NEOPLASTIC DISEASE

Immunohistochemical Study of Mixed Germ CellSex Cord Stromal Tumours in 13 Canine Testes

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Summary

Mixed germ cell sex cord stromal tumours (MGSCTs) are composed of seminiferous tubules, filled withadmixed neoplastic Sertoli cells (SCs) and germ cells (GCs). The aim of the present study was to describe13 canine testicular MGSCTs and to investigate the histochemical features and the immunophenotype ofthe neoplastic GCs and SCs. Neoplastic SCs were always diffusely labelled for vimentin (VIM), neuron specificenolase (NSE), inhibin (INH)-aand anti-M€ullerian hormone (AMH). Cytokeratins AE1/AE3 (CK) and des-min (DES) were expressed in 6/13 and 8/13 cases, respectively. Neoplastic GCs were labelled for placental alka-line phosphatase (PLAP) in 7/13 cases and for CD117 (KIT) in 8/13 cases, while 10 cases were staineduniformly by periodic acideSchiff (PAS). Immature canine SCs are known to express CK, DES, INH-aand AMH, while immature GCs are stained by PAS and express PLAP and KIT. This GC phenotype alsodistinguishes between classical and spermatocytic seminoma, with the latter being negative for these markers.The results of the present study show that both neoplastic SCs and GCs in MGSCTs have a de-differentiated phenotype.

Mixed germ cell sex cord stromal tumours(MGSCTs) are composed of tubules and cords of ad-mixed germ cells (GCs) and Sertoli cells (SCs) in var-iable proportions (Talerman and Roth, 2007).Canine MGSCTs were first described byPatnaikand Mostofi (1993), who reported MGSCTs in 7%of 262 dogs with testicular tumours. These authorsalso investigated the immunophenotype of the tu-mours, applying a limited number of markers to distinguish between mature and immature

SCs.These markers included neuron specific enolase(NSE), vimentin (VIM), desmin (DES) and cytoker-atin AE1/AE3 (CK). In the same study, periodicacideSchiff (PAS) staining was employed to differetiate gonocytes, early germ cells that contain glycogen(PAS positive), from more mature seminal cells thatdo not. In human and canine seminomas, PAS stain-ing has also been employed to distinguish betweenclassical PAS-positive seminoma and the spermatocytic PAS-negative seminoma (Masson, 1946;Griecoet al., 2007).In the last two decades, new markers such asplacental alkaline phosphatase (PLAP) and CD117(KIT) have been adopted for differentiation between immature and neoplastic GCs in human (Mostofi and Sesterhenn, 1998) and canine (Griecoet al., 2010)testes. Similarly, new markers such as inhibin(INH)-aand anti-M€ullerian hormone (AMH) havebeen employed to delineate different stages of matura-tion in normal and neoplastic SCs (Taniyamaet al.,2001; Griecoet al., 2011; Bancoet al., 2012). In arecent report on canine testicular MGSCT (Owstonand Ramos-Vara, 2007), most of these new markers,together with those used byPatnaik and Mostofi(1993)were applied; however, the results were limited to the single case reported.

