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E-cadherin in canine mast cell tumors: Decreased expression and altered subcellular localization in Grade 3 tumors

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ABSTRACT

Mast cell tumors (MCTs) are the most frequent round cell tumors in dogs and comprise approximately 21% of all canine cutaneous tumors. MCTs are highly invasive and metastatic corresponding to the histological grade. E-cadherin is an adhesion molecule expressed in epithelial cells and although it is an epithelial cellular marker, studies have shown expression of E-cadherin in canine round cell tumors. To better characterize the expression pattern of E-cadherin in several different histological grades of MCTs in dogs, the expression and localization of the adhesion molecule was investigated using immunohistochemistry. For this purpose, 18 cutaneous MCTs were classified into three histological grades, 1, 2 or 3. Clinical history and follow-up data were available for all of the dogs. Cytoplasmic and nuclear expressions of E-cadherin in all three types of tumors were verified by immunostaining using two different antibodies.

There was decreased E-cadherin expression in the more aggressive MCTs (Grade 3), suggesting an association between E-cadherin and tumor aggressiveness. Additionally, the loss of E-cadherin expression in either the cytoplasm or nucleus in more aggressive and undifferentiated tumor types confirmed the importance of cellular adhesion in tumor behavior.

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Introduction

Mast cell tumors (MCTs) are highly invasive and metastatic and are the most frequent round cell tumors in dogs, comprising 16–21% of all cutaneous tumors diagnosed (Brodey, 1970; Bostock, 1986; Rothwell et al., 1987; Misdorp, 2004). It is unclear whether multiple MCTs represent metastatic spread or new neoplasms (Kiupel et al., 2005; Mullins et al., 2006; Murphy et al., 2006; Preziosi et al., 2007). The molecular events and etiology of MCT development and progression are not well elucidated but are thought to be multifactorial (Kiupel et al., 2004; Turin et al., 2006; Scase et al., 2006; Webster et al., 2007; Thamm and Vail, 2007). The clinical and histological characteristics of cutaneous canine MCTs provide useful prognostic information. MCTs were first histologically classified as Grades 1, 2 and 3 by Patnaik et al. (1984), with each grade correlated with survival. Recently, a 2-tier classification of MCTs has been proposed by Kiupel et al. (2011) to more accurately predict biological course, particularly in the case of Grade 3 tumors.

Clinical and molecular studies of MCTs have focused on precisely identifying the molecular alterations for each grade and defining the biochemical and biological impact on cancer cell proliferation and differentiation (Bostock et al., 1989; Simoes et al., 1994; Abadie et al., 1999; Leibman et al., 2000; Lana et al., 2000; Bergmann, 2003; Scase et al., 2006; Wu et al., 2004; Webster et al., 2007; Strefezzi et al., 2009). The expression profiles of a variety of biological markers, such as Ki67, c-Kit, Ag-NOR, chymase and tryptase, have been investigated as potential prognostic markers (e.g. progression, metastasis and life expectancy) (Bostock et al., 1989; Simoes et al., 1994; Abadie et al., 1999; Bergmann, 2003; Scase et al., 2006; Wu et al., 2004; Webster et al., 2007).

Several studies involving human and canine neoplasms have demonstrated that the expression of cadherins is altered during tumor progression (Wijnhoven et al., 2000; Menke et al., 2001; Bankfalvi et al., 2002; Gama et al., 2007; Brunetti et al., 2003; De Matos et al., 2007; Polton et al., 2007), and that more aggressive phenotypes express less E-cadherin (Torres et al., 2005; Brunetti et al., 2003; De Matos et al., 2007; Gama et al., 2007). More recent studies have shown that a decrease in E-cadherin expression or its translocation from the plasma membrane to the cytoplasm and/or nucleus can be crucial events in adherent junction destabilization (Gloushankova, 2008; Salahshor et al., 2008; Elston et al., 2009; Knirsh et al., 2009). In addition, it has been shown that E-cadherin is expressed in not only canine cutaneous histiocytomas, but also multiple other canine round cell tumors, including MCTs, plasma cell tumors, histiocytic sarcomas and epitheliotropic lymphomas (Ramos-Vara and Miller, 2011).

