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Immunohistochemical expression of SOX9 protein in immature, mature, and neoplastic canine Sertoli cells

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Abstract

Sex-determining region Y box9 gene (SOX9) encodes a nuclear transcription factor highly conserved throughout vertebrates [1]. SOX9 plays crucial roles during development, being essential for chondrocytes differentiation and cartilage formation [2], notochord development [3], and maintenance of the pancreatic progenitor cell pool [4], among several developmental processes. In particular, SOX9 is involved in testicular development being a key testis-determining gene for specifying the Sertoli cell (SC) lineage [5-7].

SOX9 transcripts, initially expressed in the genital ridge of both sexes, becomes upregulated in SCs, whereas it is downregulated in the ovaries [8,9]. The inactivation of SOX9 before the sex determination stage results in XY sex reversal [3,10]. Two thirds of XY patients with a mutation in SOX9 show sex reversal indicating an essential role of this gene in the testis determination [11]. In addition, recent studies reported that SOX9 upregulates the production of anti-Müllerian hormone [12,13], secreted during the embryonic development by SCs and promoting the regression of the Müllerian ducts [14]. This latter finding supports the crucial role of SOX9 in male sex determination and testicular development. In scientific literature, few papers investigating the gene expression and protein localization of SOX9 in normal testis have been published, and most of them are focused on embryonic and fetal gonads, both in human and laboratory animals [15,16]. In human undifferentiated gonads, SOX9 protein was initially demonstrated by immunofluorescence in the cytoplasm of somatic cells and then become restricted to the nuclei of SCs when sex cord formation begins [9] and during all the normal testicular development, as demonstrated by immunohistochemistry and in situ hybridization as well [17].

Besides studies about the embryologic and fetal development of the testis, few studies explore SOX9 protein expression in adult and/or in pathologic testes [18]. In mice, the immunohistochemical pattern of SOX9 expression during testis formation and adulthood parallels humans [8,19,20]. On the other hand, in rats, SOX9 protein expression seems to be dependent on the age and stage of spermatogenesis: prominently expressed in gonadal blastemal of embryonic

testis, SOX9 gradually declines in postnatal gonads but strongly re-occurs in pubertal and adult testis [16,21].

Regarding other animal species, data are relatively scarce and mainly focused on embryonal gonads [15,22]. Concerning canine species, a polymerase chain reaction-based study investigated the expression of SOX9, both in female and male embryos from 27 to 37 days of gestation. SOX9 was found to be expressed in the urogenital ridge, in both sexes up to Day 30 of gestation, and then it becomes markedly elevated only in male gonads with little or no expression in other components of the urogenital ridge. Authors concluded that these findings similarly to those reported for humans and other domestic animals were consistent with the role of SOX9 in testis determination [23]. Nevertheless, besides its critical role in testicular determination and different timing of expression among species, the physiological relevance of SOX9 in adult testes and its possible role in the neoplastic counterpart deserve further investigation.

As a gene related to cellular proliferation and differentiation, SOX9 protein induction and its implication in the growth of neoplastic cells and extracellular matrix deposition has been recently considered [24] and SOX9's role in epithelial invasion, migration, and proliferation in various cancers and its prognostic potential has been reported [25,26]. Moreover, SOX9 deregulation and/or mutation have been found in several human cancers [27].

Recently, studies on the immunophenotype of developing, adult, and neoplastic testicular cells have been performed in dogs to prove their potential role as a relevant model for human testicular cancer [28]. Because spontaneous testicular cancers are quite common in dogs and SOX9 protein expression in normal and neoplastic SCs has never been examined in this species, the present study aimed to investigate the expression of SOX9 protein in canine SCs during testicular maturation and neoplastic transformation.