The aim ofthe present study was to characterize the immunophe-notype of neoplastic GCs and SCs in 13 canineMGSCTs.Formalin-fixed and paraffin wax-embedded tissuesfrom 13 canine testes, in which a diagnosis of ‘mixedtesticular tumour’ had been made were retrieved from the departmental archives. Three normal caninetestes, obtained during surgical neutering from dogsranging in age from 2 to 4 years, were selected as con-trols. Tumours derived from intact dogs of differentbreeds ranging in age from 7 to 15 years. Two tu-mours (cases 8 and 9) had developed within retainedtestes. Histological sections stained with haematoxylin and eosin (HE) were reviewed and tumours were classified according to the World Health Organisation classification of tumours of domestic animals(Kennedyet al., 1998).Further sections (5mm) were stained with PAS or subjected to immunohistochemistry (IHC) using theABC method (Giudiceet al., 2014). A panel of eightprimary antibodies was applied including reagentsspecific for VIM (3B4, 1 in 1,000 dilution), INH-a(R1, 1 in 40 dilution), CK (AE1/AE3, 1 in 2,000 dilu-tion), DES (NCL-L-DES-DERII, 1 in 300 dilution),AMH (C-20, 1 in 30,000 dilution), PLAP (8A9, 1 in25 dilution), KIT (CD117, 1 in 500 dilution) andNSE (1 in 25 dilution). The VIM, PLAP, KIT andNSE antibodies were from Dako (Dako Corporation,Carpinteria, California, USA), while antibodies spe-cific for INH-a, CK and DES were from Serotec (Ba-varia, Germany), Zymed Laboratories (SanFrancisco, California, USA) and Novocastra (New-castle-upon-Tyne, UK), respectively. AMH antibody was from Santa Cruz Biotechnology (Santa Cruz,California, USA).Epitope retrieval was performed in a microwave(650 W for 10 min) with citrate buffer (pH 6.0) forINH-a, AMH and KIT or with EDTA buffer (pH8.0) for PLAP. Enzymatic antigen retrieval (Pepsin,37C, 14 min) was used for CK and DES. Pepsin and

EDTA buffer were from Zymed Laboratories. Sectionswere incubated overnight at 4C. Secondary bio-tinylated horse anti-mouse immunoglobulin was usedfor detection of binding of antibodies specific for VIM, INH-a, CK, DES and PLAP. Secondary biotinylated goat antirabbit immunoglobulin was em-ployed for detection of binding of antibodies specificfor KIT and NSE, while biotinylated rabbit anti-goat immunoglobulin was employed for detection ofbinding of anti-AMH. All secondary antibodies werediluted 1 in 200 and were from Vector Laboratories (Burlingame, California, USA). These antibodieswere incubated for 30 min at room temperature(RT). After washing, the avidine biotin complex(Vectastain Standard Elite, Vector Laboratories)was added to the sections for 30 min at RT. The immunohistochemical reaction was 'developed' for 15 minwith3-amino-9-ethylcarbazole(VectorLaboratories).Sections were counterstained with Mayer's haematox-ylin.As negative controls, a serial section of each samplewas included, in which primary antibodies were re-placed with normal horse or goat serum (1 in 60 dilu-tion), respectively for monoclonal and polyclonalantibodies. For AMH, normal rabbit serum (1 in 60dilution) was used. For each immunohistochemical test, a section of neonatal canine testes and a section of a canine cutaneous mast cell tumour were includedas positive controls for AMH and KIT, respectively.For the other antibodies, internal positive controlswere available and consisted of interstitial fibroblasts(VIM), normal and/or atrophic SCs (NSE), epithe-lial cells fromrete testisand/or epididymis (CK), my-oid peritubular cells (DES and PLAP) and Leydigcells (INH-a)(Giudiceet al., 2014).For PAS staining and for each immunohistochemical marker, the percentage of stained or immunola-belled cells was assessed semiquantitatively andscored as:, negative; +,<5% of cells labelled;++, 6e30% of cells labelled; +++, 31e70% ofcells labelled; ++++, 71e100% of cells labelled.Microscopically, in the three normal testes, seminiferous tubules exhibited complete spermatogenesis.Tubular basal membranes and vascular walls werePAS positive. MGSCTs appeared as multinodularmasses, generally poorly demarcated, with expansivegrowth within the testicular parenchyma. Tumours were composed of variably sized tubular structures, surrounded by a thick basement membrane and sepa-rated by a variable amount of collagenous stroma.Intimately associated neoplastic SCs and GCs wereclearly recognizable within the neoplastic tubules.Neoplastic SCs were mainly located basally, oriented generally perpendicularly to the basement membraneand surrounded the neoplastic GCs, filling the centre of the seminiferous tubules. In 6/13 tumours, oneneoplastic population dominated over the other. Spe-cifically, 4/6 cases had a predominance of SCs (cases6, 8, 10 and 13) and 2/6 had a predominance of GSs(cases 9 and 12).Neoplastic tubules formed large cystic dilations in3/13 cases (cases 4, 12 and 13) and in 4/13 tumours intraluminal areas of necrosis were present (cases 1, 4, 5and 11). Large, coalescing areas of necrosis and wide-spread haemorrhages were evident only in case 1.In

