and the androgen receptor, which may be indicative of differentiation induced by the IGF system.

Materials and Methods
Material
Twenty-four dogs with testis tumors and 6 dogs with normal testes were studied. Approval of the ethical committee of the University of Utrecht for the use of the animals for research was obtained. The testes were collected from several veterinary clinics and a hign number of different breeds of dogs were included in this study. The age of the dogs varied from 3 to 14 years. Bilateral orchidectomy was performed except in 2 dogs in which only one testis was present. Four dogs had signs of feminization. Tumors were dissected free as well as possible, cut in pieces, and immediately placed in liquid nitrogen. One piece of tumor material was fixed by immersion in Bouin's solution for at least 24 hours. If a (macroscopically) nonhomogenous tumor was present, more pieces of tissue were fixed. The frozen pieces were stored at -70°C until further processing.

Characterization of tumors
The pieces of tissue fixed by immersion were embedded in paraffin and sections were cut at 5 μm. Sections were stained with Mayer's hematoxylin and eosin for

Table 1: Primer sequences

Primer	Size	Sense	Antisense
Aro	419	CCCACTTCAGGTTCCTCTGGATGG	TGTTAGAGGTGTCCAGCATG
AR	598	CCGTGAGCGCAGCACCTCCCGGTG	TGCTCTCCCGCTGCTGCTACCTTCTG
IGF-I	350	ATGTCCTCCTCGCATCTCTT	TCCCTCTACTTGCGTTCTTC
IGF-II	450	TTGGCCTTCGCCTCGTGCTG	GGACGGTGACGCTTGGCCTCTCTG
IGF-IR	437	AAATGTGCCCGAGCGTGTG	TGCCCTTGAAGATGGTGCATC
Red 1	213	CTGAGGAATCTCCGAAAACC	TCTAAGGTACCACCGGTGAT
Red 2	250	TCACTAGAGGGAGGCCTTTTC	ACAAGCCACCTTGTGGAATC
IGFBP1	240	ATAACTCAGGAGCAGCTTCTGG	TCTTGTTGCAGTTTGGCAG
IGFBP2	553	AACGGCGAGGAGCACTCTGA	AGGCACCGGCTGGCTATGTT
IGFBP3	200	AGTGAGTCCGAGGAGGACC	GACTCAGAGGAGAAGTTCTGGG
IGFBP4	513	CTGCGGTTGTTGTGCCACTTGC	CAGCATTTGCCACGCTGTCCATC
IGFBP5	484	GCAGGACGAGGAGAAGCCGC	TCCACGCACCAGCAGATGCC

Aro = p450-aromatase enzyme, AR = androgen receptor, IGF = insulin-like growth factor, IR = receptor type I, Red 1 = type I 5α-reductase enzyme, Red 2 = type II 5 α reductase enzyme, IGFBP = binding protein.

histological examination. Paraffin sections were also stained with an antibody against the LH receptor which is characteristic for Leydig cells in the testis [21]. An antibody against vimentin was used to identify Sertoli cells. This antibody does not stain seminoma cells in dog testes and can, therefore, be used to discriminate between Sertoli cells and seminoma cells [22] as was described in an earlier study [21].

Immunohistochemical staining was also performed with frozen material. Frozen pieces of tumorous testis tissue were cleaved, one half being used to prepare cryostat sections of 5 μm, which were stained with the antibody against LH receptor, and the other half being used to study gene expression.

Tissue processing for RNA isolation and RT-PCR
Frozen pieces of testicular and tumorous tissue were weighted. About 1 g of frozen material was transferred to a liquid-nitrogen-cooled container and ground thoroughly with a dismembrator (Braun Biotech Int., Melsungen, Germany) for 45 s at 2200 rpm. Tissue powder was decanted into another liquid-nitrogen-cooled tube and stored at -70C°. When necessary the remaining material was homogenized again for 45 s with the dismembrator. This step was repeated until all pieces were completely ground into powder.