2. Materials and methods

2.1. Samples

No animals were killed for this study, and all selected samples were originally submitted for diagnostic purposes to departmental routinary biopsy or necropsy service. For this retrospective study, paraffin blocks of formalin-fixed normal pairs of testes from three canine fetuses (50 days of gestation), four newborn dogs (from 0 to 20 days of age), and four prepubertal puppies (56, 90, 120, and 180 days of age) and from five adult/aged dogs (2, 4, 6, 10 and 13 year old) were selected from departmental archives. During sample selection, particular attention was posed to consider well-preserved testes: not macerated fetal testes, adult normal testes from routinary spaying, immature testes from well-conserved puppies, submitted to necropsy after sudden traumatic death. Regarding neoplastic testes, from the same diagnostic archive, 31 SC tumors and five Leydig cell tumors (LCTs) were selected. Sertoli cell tumors (SCTs) were from dogs ranging in age from 4 to 15 years.

One of these tumors was malignant, with metastases to the lumboaortic lymph nodes 2 years after surgical castration; 10 of 31 SCTs developed within cryptorchid testes. LCTs were from dogs ranging in age 9 to 13 years and all developed in eutopic testes.

2.2. Histology

For each sample, 5-mm-thick sections were obtained and stained with hematoxylin and eosin. As in previous studies, the histologic variants of canine SCTs were recorded as “typical” and “lipid rich” [28]. The growth pattern of the SCTs was also recorded and defined as“intratubular” or “diffuse.” Tumors showing both intratubular and diffuse neoplastic growth were defined as “intratubular/diffuse.” The histologic variants of LCTs were classified as “solid-diffuse” and “cystic vascular” as reported in the “World Health Organization International Histological Classification of Tumors of Domestic Animals” [29]. For all the neoplastic testes, the mitotic index (MI) was

assessed by counting the mitotic figures in 10 high-power fields (x 400), and the mean value for each sample was then recorded.

2.3. Immunohistochemistry

The immunohistochemical staining of all samples was performed using the avidin-biotin-peroxidase complex procedure [30] with a commercial immunoperoxidase kit (Vectastain Standard Elite; Vector Laboratories, Inc., Burlingame, CA, USA).

Sections were dewaxed, treated with hydrogen peroxide 0.5% in methanol for 20 min, and rehydrated. Antigen retrieval was carried out by microwave in citrate buffer, pH 6.0 (10 min, 650 W). After pretreatment, the sections were incubated for 30 min in normal goat serum (diluted 1:60). As a primary antibody, a rabbit anti-human polyclonal antibody directed against SOX9 (Sigma-Aldrich Corporation, St. Louis, Missouri, USA) was applied and diluted 1:150 in Tris buffer. According to the manufacturer's instructions, the antibody was raised against a human SOX9 synthetic peptide sharing 96% amino acid homology with Canis familiaris SOX9. Sections were incubated overnight in a humid chamber at 4 C. After incubation with the secondary biotinylated anti-rabbit immunoglobulin (diluted 1:200; Vector Laboratories, Inc.) for 30 min, the avidin-biotin-peroxidase complex method (Vector Laboratories, Inc.) was performed.

The peroxidase reaction was developed by 3-amino 9-ethyl carbazole (Vector Laboratories, Inc.), and sections were counterstained with Mayer's hematoxylin. Negative controls were obtained replacing the primary antibody with normal goat serum.

2.4. Assessment of the immunohistochemical labeling

The percentage of positive cells was semiquantitatively assessed and scored as follows: - 0; + 20% or less; ++ 21% to 50%; +++ 51% to 80%; ++++ 81% to 100%.

3. Results

3.1. Histology

Both in fetuses and newborns, the seminiferous tubules were small, with no recognizable lumen, and lined by undifferentiated SCs and rare early germ cells.

In the testes from prepubertal dogs, spermatogonia were recognizable in all samples together with few spermatocytes in the oldest one (180 day old). In this latter dog, a small central lumen was also evident.

In the testes from adult dogs, complete seminal line, including spermatozoa, was evident into the seminiferous tubules.

Regarding SCTs, on the basis of their pattern of growth,17 of 31 tumors were classified as "intratubular" and nine of31 as "intratubular/diffuse." Five cases were characterized by a "diffuse" pattern of growth; 18 of the 31 tumors examined (12 intratubular, three "intratubular/diffuse," and three "diffuse," respectively) were classified as typical SCT. These tumors comprised neoplastic tubules, irregular in shape and diameter, and surrounded by thick basal membranes. Neoplastic tubules were separated by a variable amount of dense fibrous stroma and lined by one to two layers of tall and slender cells, oriented perpendicularly to the basal membrane in a palisading arrangement. Neoplastic cells were characterized by indistinct cell borders, oval to spindle basal nuclei with stippled chromatin, and scant to moderate, faintly eosinophilic cytoplasm.

