**Chondroitin sulfate proteoglycan 4: A biomarker and potential immunotherapeutic target for canine malignant melanoma**

# Chondroitin sulfate proteoglycan-4: A biomarker and a potential immunotherapeutic target for canine malignant melanoma*′**

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A B S T R A C T

Chondroitin sulfate proteoglycan-4 (CSPG4), also known as high molecular weight-melanoma associated antigen (HMW-MAA), is a membrane-bound chondroitin sulfate proteoglycan highly expressed by human melanoma cells. This phylogenetically conserved tumour antigen plays an important biological role in human melanoma, where it is used as a marker to diagnose forms with unusual characteristics, such as desmoplastic melanoma, and to detect melanoma cells in lymph nodes and peripheral blood, and as a target for immunotherapy because of its restricted distribution in normal tissues. To identify suitable targets to develop novel approaches of treating canine melanoma, CSPG4 was studies to see whether it is expressed in canine malignant melanomas.

Immunohistochemical staining of 65 canine malignant melanomas with an anti-human CSPG4-specific antibody detected CSPG4 in 37 cases (56.9%). Positive staining was more frequent, albeit not significantly, in amelanotic compared to melanotic tumours and was statistically associated with tumours having both melanin and the epithelioid histotype. The frequency of CSPG4 expression was similar to that of other melanoma antigens used as diagnostic markers for canine malignant melanoma, such as Melan A and the protein recognized by the PNL2 monoclonal antibody. The results suggest that CSPG4 constitutes a new potential immunohistochemical marker of canine malignant melanoma and may represent an immunotherapeutic target as in humans.

## Introduction

Canine malignant melanoma (MM) shares many characteristics with its human counterpart, including histological phenotype, tumour genetics, molecular targets, and clinical biological behaviour (Bostock, 1979; Bergman et al., 2003; Ottensmeier and Gore, 2008). Commonly affected sites are the oral cavity (56%), lip (23%), skin (11%), and digits (8%) (Smith et al., 2002). Clinical biological malignancy is mainly attributed to both oral and digit (subungueal or nail bed) melanomas. However, some differences exist between these two locations. Practically all oral melanomas are considered malignant (metastatic rate up to 80%), whereas the subungueal or nail bed forms spread to regional lymph nodes and other organs, including lungs, in up to 50-58% of cases (Smith et al., 2002; Vail and Withrow, 2007). Histotype (epithelioid, fusiform and mixed) and degree of pigmentation are not prognostic factors. Clinical stage and localization alone influence the outcome (the latter also influencing the possibility of en bloc surgical excision) (Liptak and Withrow, 2007).

As in humans, conventional management of canine oral MM is often disappointing. Surgery, radiotherapy and chemotherapy, alone or in combination, control the tumour locally in up to 75% of animals; however the 1-year survival rate does not exceed 30% because of metastasis (Freeman et al., 2003; Proulx et al., 2003; Boria et al., 2004; Murphy et al., 2005; Liptak and Withrow, 2007). The reported median survival for digital MM in dogs is 12 months (Marino et al., 1995; Vail and Withrow, 2007). These results highlight the need for new therapeutic strategies. Immunotherapy is an attractive option due to the potential role of immunological events in the clinical course of MM, at least in humans (Guerry, 1998), and the availability of well-characterized melanoma antigens and corresponding probes to target MM with immunological effector mechanisms (Ribas et al., 2003; Campoli and Ferrone, 2009; Rosenberg and Dudley, 2009).

Immunization of dogs with human melanoma antigens, such as tyrosinase (Bergman et al., 2003, 2006) and gplOO (Gyorffy et al., 2005; Alexander et al., 2006), which display homology with their canine counterparts, induces a humoral and cellular immune response to the immunizing antigens and has a beneficial effect on clinical course of the disease. Most responders to tyrosinase vaccination develop antibodies cross-reacting with the syngeneic canine tyrosinase (Liao et al., 2006). However, both melanoma antigens cannot always be used as targets since they are expressed in about 50% of canine MMs (Bergman et al., 2003). Furthermore, clinical responses have been observed in only some animals immunized with these antigens.