10 tumours neoplastic GCs were diffusely PASpositive, with the percentage of PAS-stained cells ranging from<5% to 70% (Table 1). IHC revealed scattered spermatogonia positive for KIT in normalCanine Testicular Tumours testes, while SCs in these samples were exclusively anddiffusely positive for VIM and NSE. All othermarkers were not expressed by either GCs or SCs.Within MGSCTs (Table 1), neoplastic SCs werediffusely immunolabelled for expression of VIM in12/13 cases. In the majority of the MGSCTs, the percentage of positively labelled SCs ranged from 6 to100%. In case 11, the percentage of labelled SCswas<5%. NSE was expressed in 11 tumours and the percentage of labelled SCs ranged from<5% to100%. For case 4, no more neoplastic tissue was avail-able in the tissue block and the immunohistochemicaltesting for this antibody was not possible. CK was ex-pressed in 6/13 tumours with the percentage oflabelled SCs ranging from<5% to 70%. DES was ex-pressed by SCs in 8/13 cases and the percentage oflabelled cells was<5% to 30%. INH-awas expressedby SCs in all cases with positively-labelled cellsranging from<5% to 100%. AMH was expressed in 11/13 cases and labelled SCs ranged from<5%to 100% (Fig. 1).Neoplastic GCs expressed PLAP in 7/13 cases andpositively-labelled cells ranged from<5% to 30%(Fig. 2). KIT was expressed in 8/13 cases and labelledcells ranged from 6 to 100% (Fig. 3).All of the tumours were histologically consistentwith the diagnosis of MGSCT according to the description of Patnaik and Mostofi (1993)andKennedyet al.(1998).Moreover, as reported by the same authors, theproportion of the two different neoplastic components varied greatly. Two of the 13 MGSCTs had a pre-dominance of GCs; four had a predominance ofSCs, while in seven cases neoplastic GCs and SCs were similarly represented. This latter finding wasnot observed byPatnaik and Mostofi (1993)who re-ported a predominant cellular population, mostfrequently SCs, in all the MGSCTs described in theirstudy.All cases showed positive immunolabelling of inter-nal control structures. The presence of an internalpositive control in testicular sections is important inorder to avoid false-negative results, which are re-ported frequently in studies of canine testes and maybe related to delayed fixation, over fixation and underfixation (Hewittet al., 2011). For the testes, longitudi-nal gross sectioning before fixation is highly recom-mended.

All cases showed positive immunolabelling of inter- nal control structures. The presence of an internal positive control in testicular sections is important in order to avoid false-negative results, which are re- ported frequently in studies of canine testes and may be related to delayed fixation, over fixation and under fixation (Hewitt et al., 2011). For the testes, longitudi- nal gross sectioning before fixation is highly recom- mended.

In the present study, consistent with previous re- ports (Patnaik and Mostofi, 1993; Owston and Ramos-Vara, 2007), SCs in most of the MGSCTs examined were labelled for VIM and this marker was also expressed by SCs in control testes. VIM therefore appears to represent a reliable marker

for canine SCs, paralleling the situation in human pathology. Similar results were obtained with NSE, which consistently labelled SCs in MGSCTs (Patnaik and Mostofi, 1993; Owston and Ramos- Vara, 2007). CK, DES, INH-a and AMH, all reported as markers of immature SCs (Banco et al., 2010; Grieco et al., 2011; Banco et al., 2012), were not expressed in normal testes (Giudice et al., 2014). In contrast, these markers were expressed diffusely by neoplastic SCs within MGSCTs, consistent with neoplastic SCs in canine Sertoli cell tumours (SCTs) (Banco et al., 2010). In the majority of MGSCTs, SCs expressed at least two of these markers and in 4/ 12 cases, SCs were positive for all of them. The per- centage of positively-labelled neoplastic cells was highly variable, ranging from 31 to 100% of the neoplastic SCs. Specifically, AMH and INH-a were expressed in a higher number of MGSCTs and in a higher percentage of neoplastic SCs than CK and DES (Table 1).