In 13 of 31 SCTs (five intratubular, six "intratubular/ diffuse," and two "diffuse", respectively), neoplastic SCs contained large intracytoplasmic, round, smooth contoured vacuoles displacing the nucleus at the periphery, giving the cell a signet-ring appearance. These cases, according with a previous report in the canine species [28], were classified as "lipid-rich" SCTs.

Three cases out 31 SCTs, two intratubular typical, and one intratubular lipid rich were additionally characterized by rare Call-Exner bodies, represented by neoplastic SCs arranged in rosettes surrounding microcavities filled with hyaline eosinophilic amorphous material.

In nine of 31 SCTs (four typical and five lipid rich), scattered areas of intratubular necrosis, cystic cavities, and hemorrhages were present. In three of these nine cases, neoplastic SCs were characterized by severe anisokaryosis and anisocytosis, infiltration of the albuginea, and large areas of necrosis. One case was associated with lumboaortic lymph node metastasis and, thus, was malignant.

In most SCTs (24 of 31), MI was low, ranging from 0 to0.5. The remaining seven cases had high MI (0.7-2.4).

Concerning LCTs, three cases, composed by sheets and cords of polygonal cells separated by scant amount of fibrovascular stroma, were classified as "solid-diffuse" LCTs. One case, characterized by interconnecting cords of neoplastic Leydig cells (LCs), surrounding and delineating large lacunae filled with erythrocytes, was classified as "cystic vascular" LCT. Finally, in one case, both growth patterns were present and it was, therefore, classified as "solid-diffuse"/"cystic vascular" LCT. Neoplastic cells were polyhedral to cuboidal, with low N/C ratio, finely granular eosinophilic cytoplasm, variably distinct cell borders, small and round eccentric nucleus, and an indistinct nucleolus. In two cases, large clusters of cells had numerous vacuoles within the cytoplasm, compressing the nucleus to the periphery (signet ring cells). In three cases, focal hemorrhages were observed. In all the LCTs, MI was less than one.

3.2. Immunohistochemistry

In fetal and neonatal testes, a strong positive and exclusively nuclear immunolabeling for SOX9 protein was detected in all SCs (++++) (Fig. 1A and B).

In the testes of the four prepubertal puppies, the immunohistochemical staining was variable. In the youngest and oldest dogs, belonging to this category 56 and 180 days old, intense nuclear expression of SOX9 was detected in an high percentage of SCs (+++ and ++++, respectively), similarly to fetal, neonatal, and adult testes.

Conversely, the SCs of the 90-day-old puppy exhibited both strictly nuclear or nuclear and cytoplasmic expression of SOX9 (Fig. 1C). In this case, independently from the localization of the immunohistochemical signal, the percentage of labeled SCs was assessed as +++. In the 120-day-old puppy, all SCs were negative.

In adult testes, all SCs exhibited a strong nuclear immunolabeling (++++) for SOX9 (Fig. 1D). In all testes examined, LCs and germ cells were constantly negative for SOX9.

Regarding the testicular tumors examined, LCTs were always negative for SOX9 protein, whereas in 31 of 31 SC tumors, SOX9 was constantly expressed. In 28 of 31, the percentage of stained SCs was high, ranging from+++/++++. In only three cases, the percentage of positive cells was low, less than 50% (++). The signal varied from nuclear (Fig. 2A), nuclear and cytoplasmic (Fig. 2B), and exclusively cytoplasmic in 18 of 31, 11 of 31, and two of 31 SCTs, respectively.

Nuclear labeling was observed in 12 typical and six lipid-rich SCTs; nuclear and cytoplasmic labeling was evident in six typical and in five lipid-rich SCTs, whereas a strictly cytoplasmic signal was recorded in two lipid-rich SCTs. Interestingly, in one lipid-rich SCT, exhibiting both nuclear and cytoplasmic signal, a strong positive staining of the membrane of neoplastic cells was observed (Fig. 2C).