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E-cadherin is a transmembrane glycoprotein with an extracellular domain that interacts with other E-cadherin molecules on adjacent cells. It has an intracellular domain associated with a protein complex that is comprised of α-catenin, β-catenin, γ-catenin and p120-catenin. Therefore, events that disrupt the complex cadherin–catenin–cytoskeleton lead to a destabilization of cell–cell adhesion and ultimately a reorganization of the actin cytoskeleton (Mareel and Leroy, 2003). It has been hypothesized that alterations in E-cadherin expression might be a crucial event in malignant tumor progression and growth (Wijnhoven et al., 2000; Menke et al., 2001; Bankfalvi et al., 2002; Nowak et al., 2008).

As E-cadherin is an important adhesion molecule, we hypothesized that more aggressive MCTs would express decreased E-cadherin. In this study, we sought to investigate the association between the expression and localization of E-cadherin and histological grade in cutaneous MCTs in dogs.

Materials and methods

Specimens

Eighteen cutaneous canine MCTs that had been submitted for diagnosis at the Department of Pathology of the School of Veterinary Medicine and Animal Science of the University of São Paulo (USP) between 2004 and 2010 were used. Each tumor was obtained from a different dog and surgical excision was the only treatment modality used in each case. The cases were diagnosed into three histological grades (1, 2 or 3; $n = 6$ cases/grade). Clinical history and follow-up data were available for all dogs.

Tissues were fixed in 10% buffered formalin for 24–30 h and were dehydrated in alcohol and embedded in paraffin. The initial diagnosis of cutaneous MCT was confirmed independently by two pathologists and classified according to the established histological grading system (Patnaik et al., 1984). Medical records were examined for each case and data recorded included dog signalment, number and location of tumors, presence or absence of lymph node metastasis, evolution time, recurrence interval, survival time, and cause of death. For the purposes of the study, recurrence was defined as clinical reappearance of a mass at the initial tumor site. Metastasis was defined as the development of an additional mass at a site distant from where the original mass was resected.

Table 1
Summary of clinical findings.

Identification number	Tumor grade	Dog breed	Age (years)	Gender	Number of tumors	Tumor site	Lymph nodes with metastasis	Tumor evolution time (months)	Recurrence interval (months)	Death	Time survival (months)
1	1	Labrador Retriever	8	M	2	LA, S	No	1	No	No	26-Alive
2	1	Boxer	10	M	4	LA, C, L, EC	No	8	No	22-NA	22
3	1	Mixed-breed	11	M	1	LA	No	6	7	8-NA	8
4	1	Golden Retriever	8	M	2	H/N, L	No	3	No	No	12-Alive
5	1	Boxer	9	F	2	H/N, L	No	6	2	2-A	2
6	1	Golden Retriever	8	M	1	PT	No	20	No	No	20-Alive
7	2	Dachshund	11	F	1	L	Popliteal	24	10	12-A	12
8	2	Boxer	10	F	9	LA, C, L, H/N	Submandibular	24	No	12-NA	12
9	2	Golden Retriever	6	M	1	C	No	6	No	No	14-Alive
10	2	Boxer	9	F	1	C	No	2	5	9-A	9
11	2	Boxer	12	F	5	D, LT, PT	No	6	No	No	22-Alive
12	2	Boxer	7	M	1	LA	No	6	No	No	20-Alive
13	3	Doberman	7	F	2	EC	Prescapular	4	1	1-A	1
14	3	Doberman	11	F	1	C	No	30	2	7-A	7
15	3	Mixed-breed	13	M	3	L, C	Inguinal	24	1	6-A	6
16	3	Dachshund	13	M	2	LA, S	No	1	2	4-A	4
17	3	Boxer	10	M	1	C	No	6	5	5-A	5
18	3	Shar-Pei	11	M	1	C	Inguinal, sublumbar	2	2	2-A	2