These results paralleled previous ob- servations in canine immature normal SCs and neoplastic SCs in SCTs (Grieco et al., 2011; Banco et al., 2012). Only in one case (case 12), were neoplastic SCs positive for a marker of immaturity (INH-a), with faint expression observed in scattered cells. DES was expressed in 8/13 MGSCTs, consistent with the results of Patnaik and Mostofi (1993). CK was expressed in 6/13 cases, similar to the case reported by Owston and Ramos-Vara (2007), but different to the study of Patnaik and Mostofi (1993), in which all MGSCTs were negative for CK. GCs in normal testes were negative for PAS and PLAP, consistent with previous studies (Grieco et al., 2007; Yu et al., 2009). PAS staining and positive immunolabelling for PLAP are typical of the progenitors of GCs, the gonocytes, and this phenotype is lost during prenatal maturation, therefore pre-spermatogonia are both PAS and PLAP negative (Gaskell et al., 2004). Gonocytes also express KIT and the expression of this protein is maintained during the maturation of GCs until the stage of spermatogonium (Owston and Ramos- Vara, 2007; Grieco et al., 2010). Consistently, in the normal testes included in the present study, spermatogonia were positive for KIT.

Neoplastic GCs have been characterized previously in seminomas. In man and dogs, there are two types of seminoma: classical and spermatocytic (Emerson and Ulbright, 2005; Grieco et al., 2007; Yu et al., 2009; Saegusa et al., 2011). In men, classical seminomas are regarded as the most frequent type, while recent reports describe spermatocytic seminoma as the most common type in dogs (Bush et al., 2011; Thorvaldsen et al., 2012). In both species, these tumours can be differentiated by PAS staining, which is diffusely positive in classical seminomas and negative in spermatocytic tumours (Masson, 1946; Grieco et al., 2007). In addition, classical seminomas are characterized immunohistochemically by the expression of PLAP and KIT (Kraggerud et al., 1999; Grieco et al., 2010), both of which are negative in spermatocytic seminomas.

In the present study, the neoplastic GC component was characterized in 6/13 cases by the immunohisto- chemical expression of both PLAP and KIT and by a PAS staining of most cells. In three cases, GCs were PAS and PLAP negative and two of these cases were exclusively positive for KIT expression. These find- ings suggest that, in these tumours, neoplastic GCs shared a phenotype similar to that of classical semino- mas. In case 10, GCs were labelled diffusely for PLAP, were KIT negative and stained occasionally with PAS. In the remaining 3/13 MGSCTs, the GCs exhibited only few PAS-positive granules in occasional neoplastic cells and were constantly KIT and PLAP negative, suggesting that the GC population of these three cases was more differentiated and similar to the cells in spermatocytic seminomas. These results parallel those of Talerman and Roth (2007) who observed, in human MGSCTs, a heterogeneous neoplastic GC population composed of both imma- ture and mature GCs, frequently coexisting in the same case.

In conclusion, in the present study the expression of immunohistochemical markers of immaturity has been demonstrated in both SCs and GCs in canine MGSCTs. Further studies of the clinical follow up of MGSCTs are required in order to investigate if the expression of such markers may influence the bio- logical behaviour of these tumours.