Although all the SCTs were positively labeled for SOX9, they were characterized by an heterogeneous pattern of expression, particularly evident in the lipid-rich cases: within a single tubule, most cells could be immunolabeled, or the positive immunostaining could be restricted to a small number of cells. Moreover, strongly immunostained tubules could be adjacent to weakly positive or completely negative ones (Fig. 2D).

Within the atrophic testicular parenchyma surrounding both SCTs and LCTs, SOX9 immunolabeling was confirmed to be strictly limited to SC nuclei (Fig. 1E).

4. Discussion

In mammals, numerous studies focusing on the genetic regulation of mammalian gonads reported the pivotal role of SOX9 gene in male gonadal development. Highly conserved among vertebrates, SOX9 gene expression was already demonstrated by polymerase chain reaction in testes of canine fetuses from 27 to 37 days of gestation [23].

On the other hand, scarce information about immunohistochemical SOX9 protein expression and localization in postnatal testes is present in literature, and data about its expression in testicular tumors are lacking. The present study investigated the immunohistochemical expression of SOX9 in normal canine testes, from fetal to adult age, and in tumors derived from SCs and LCs.

In the dog, SOX9 immunolabeling was confirmed to be limited to the nucleus of SCs in fetal, neonatal, and normal adult testes, as already described in human species and laboratory animals [8,9,17,18,20,21]. Interestingly, in the testes from the four prepubertal dogs, the immunohistochemical expression of SOX9 protein was highly variable. In the youngest and oldest dogs (56 and 180 days old), SOX9 was restricted to the nucleus of SCs, as observed in fetal and neonatal testes, whereas in the 120-day-old pup no expression of SOX9 was observed. In the 90-day-old dog, SOX9 expression reappeared and was detectable both in the nucleus and in the cytoplasm. Similar variations in SOX9 protein expression have been reported in rat gonads.

In rats, nuclear SOX9 expression was described in SCs of fetal testes and then the immunoreaction gradually declined, becoming faintly positive to negative in postnatal rats. From the onset of puberty until 15-day-old rats, SOX9 protein expression returned and increased [16,21]. These authors suggested that the presence and amount of SOX9 protein could be dependent on the age and the spermatogenetic stage within the seminiferous tubules. Although the present study only

examined four prepubertal dogs and, in particular only one of 120 days, the results seem to parallel what is observed in rats, suggesting a similar biologic role. However, these results deserve to be verified on a larger number of samples.

In all testes examined, seminal and LCs were always negative, consistently with results obtained in other mammals [9,18]. In addition, SOX9 immunohistochemical signal was absent in all LCTs, whereas SCTs were constantly positive. These results confirmed that in canine testis SOX9 protein could be considered a reliable marker for normal and neoplastic SC and could be used to discriminate between LCTs and SCTs, in particular the lipid-rich variant. In fact, LCTs and SCTs share morphologic features although having different clinical outcomes: LCT is a benign tumor, whereas malignant and metastasizing SCTs have been described [31].

In the present study, all the SCTs were positively labeled. A strong immunohistochemical signal, varying from 51% to 100% of neoplastic cells was observed, confirming that SOX9 protein remains a specific marker of SCs, also in the neoplastic counterpart. In most SCTs (18 of 31), SOX9 labeling was exclusively nuclear, as observed in normal testes, whereas in 11 of 31 cases the signal was nuclear and cytoplasmic and in two of 31 exclusively cytoplasmic.

The cytoplasmic expression of SOX9 protein observed both in neoplastic samples and in one prepubertal pair of testes is an interesting finding that deserves some consid-eration. Cytoplasmic SOX9 protein expression has been described in the early stage of testicular development. In both murine and human species, during early gestation, SOX9 gene is upregulated in the male. Simultaneously, SOX9 protein, which appears cytoplasmic in the undifferentiated gonads, becomes nuclear in pre-SCs [8,9]. On the basis of these observations, we hypothesize that the cytoplasmic expression of SOX9 protein in prepubertal and neoplastic SCs could be related to functional immaturity or alternatively could represent an undifferentiated, not fully developed SC transient phenotype, similarly to what observed during the testicular development. The dynamic subcellular redistribution of SOX9 protein in the gonads at the time of sexual differentiation has been described both in humans and mice [8,9] demonstrating that SOX9 is able to shuttle between the nucleus and

the cytoplasm, hypothesizing the existence of a nuclear export signal triggering male-specific sexual differentiation [32]. In human medicine, no data regarding testicular neoplasm and SOX9 protein expression are present, but this marker has been used on other neoplastic tissues (mammary gland and ovaries) revealing interesting results in a subset of cases, such as cytoplasmic and/or membranous labeling [27,33,34].