Total RNA isolation was performed using the Qiagen RNeasy Maxi Kit (Westburg, Leusden, The Netherlands). Reverse transcription of the poly (A)+ RNA was performed with a reverse transcription system (Promega Corporation, Madison, WI). Five μg of total RNA was used for this reaction in a mixture of 30 μl 25 mM $MgCl_2$, 10 μl 10 × AMV-RT buffer, 10 μl 10 mM dNTPs, 100 units RNasin RNase inhibitor, 75 units AMV reverse transcriptase, 2.5 μg oligo 15(dT) primer, and RNase free water to a final volume of 100 μl. The reaction occurred for 60 min at 42°C followed by 5 min at 95°C. For the PCR reaction 10 μl of the RT-PCR mixture together with 1.25 units Taq DNA polymerase (Promega), 4 μl 10 × PCR buffer (Promega), and 10 pmol of each primer were combined. Sterile water was added to a final volume of 50 μl. Primers for aromatase, 5α-reductase, androgen receptor, IGF-I, IGF-II, IGF-IR, and IGFBP1 to IGFBP5 were used for the different reactions (Table 1).

A PCR with sterile water with or without RT mixture was used as a negative control. The numbers of cycles were chosen in such a way that the reactions were in the exponential phase of the amplification reactions, enabling a semiquantitative evaluation of the results. For this purpose several tests were carried out with 20, 25, 30, and 35 cycles to estimate the linear section. PCR was performed in a Perkin Elmer Cetus or a MJ Biozym thermal cycler using 30 or 35 cycles of denaturation (94°C, 1 min; first cycle 94°C, 5 min), annealing (55–60°C, 1 min) and extension (72°C, 1 min) followed by a final extension of 10 min at 72°C (Table 2).

Fifteen μl of all PCR products were separated on 1.2% agarose gels stained with ethidium bromide and photographed under UV illumination using a CCD camera. The DNA molecular weight marker VI was used as a size marker (Boehringer, Mannheim, Germany). Photographs were scanned and band intensities were measured using Molecular Analyst software (BioRad Laboratories, Veenendaal, The Netherlands). Fragment sizes were quantified and compared with that of negative controls. Per pair of primers all samples were separated on one gel and each gel contained a negative control.

Table 2: PCR schedule

Primer	Temperature	Cycles	Magnesium	Gel %
Aro	MJ 55°C	35	1.5 mM	1.2
AR	PE 55°C	30	1.5 mM	1.2
IGF-I	PE55°C	35	1.5 mM	1.2
IGF-II	PE55°C	35	1.5 mM	1.2
IGF-IR	PE58→50°C	35	1.5 mM	1.2
Red 1	55°C	35	3.0 mM	1.0
Red 2	55°C	35	3.0 mM	1.5
IGFBP1	58→50°C	35	1.5 mM	1.5
IGFBP2	60→55°C	35	1.5 mM	1.2
IGFBP3	55°C	35	4.0 mM	1.5
IGFBP4	55°C	30	1.5 mM	1.2
IGFBP5	55°C	30	1.5 mM	1.2

Aro = p450-aromatase enzyme, AR = androgen receptor, IGF = insulin-like growth factor, IR = receptor type I, Red 1 = type I 5α-reductase enzyme, Red 2 = type II 5α reductase enzyme, IGFBP = binding protein, MJ = MJ biozym thermal cycler, PE = Perkin Elmer Cetus.

All PCR products were sequenced with the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). For sequence analysis an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA) was used. Sequences were matched with the Gen-Bank using the Blast Search program in order to be certain that the correct products were identified.

Statistical Analysis
Dogs with different types of tumors were compared separately with dogs with normal testes. Dogs with mixed tumors were not evaluated according to the type of tumor but as one group. Differences were evaluated statistically using SSPS 7.5 software. A one-way Anova was used with *post hoc* tests (LSD) to analyze differences between tumors and normal testes. Differences were considered to be significant when $p < 0.05$.

Results
Characterization of testis tumors
Immunohistochemical characterization of the tumors with Leydig cell specific antibodies against the LH-receptor and 3β-hydroxy steroid dehydrogenase (3β-HSD) and the Sertoli cell specific vimentin antibody revealed that 7 dogs had Leydig cell tumors, 7 had seminomas, 6 had Sertoli cell tumors and 4 had mixed tumors. The mixed tumors consisted of 2 combinations of Sertoli cell tumor and Leydig cell tumor and 2 combinations of seminoma and Leydig cell tumor. There were bilateral tumors in 9 dogs. Seven of these dogs had identical tumors in both testes. These were Sertoli cell tumors (4 dogs), seminomas (1 dog), Leydig cell tumors (1 dog), and mixed Sertoli cell tumor and Leydig cell tumor (1 dog). Two dogs had a Sertoli cell tumor in the left testis and either a Leydig cell tumor or a seminoma in the right testis.