In view of these results, we determined whether the counterpart of human chondroitin sulfate proteoglycan-4 (CSPG4) is also expressed by canine MM. In humans, this phylogenetically conserved, membrane-bound melanoma antigen is expressed in at least 85% of surgically removed melanomas (Natali et al., 1981; Campoli et al., 2004, 2010). CSPG4, also known as high molecular weight-melanoma associated antigen (HMW-MAA), is implicated in numerous aspects of melanoma cell biology, including adhesion, motility and invasion (Campoli et al., 2010), while its high expression by pericytes in the tumour microenvironment (Schlingemann et al., 1990) suggests an involvement in tumour neovascularisation (Campoli et al., 2010).

CSPG4 is an effective target of T cell- and antibody-based immunotherapy both in humans with melanoma and in animal models (Mittelman et al., 1992; Peng et al., 2006; Maciag et al., 2008; Wang et al., 2010a,b). In the present study, we stained surgically removed samples or biopsies of canine MM using CSPG4 antibody. Furthermore, since CSPG4 plays a role in the biology of human melanoma cells (Campoli et al., 2010), we evaluated potential correlations between immunohistochemical results and histo-pathological characteristics, as well as clinical stages.

#### Materials and methods

Tissue samples from 65 canine oral (n = 55) and subungueal (n = 10) MMs collected between 2000 and 2010 at the Diagnostic Laboratory of the Department of Animal Pathology of the University of Turin were examined. Data regarding breed, sex, age and tumour localization were available for all dogs. Clinical TNM staging (Owen, 1980) was available for 29 dogs.

All samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 µm and stained with haematoxylin and eosin. Sections were examined and classified as MM following the criteria of the World Health Organization (WHO) classification for tumours of domestic animals (Goldschmidt et al., 1998). Histotype (epithelioid, fusiform or combination), presence of melanin (melanotic vs. amelanotic), nuclear and cellular pleomorphism, and mitotic counts were recorded. When a morphological diagnosis was uncertain (18 amelanotic cases), immunohistochemistry (IHC) with Melan A monoclonal antibody (mAb) (mouse monoclonal anti-human Melan-A, Clone A103, diluted 1:50, DakoCytomation) and S100 (Rabbit polyclonal anti-cow S100, diluted 1:300, DakoCytomation) was used to confirm the diagnosis (Koenig et al., 2001).

IIHC for CSPG4 in the 65 samples was performed on 4 µm sections of formalin-fixed, paraffin-embedded tissues. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 30 min at room temperature. Sections were exposed to high-temperature antigen unmasking by incubation at 98 °C with citric acid buffer, pH 6.0. Tissue sections were incubated for 12 h at room temperature with a polyclonal anti-CSPG4 antibody (diluted 1:40, Sigma Aldrich) and revealed with the Vector VIP Substrate kit for peroxidase (Vector Laboratories).

Human skin samples expressing the antigen (kindly provided by Dr. M.G. Bernengo, University of Turin, Italy) were used as a positive control and samples of normal canine lung tissue were used as a negative control.

A modified semi-quantitative scoring system was adopted to evaluate membrane staining on 10 high-power fields (x400) randomly selected in the tumour as suggested by Allred et al. (1998) and Yamashita et al. (2006). A score representing the estimated proportion of positively stained tumour cells was assigned as follows: 0 (none); 1 (<l/100); 2 (1/100-1/10); 3 (1/10-1/3); 4 (1/3-2/3); and 5 (>2/3). An intensity score was also assigned, which represented the estimated average staining intensity of positive tumour cells (0, none; 1, weak; 2, intermediate; 3, strong). The proportion and intensity scores were then added to each other to obtain a total score ranging from 0 to 8. Samples with a total score 55 3 were classed as positive.