SOX9 is a transcription factor; thus, the nuclear distribution of this protein is an expected finding, compared with cytoplasmic and membranous staining. According to Chakravarty et al. [26], cytoplasmic SOX9 in breast cancer is associated with invasive and metastatic breast carcinomas and it is related to increased cell proliferation. Similarly, in two thirds of ductal breast lesions, SOX9 protein was located in the cytoplasm and not in the nucleus [34].

Moreover, cytoplasmic and membranous SOX9 staining has been found in "borderline" ovarian tumors and not in well-differentiated ones [33]. Therefore, the results of our study could support the hypothesis that the cytoplasmic expression of SOX9 in neoplastic SCs reflects a less differentiated phenotype. This point could be relevant and should be explored in future studies. Moreover, although all SCTs were SOX9 positive, a variable number of negative neoplastic cells were evident in all the SCTs examined. In addition, in this study, one prepubertal pair of testes was characterized by the lack of SOX9 expression, similarly to a minority of neoplastic SCs not labeled in the SCTs. Regarding normal canine prepubertal SCs, we hypothesize that the negative SOX9 labeling parallels the results observed in rat gonads [21], suggesting that SOX9 expression, before adulthood, is unstable and physiologically reduced. In the same way, negative neoplastic SCs could reflect a more immature or dedifferentiated immunophenotype.

The results obtained from this first study investigating SOX9 protein expression in normal and neoplastic canine testes suggest that this protein may play a role in SC proliferation and neoplastic transformation. However, further investigations on a larger number of samples are required to confirm these hypotheses.

On the other hand, the consistent expression of SOX9 in the SCTs, together with the consistent negative results obtained in LCTs, show that SOX9 is a reliable marker for confirmatory diagnosis of these tumors in dogs.

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Table 1

Expression of SOX9 in fetal, neonatal, prepubertal and adult testes.

No.	Breed	Age	SOX9 immunolabeling	
			Percentage of labeled cells	Signal localization
1	Mixed breed	Fetus (50 d gestation)	++++	N
2	Mixed breed	Fetus (50 d gestation)	++++	N
3	Mixed breed	Fetus (50 d gestation)	++++	N
4	West Highland White Terrier	Stillborn	++++	N
5	Boxer	Stillborn	++++	N
6	Mexican hairless dog	3d	++++	N
7	Mexican hairless dog	20 d	++++	N
8	Rough Collie	56 d	+++	N
9	Mixed breed	90 d	+++	N/C
10	English Bulldog	120 d	-	-
11	American Staffordshire Terrier	180 d	++++	N
12	Golden Retriever	2y	++++	N
13	German shepherd	4y	++++	N
14	Mixed breed	6y	++++	N
15	Dachshund	10 y	++++	N
16	Mixed breed	13 y	++++	N

- 0; +++ 51%-80%; ++++ 81%-100%.

Abbreviations: C, cytoplasmic; N, nuclear; N/C, nuclear and cytoplasmic.

Table 2

Expression of SOX9 in neoplastic testes.