In 3 dogs with a Sertoli cell tumor and 1 dog with a Leydig cell tumor there were signs of feminization.
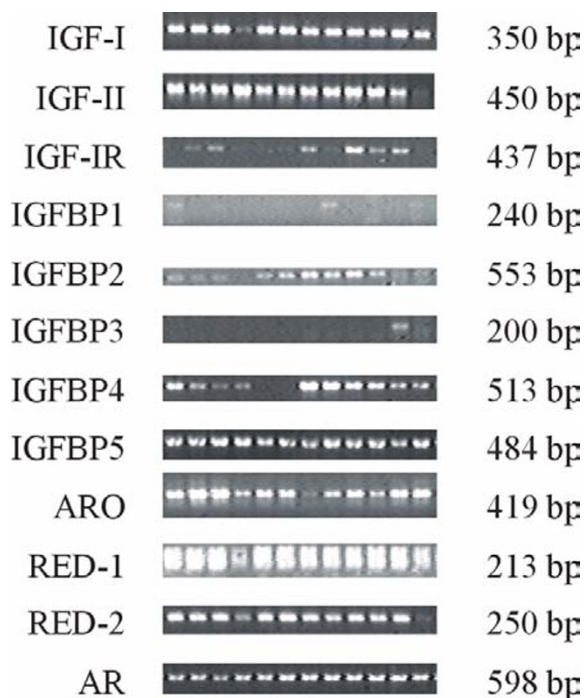
Expression of genes
In the normal testes as well as in testis tumors, a clear expression was found by RT-PCR analysis of IGF-I, IGF-II, IGF-R and the binding proteins IGFBP2, IGFBP4, and IGFBP5. The expression of IGFBP1 and IGFBP3 was relatively low or absent (Fig. 1). There was also a clear expression of both types of the 5α-reductase enzyme (types I and II), the androgen receptor, and p450-aromatase (Fig. 1). Compared to normal testes Sertoli cell tumors and seminomas had significantly lower expression of IGFBP1, IGF-I, and 5α-reductase type 1. Seminomas also had a significantly lower expression of aromatase (Table 3). No differences were found with regard to the other genes investigated. Thus, except for aromatase the expression patterns of these two different types of tumors were very similar. Compared to normal testes Leydig cell tumors and mixed tumors had a significantly higher expression of IGFBP4 and IGF-IR (Table 3). The expression pattern of the mixed tumors was quite similar to that of the Leydig cell tumors. The expression of aromatase was significantly lower in dogs with mixed tumors than in dogs with normal testes (Table 3).

In three Sertoli cell tumors and one Leydig cell tumor of dogs that showed feminization there was no increase in the aromatase expression.

Discussion
RT-PCR analysis revealed a clear expression of the androgen receptor (AR), aromatase, IGF-I, IGF-II, 5α-reductase type I and II, and IGFBP5 in almost all samples from dogs with testis tumors and from dogs with normal testes. There was a less pronounced but still visible expression in

Figure 1
RT-PCR products of canine testis tumors. IGF = insulin-like growth factor; IGF-1R = insulin-like growth factor receptor type 1; IGF-BP = insulin-like growth factor binding protein; ARO = p450-aromatase enzyme; RED = 5α-reductase enzyme type I (RED-1) and type II (REDII); AR = androgen receptor; Lanes 1–12: random samples of canine testicular tumours.

In the present study differences in the expression of several genes of the IGF system were observed between normal testis tissue and the different types of testicular tumors. IGF-I and IGFBP1 expression were reduced in Sertoli cell tumors and seminomas. IGFBP4 and IGF-RI mRNA levels were not different from normal dog testes. In contrast to these observations, IGF-I and IGFBP1 mRNA levels were increased in human neoplastic tissues, where the IGF system is believed to play a role in tumorgenesis [26–28]. Although it does not seem likely that the IGF system plays an important role in the development of canine Sertoli cell tumors and seminomas, measurement of IGF, IGF-RI and IGFBP proteins in tumor tissue should be carried out to elucidate a possible role of this system in development of these two types of tumors.