Statistical analysis was performed using freeware software R 2.3.0 (The R development Core Team-2006). A Wilcoxon signed rank sum test was used to compare age of dogs in CSPG4 positive vs. CSPG4 negative groups. Cross tabulations were performed using Fisher's exact test and Chi square test for all the others variables. The significance cut-off was $P < 0.05$.

### Results

Forty-seven (72.3%) dogs were males and 18 (27.7%) were females. The mean age was 9.9 years (SD: ±2.3; median 10). Twenty-five (38.5%) dogs were mixed breed and 40 (61.5%) were pure breed (7 Rottweilers, 5 Dachshunds, 4 Schnauzers, 4 Cocker spaniels, 4 German shepherds, 3 Boxers, 2 Yorkshire terriers, 2 Labrador retrievers, 2 Bull Mastiffs, and 1 of the following breeds: Akita Inu, Scottish Terrier, Siberian Husky, Maremman Shepherd, Golden Retriever, Dogue de Bordeaux, and Bloodhound).

MM localization was oral in 55 cases (84.6%) and digital in 10 (15.4%). Histologically, there were 47 (72.3%) melanotic and 18 (27.7%) amelanotic MMs. Twenty-six (40.0%) were classified as epithelioid, 15 (23.1%) as fusiform, and 24 (36.9%) as mixed (i.e. presenting both patterns).

Immunohistochemical results for CSPG4 are summarized in Table 1. There were 37 (56.9%) positive and 28 (43.1%) negative MMs. CSPG4 expression was observed in 31 oral (22 melanotic and 9 amelanotic) and 6 digital tumours (all melanotic). Staining was mostly restricted to the cell membrane. No staining was detected in the surrounding stroma and inflammatory infiltrate, when present. Representative IHC examples are shown in Fig. 1.

No statistical association was found between CSPG4 positive staining and age ($P > 0.05$, Wilcoxon test) and between CSPG4 staining and sex ($P > 0.05$, Fisher's test). The frequency of CSPG4 staining was higher in amelanotic (66.7%, 12/18 cases) than in melanotic (53.2%, 25/47 cases) tumours, but this difference was not significant ($P > 0.05$, Fisher's exact test). Of the 37 CSPG4 positive MMs, 21 (56.7%) were epithelioid, 10 (27.1%) were mixed and 6 (16.2%) were fusiform. The frequency of CSPG4 expression in epithelioid lesions was higher than in the other two histotypes and this was statistically significant ($P = 0.02$, Chi square test). Furthermore pigmented MMs with the epithelioid histotype expressed CSPG4 more frequently ($P = 0.002$, Chi square test).

Of the 29 dogs in which TNM clinical staging was available (26 oral and 3 digital MMs), 4 (13.8%) were in stage I, 2 (6.9%) in stage II, 21 (72.4%) in stage III, and 2 (6.9%) in stage IV. Of these 29 MMs, 16 were CSPG4 positive (Table 2): 25% (1/4) in stage I, 50% (1/2) in stage II, 57% (12/21) in stage III, and 100% (2/2) in stage IV. There was no association between CSPG4 staining and clinical stage ($p>0.05$, Chi square test).

### Discussion

Diagnosis of canine MM can be difficult, in particular with amelanotic tumours, and IHC with antibodies against Melan A, S100 and PNL2 is useful to confirm a diagnosis (Koenig et al., 2001; Giudice et al., 2010). In humans, CSPG4 is more sensitive than HMB-45 and MART-1 for immunohistochemical diagnosis of primary and metastatic MM lesions, including desmoplastic melanoma (Goto et al.,

Table 1
CSPG4 expression in canine MMs.