No.	Breed	Age	Growth	Variant	Necrosis and hemorrhages	MI	SOX9 immunolabelling	
						Percentage of labeled cells		Signal localization
1	German Shepherd	4	D	T	X	2, 4	+++	N/C
2	German Shorthaired Pointer	4	D	LR	X	1, 1	++++	N/C
3	Boxer	5	D	T	X	1 ,6	+++	N/C
4	Chihuahua	6	I	T		0, 1	++++	N
5	Mixed breed	6	I/D	T	X	1	++	N/C
6	Rough Collie	7	I	T		0, 1	++++	N
7	Cocker spaniel	7	I	LR		0, 1	++++	N
8	Alaskan malamute	7	I	T		0, 3	++++	N
9	Mixed breed	7	I	T		0, 3	++++	N
10	Mixed breed	8	I	LR		0	++++	N
11	Dalmatian	8	D	T		1, 2	+++	N/C
12	German Shorthaired Pointer	8	I	LR		0, 2	++++	N
13	Mixed breed	9	I	T		0	++++	N
14	Belgian Shepherd	9	I	T		0	++++	N
15	German Shepherd	10	I	T		0, 1	+++	N
16	Labrador Retriever	10	I/D	LR		0, 1	+++	C
17	Doberman Pinscher	10	I/D	LR	X	0, 8	+++	N/C
18	Springer Spaniel	10	I	T		0, 2	+++	N
19	Bergamasco Shepherd	11	I	T		0, 3	+++	N/C
20	Mixed breed	11	D	LR	X	0, 1	++++	C
21	German shepherd	12	I/D	T		0, 3	++++	N
22	Beagle	13	I	T		0, 1	++++	N
23	Mixed breed	14	I	LR	X	0, 3	+++	N/C
24	Yorkshire Terrier	14	I/D	LR	X	0, 2	+++	N/C
25	Mixed breed	15	I/D	LR		0,3	++++	N
26	Mixed breed	NR	I	T		0, 2	+++	N/C
27	Shetland Sheepdog	7	I	LR		0, 5	+++	N
28	Boxer	5	I/D	LR		0, 4	++	N
29	German Shepherd	4	I/D	T		0, 4	+++	N
30	Airedale Terrier	5	I/D	LR		0, 5	þþ	N/C Membrane
31	German Shepherd	NR	I	T	X	0, 7	++++	N
32	Beagle	9	SD			0	-	-
33	American Staffordshire Terrier	10	CV	L	X	0	-	-
34	Dalmatian	11	SD			0	-	-
35	Mixed breed	12	SD/CV	L	X	0	-	-
36	Yorkshire Terrier	13	SD		X	0	-	-

- 0; ++ 21%-50%; +++ 51%-80%; ++++ 81%-100%.

Abbreviations: C, cytoplasmic; CV, cystic vascular; D, diffuse; I, intratubular; I/D, intratubular/diffuse; LR, lipid rich; MI, mitotic index; N, nuclear; NR, not reported; SD, solid diffuse; SD/CV, solid-diffuse/cystic vascular; T, Typical.

Legends

Fig. 1. SOX9 protein immunohistochemical expression in non-neoplastic Sertoli cells. (A) Canine fetal testis (SOX9 immunolabeling). Strong nuclear staining detectable in all Sertoli cells lining the seminiferous tubules. Negative Rete testis epithelial cells are evident at the center of the image. Magnification 100. Scale bar 70 μm. (B) Canine neonatal testis (SOX9 immunolabeling). Strong nuclear staining detectable in all Sertoli cells. Magnification 200. Scale bar 35 μm. (C) Canine prepubertal testis (SOX9 immunolabeling). Sertoli cells in seminiferous tubules from 90-day-old puppy were characterized by both nuclear and cytoplasmic staining. Magnification 100. Scale bar 70 μm. (D) Canine adult testis (SOX9 immunolabeling). Strong nuclear staining detectable in all mature Sertoli cells. Magnification 100. Scale bar ¼ 70 mm. (E) Canine adult testis. Atrophic testicular parenchyma (SOX9 immunolabeling). Atrophic tubules, generally at the periphery of the Sertoli cell tumors, were lined by Sertoli cells characterized by a strong nuclear labeling. Magnification 100. Scale bar 70 μm.