In dogs with a Leydig cell tumor, or mixed tumors with a Leydig cell component, significantly higher IGF-IR and IGFBP4 expression was observed. IGFBP's may compete with the IGF-R's for binding of the available IGF. In cultured porcine Sertoli cells an increase in IGFBP3 has been shown to result in a decrease in bioactive IGF, suggesting that IGF binds with higher affinity to the binding protein than to its receptor [29]. Hence, although IGF-I mRNA levels were not different from normal testes tissue in dogs with Leydig cell tumors and mixed tumors, we can not exclude that the modulation of IGF-I action may be different among these groups of dogs, due to changes in IGFBP4 and IGF-IR mRNA levels. Moreover, we have only measured the IGF-I, IGF-RI and IGFPB mRNA levels in testes of dog with large tumors. We can not exclude the possibility that the initial growth phase of the tumors coincides with elevated levels of some of the members of the IGF system, followed by a decrease when the tumor ceases to grow or when tumor growth is reduced. This needs to be further investigated.

In general the changes observed in the expression of the IGF system among the different types of tumors is not very dramatic when compared to normal testis tissue. This does not make it likely that this system plays an important role in tumor maintenance and growth. In order to determine whether the IGF system is involved in the initiation of tumor development in the canine testis, mRNA levels should be determined from middle aged dogs to old dogs when the chance of tumor development increases dramatically. IGFs are also known to contribute to the process of tumor metastasis by stimulating the motility of malignant cells [30]. Since testicular tumors in dogs are usually not malignant [2], it could be argued that the relatively normal expression levels of the IGF system contributes to the low tendency to metastasize in this species.

The lower expression of 5α-reductase type I in dogs with Sertoli cell tumors and seminomas compared to dogs with

almost all samples for IGFBP2, IGFBP4, and IGF-1R. In only a few samples there was a clear expression of IGFBP1 and IGFBP3. Care was taken to limit the number of cycles for the RT-PCR in order to remain within the linear part of the amplification reaction. It should be stressed that the results have to be interpreted as semiquantitative and with caution. Nevertheless, using the RT-PCR technology, indications of changes in gene expression patterns can be evaluated.

Earlier studies have shown expression of most genes of the IGF system in mammary gland tissue of dogs [23,24]. To the best of our knowledge, this is the first report of the expression of genes of the IGF system in the canine testis, implicating that this system may play a role in the adult canine testis. In the human and rat testis it has been shown that the IGF system is expressed and plays an important role in testicular functioning [9,25].

Table 3: RT-PCR analysis of canine testis tumors. PCR products were separated on agarose gels containing ethidium bromide. The gels were photographed and scanned and then relative band intensities were measured with software from Molecular Analyzer and expressed as volume counts per mm^2 ± the standard error of means.

Diagnosis	ARO	IGFBP1	IGFBP4	IGF-1	IGF1-R	RED 1
SCT (n = 15)	20.4 ± 8.8	10.7 ± 5.4*	17.9 ± 12.2	26.3 ± 12.1*	5.6 ± 2.2	41.3 ± 23.3*
SEM (n = 7)	17.0 ± 2.9*	8.7 ± 6.5*	17.0 ± 15.1	27.1 ± 8.0*	10.4 ± 4.1	38.3 ± 17.9*
LCT (n = 9)	19.4 ± 10.6	16.0 ± 4.9	33.3 ± 18.5*	35.4 ± 11.5	21.3 ± 3.2*	66.7 ± 20.6
MIXED (n = 5)	13.4 ± 5.3*	20.8 ± 9.4	36.8 ± 17.3*	30.8 ± 10.4	22.3 ± 8.4*	66.6 ± 6.7
NORMAL (n= 13)	27.6 ± 2.8	17.4 ± 9.4	19.5 ± 11.3	37.3 ± 9.3	7.9 ± 1.2	69.0 ± 15.9