| Histotypes | Melanotic (positive/total) | | Amelanotic (positive/total) | | All tumours (positive/total) |
|---|---|---|---|---|---|
| | Oral | Digital | Oral | Digital | |
| Epithelioid | 12/15 | 3/3 | 6/8 | 0/0 | 21/26 (80.8%) |
| Fusiform | 2 / 6 | 1/2 | 3/6 | 0/1 | 6/15 (40.0%) |
| Mixed | 5 /17 | 2/4 | 3/3 | 0/0 | 10/24 (41.7%) |
| Proportion of CSPG4-stained tumours | 19/38 (50%) | 6/9 (66.7%) | 12/17 (70.5%) | 0/1 (0) | 37/65 (56.9%) |

2010). It is also used to detect melanoma cells in lymph nodes and peripheral blood of MM patients (Goto et al., 2008; Kitago et al., 2009) and as a target for immunotherapy (Mittelman et al., 1992; Murray et al., 2004).

This is the first study that investigates CSPG4 expression in canine MM. CSPG4 positive staining was present in 56.9% of the samples analyzed. The fact that a major expression was found in amelanotic MMs suggests that this antibody may represent a further supportive diagnostic marker for these cases.

The 56.9% frequency of expression in canine MM is lower than in human MM (Natali et al., 1981; Campoli et al., 2004). This higher frequency may reflect a difference in the biology of this antigen and/or specific characteristics of the canine lesions. The lower frequency in canine MM may also be due to a lower affinity of the antibody used for the immunohistochemical staining. Epitopes of CSPG4 are known to be heterogeneous in their expression (Morgan et al., 1986; Ziai et al., 1987) and CSPG4 expression in canine MM might have been underestimated due to the lack of expression of
the determinant recognized by the antibody used. On the other hand, the frequency of CSPG4 expression in canine MMs is similar to that of Melan A (Koenig et al., 2001) and PNL2 (Giudice et al., 2010), and all three antigens are more frequently expressed in amelanotic canine MMs.

Human CSPG4 expression is different from its canine counterpart since no association has been found with pigmentation of MM (Campoli et al., 2004). In human melanoma cell lines, CSPG4 is preferentially expressed in amelanotic cell lines (Houghton et al., 1987). This association may reflect expression by melanoma cells at an early stage of differentiation and possibly a role for CSPG4 in the malignant phenotype of melanoma cells.

CSPG4 may represent a target for immunotherapy in canine MM. The provisional data of our ongoing pilot study based on vaccination of dogs with CSPG4-positive MM with a plasmid coding for human CSPG4 support the validity of this target (Prestigio et al., 2009). Besides the potential therapeutic relevance, immunotherapy studies in dogs may yield clinical information more relevant for humans compared to experiments in immunodeficient mice grafted with human melanoma cells. Canine and human melanomas have many similarities, including histological appearance, tumour genetics, molecular targets, biological behaviour and response to conventional therapies. Studying dogs with MM is likely to provide a valuable perspective distinct from that generated by studying rodent melanoma models (Paoloni and Khanna, 2008; Khanna et al., 2009).

Pet dogs are large and are relatively outbred compared to laboratory rodents. In addition, inclusion of dogs of different breeds in clinical trials provides a background genetic diversity similar to that seen in humans (Paoloni and Khanna, 2008). Furthermore, naturally occurring MMs in dogs develop in the context of an intact immune system. Therefore it is logical to believe that, both in human and canine cancers, tumour initiation and progression are influenced by similar factors, including age, nutrition, sex, reproductive status and environmental exposures (Paoloni and Khanna, 2008).