Table 1
Histological and immunohistochemical features of canine MGSCTs

Case	*Breed*	*Age (years)*	*Sertoli cell markers*						*Germ cell markers*		
			VIM	*NSE*	*CK*	*DES*	*AMH*	*INH-α*	*PLAP*	*C KIT*	*PAS*
1	Poodle	10	+ +	+ + +	+ + +	–	+ + +	+ + + +	+	+ +	+ + +
2	German shepherd dog	10	+ + +	+ +	+	+ +	+ + + +	+ +	+	+ + +	+ + +
3	Boxer	15	+ + +	–	–	–	+	+	–	+ + + +	–
4	Siberian husky	9	+ + +	NA	–	+	+	+ + + +	+ +	+ + +	+ +
5	NR	8	–	+ +	–	+	+ + + +	+	+	+	+
6	Poodle	9	+ + +	+ + + +	+ +	+ +	+ + +	+ + +	–	–	+
7	Mixed breed	NR	+ + +	+ + +	–	–	+ +	+ + + +	+ +	+ +	+ + +
8	Belgian sheepdog	8	+ +	+ + + +	+ + +	+ +	+ + +	+ +	+	+ + + +	+ +
9	Mixed breed	13	+ + +	+ + + +	+ +	+ +	+	+ +	–	–	+
10	Welsh terrier	7	+ + +	+ + + +	–	+	+ + + +	+ + +	+ + +	–	+
11	Mixed breed	9	+	+	+	–	–	+ + + +	–	–	–
12	English setter	12	+ + +	+ + +	–	–	–	+	–	–	+
13	Giant schnauzer	12	+ + + +	+ + +	–	+ +	+ +	+ + +	–	+ +	–

VIM, vimentin; CK, cytokeratin AE1/AE3; DES, desmin; INH, inhibin-a; AMH, anti-Mu∈llerian hormone; NSE, neuron specific enolase; PLAP, placental alkaline phosphatase; PAS, periodic acideSchiff; NA, not assessed; NR, not recorded; _, negative; +, <5% of cells labelled; ++, 6e30% of cells labelled; +++, 31e70% of cells labelled; ++++, 71e100% of cells labelled.

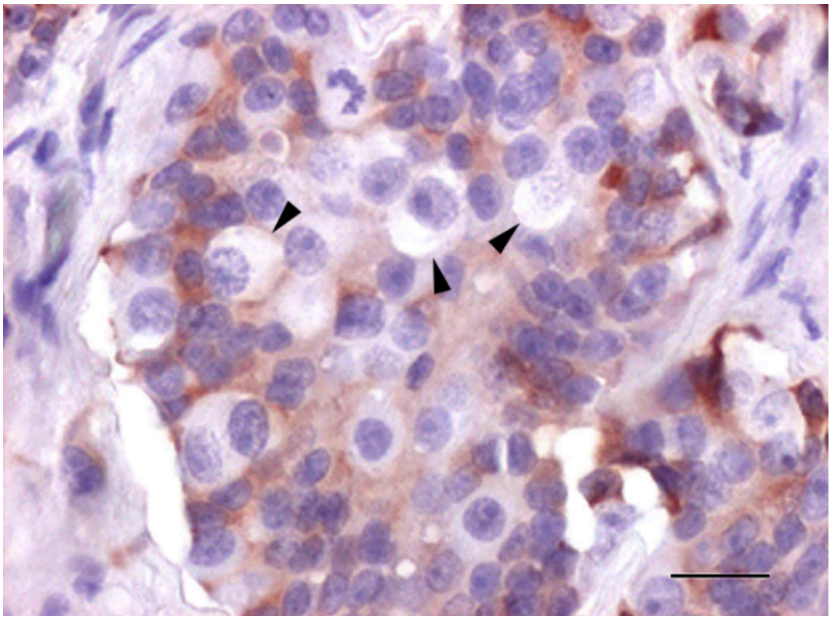

Fig. 1.

Canine MGSCT. Labelling for AMH. SCs lining the pe- riphery of the tubules are positively labelled, while GCs located centrally are negative (arrowheads). IHC. Bar, 20 mm.