Fig. 2. SOX9 protein immunohistochemical expression in neoplastic Sertoli cells. (A) Canine intratubular typical Sertoli cell tumor (SOX9 immunolabeling). Strong nuclear staining in neoplastic SCs. Magnification 50. Scale bar 130 μm. (B) Canine intratubular typical Sertoli cell tumor (SOX9 immunolabeling). Strong nuclear and cytoplasmic staining in neoplastic SCs. Magnification 200. Scale bar 35 μm. (C) Canine intratubular/diffuse Sertoli cell tumor (SOX9 immunolabeling). Neoplastic tubules filled with Sertoli cells showing a strong membrane immunostaining. Magnification 400. Scale bar 20 μm. (D) Canine intratubular, lipid-rich Sertoli cell tumor (SOX9 immunolabeling). Heterogeneous pattern of expression of SOX9. Neoplastic tubules at the periphery of the image are lined by neoplastic Sertoli cells characterized by diffuse nuclear labeling, whereas the centrally located tubule is characterized by lesser number of positive

Sertoli cells. Strongly immunostained cells are closely opposed to weakly positive or completely negative ones. Magnification 200. Scale bar 35 μm.

Figure 1

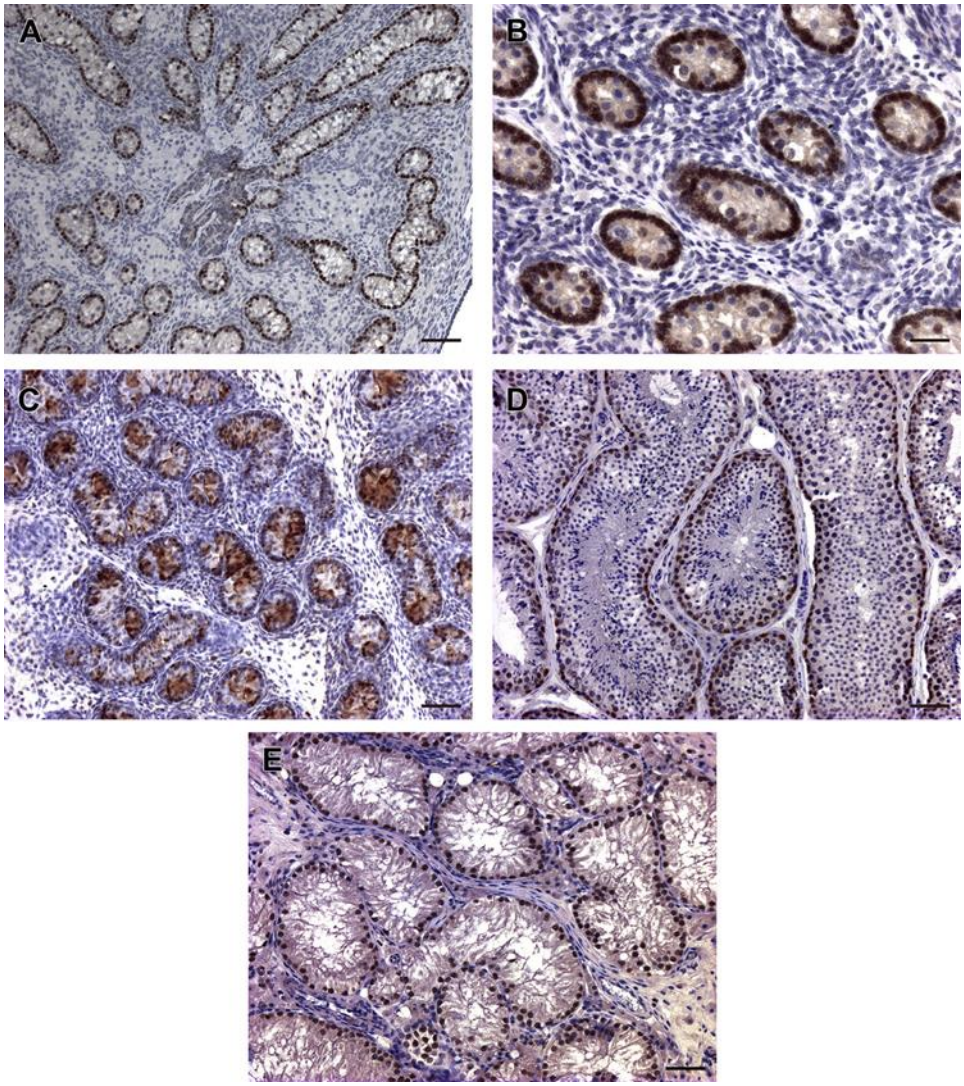

Figure 2