S, scrotum; LA, lateral abdomen; LT, lateral thigh; PT, posterior thigh; H/N, head/neck; D, digits; L, limbs; C, chest; EC, ear canal; MB, mixed-breed dog; NA, death not associated with MCT; A, death associated with MCT.
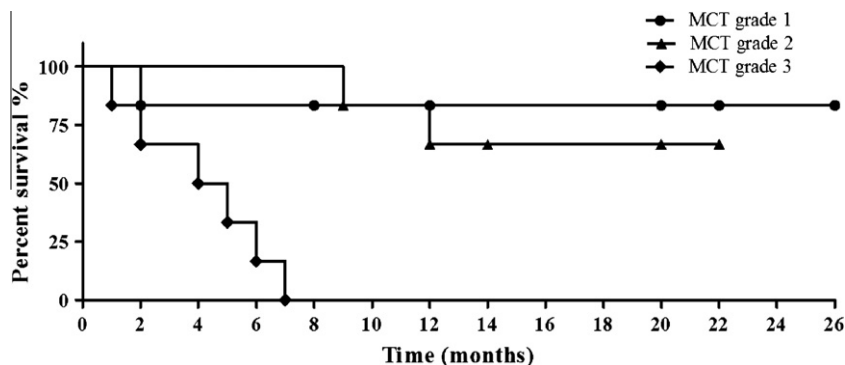

Fig. 1. Comparative analysis of survival by MCT grade. There was no significant difference between the survival time of animals with Grades 1 and 2 MCT, but survival times of animals with Grade 3 MCT were shorter than those with Grades 1 and 2 MCT ($P = 0.0008$ and $P = 0.0018$, respectively).

Histological analysis of canine cutaneous mast cells

Two 5 μm tissue sections were stained with hematoxylin/eosin and toluidine blue for histological examination and grading according to Patnaik et al. (1984). Staining was performed according to an established protocol (Behmer et al., 1976a,b).

Antibodies and immunohistochemistry

All MCTs were fixed in 10% buffered formalin and paraffinized following the standard protocol. Two mouse monoclonal antibodies were used for E-cadherin detection – clone NCH38 (Dako) and clone HECD-1 (Invitrogen). Each of these antibodies recognizes the extracellular domain of human E-cadherin (Nowak et al., 2007; Huiping et al., 1999).