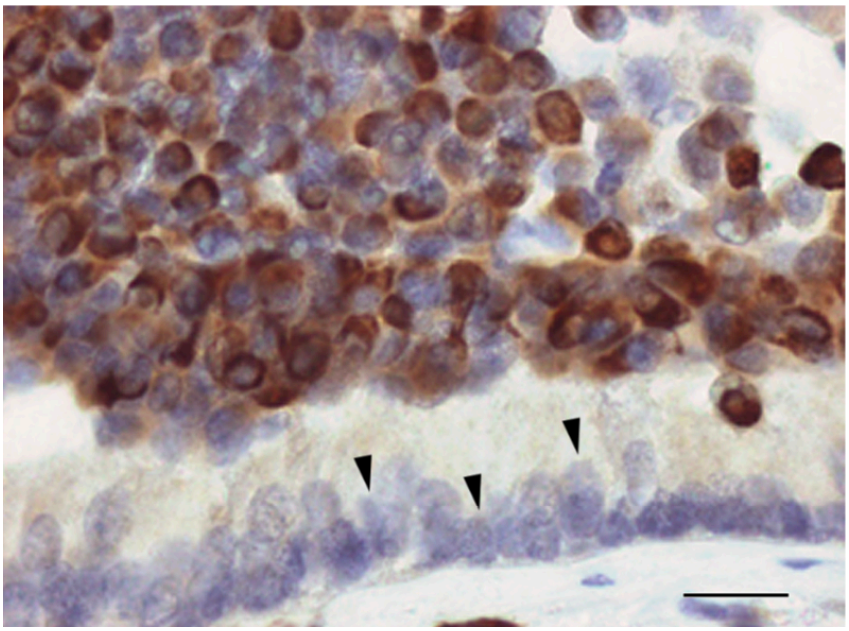

Fig. 2. Canine MGSCT. Labelling for PLAP. Within the tubule, centrally located GCs are labelled for PLAP, while SCs lin- ing the tubule are negative (arrowheads). IHC. Bar, 20 mm.

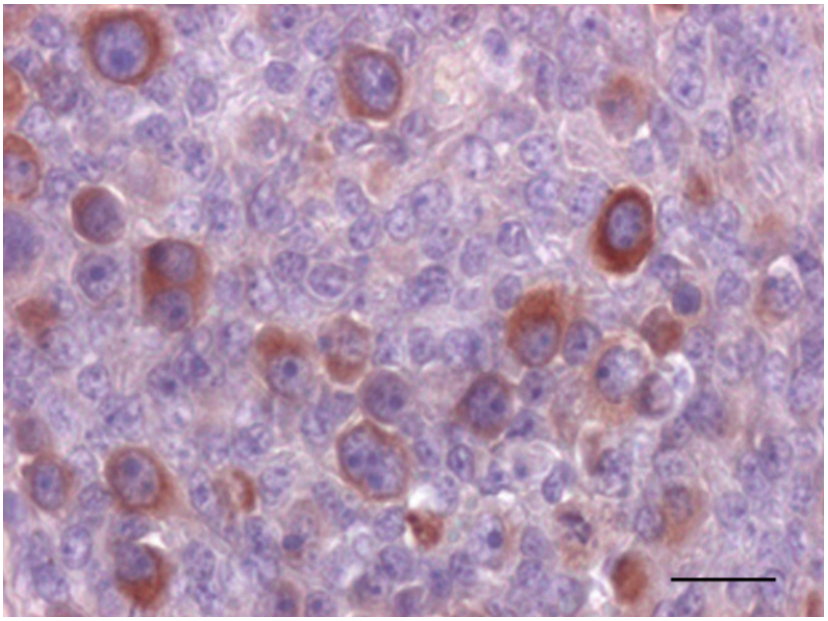

Fig. 3. Canine MGSCT. Labelling for KIT. Scattered neoplastic cells, positive for KIT, are observed within the GC compo- nent of the tumour. IHC. Bar, 20 mm.

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