SCT = Sertoli cell tumor, SEM = seminoma, LCT = Leydig cell tumor, mixed = a combination of testis tumors, ARO = p450-aromatase enzyme, IGFBP = insulin-like growth factor binding protein, IGF = insulin-like growth factor, IGF1-R = insulin-like growth factor receptor and RED 1 = 5α reductase type I. The number of tumors included in the measurements is indicated between brackets. Asterisk indicates significant difference between the tumor and the normal testis (p < 0.05).

normal testes, suggests a reduced capacity to form androgens. The 5α-reductase enzymes type I and type II which convert testosterone to the metabolically more active form dihydroxytestosterone (DHT), are localized in testicular tissues [31,32]. Since the conversion of testosterone in DHT is IGF-1 dependent [20]. The reduction of 5α-reductase expression in Sertoli cell tumors could be a result of the lower IGF-1 expression. In human skin fibroblasts an increase in IGF-I levels has been shown to cause a rise in 5α-reductase activity [33]. It is not likely that the seminoma cells themselves express 5α-reductase, hence, the decreased 5α-reductase expression in these tumors is presumably the result of the reduced number of Sertoli cells and Leydig cells present in the tumor.

Seminomas and mixed tumors had a lower expression of aromatase than normal testes tissue, while the expression in Sertoli cell tumors and Leydig cell tumors was comparable to that in normal testes even when signs of feminization were present. A comparable expression of aromatase in these tumors was a rather unexpected finding, since the presence of both Sertoli cell tumors and Leydig cell tumors in dogs can result in elevated estradiol levels, sometimes even causing feminization [34]. In other mammals, including man, aromatase has been found in Leydig and Sertoli cells as well as in germ cells [35], while in the equine testis only Leydig cells have been shown to contain aromatase activity [36]. There are several possible explanations why aromatase expression in dogs with Sertoli cell tumors and Leydig cell tumors is comparable to that in normal dogs, although the level of estradiol in peripheral and testicular venous blood was elevated [37]. First, the aromatase enzyme is not the rate-limiting step in the formation of estrogens unless it becomes saturated. A condition that is very unlikely to happen since the level of testosterone in dogs with Sertoli cell tumors is very low [37]. Second, due to the reduced 5α-reductase type I expression, there is more testosterone

substrate available for the aromatase enzyme to convert to estradiol, since both enzymes use androgens as substrate. Third, there may be no correlation between aromatase activity and thus estrogen synthesis, and aromatase mRNA expression in canine testicular tumors, as was demonstrated in the epididymis of monkeys[38]. Fourth, the level of the catabolic 17beta-HSD enzyme, that is able to convert estradiol to estrone, can become down-regulated in pathological testis tissue leading to an accumulation of estradiol. This issue obviously needs more research in order to clarify the discrepancy between aromatase mRNA expression and estradiol levels in serum.

We did not observe differences in the expression of the androgen receptor among the different types of tumors. The androgen receptor has been implicated to play a role in neoplastic transformation of the prostate in men [39] and by its presence in neoplastic germ cells, a role in the pathogenesis of male germ cell tumors has also been suggested [40]. Expression of the androgen receptor in the canine testis has been examined in a previous study. These authors were unable to detect androgen receptors in benign Leydig cell tumors, while malignant Sertoli cell tumors and seminomas were characterized by higher androgen receptor levels [41]. We investigated only benign tumors, which may explain the differences between our findings and the study mentioned above.

In conclusion, the expression of genes of the IGF system has some different features in canine testis tumors compared to normal testis tissue. Similar expression patterns for IGF-I and IGFBP1 were found for Sertoli cell tumors and seminomas, suggesting the possibility of a common pathobiology. Leydig cell tumors and mixed tumors, all of which contained Leydig cell tumor components had a different expression pattern, in these tumors the expression of IGF-RI and IGF-BP4 was increased. However, the observed changes in gene expression were all relatively

small, and thus it does not seem likely that the IGF system is important for tumor maintenance and growth in the dog testis. Whether this system is involved in the initiation of tumor growth is subject to further investigation. Furthermore, signs of feminization cannot be attributed to increased expression of aromatase mRNA levels. In order to obtain more insight in this complicated model of multiple tumor induction in the canine testis, additional research in necessary.

Acknowledgments

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