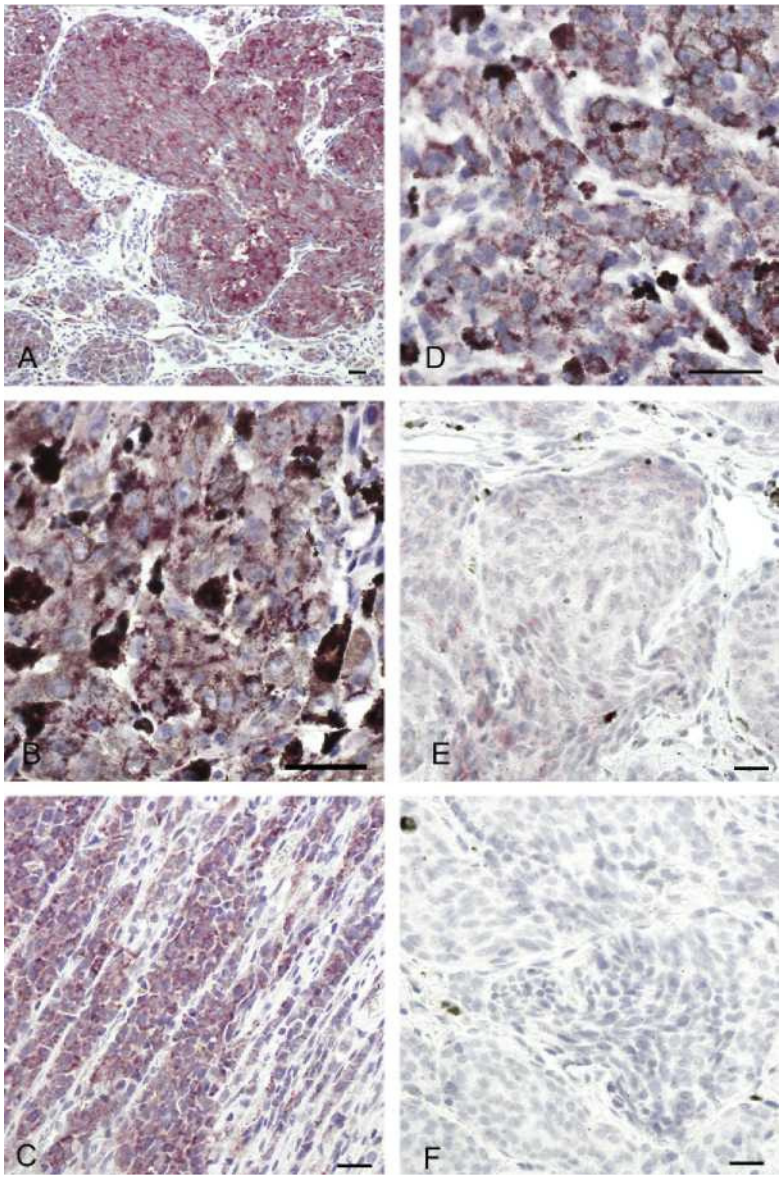


Fig. 1. Immunohistochemical staining of canine MM with an anti-CSPG4 antibody. (A-C) High expression in the majority of tumour cells (score = 8, 7, 6); (D and E) low expression (score = 4,3) in scattered neoplastic cells; (F) no expression (score = 0) in neoplastic tissue. Melanin (stained in brown) is clearly distinguishable (B and D) from the immunohistochemical reaction (purple). Mayer's haematoxylin counterstain; scale bar = 20 μm.

**Table 2**
CSPG4 expression and TNM staging (29/65 cases).

| | I grade (positive/tot) | | II grade (positive/tot) | | III grade (positive/tot) | | IV grade (positive/tot) | | Total |
|---|---|---|---|---|---|---|---|---|---|
| | oral | digital | oral | digital | oral | digital | oral | digital | |
| melanotic | 1/3 | 0/1 | 1/2 | 0 | 5/12 | 2/2 | 1/1 | 0 | 10/21 |
| amelanotic | 0 | 0 | 0 | 0 | 5/7 | 0 | 1/1 | 0 | 6/8 |
| Total (%/n) | 1/4 (25%) | | 1/2 (50%) | | 12/21 (57%) | | 2/2 (100%) | | 16/29 (55%) |

## Conclusions

CSPG4 constitutes a new potential IHC marker to confirm a diagnosis of canine MM and may represent a promising candidate antigen for immunotherapy in dogs. Further clinical studies are warranted.

## Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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