Immunohistochemical analysis was performed on 5 μm thick sections with monoclonal antibodies against human E-cadherin (NCH-38 and HECD-1, both diluted at 1:100) by the peroxidase-conjugated streptavidin complex method (LSAB, Dako), following the manufacturer's instructions. Antigen retrieval for E-cadherin was carried out by microwave treatment (3 × 4 min) in 10 mM citrate buffer at pH 6.0. After an antigen retrieval step, sections were treated with 0.3% H_2O_2 in methanol for 30 min to block endogenous peroxidase activity. Slides were then rinsed in phosphate buffered saline (PBS)/Tween-20 and blocked for 30 min with 5% skimmed milk diluted in PBS/Tween-20. Sections were incubated overnight at 4 °C with the primary antibody (dilution 1:100). The slides were then rinsed with PBS/Tween-20 and incubated for 45 min with LSAB. Adjacent normal epithelial tissues were used as internal positive controls. The primary antibody was replaced with PBS for negative controls. The reaction was developed with the chromogen diaminobenzidine tetrahydrochloride (DAB kit, Dako) and the sections were coun-
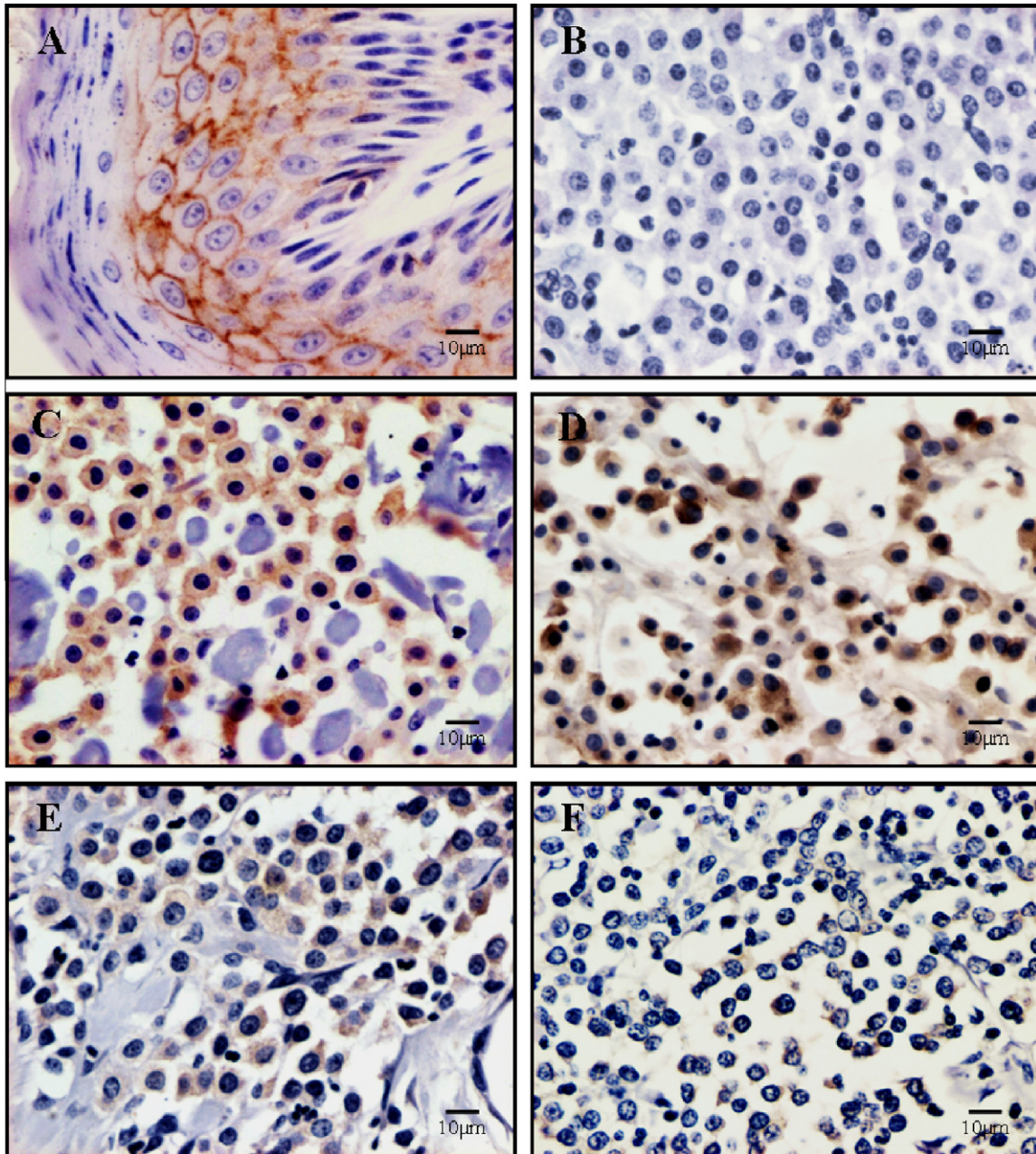

Fig. 2. (A) Photomicrograph immunostaining of E-cadherin (NCH-38) in all the MCT grades. Canine epithelial cells were highly positive for E-cadherin, and the immunostaining was strictly localized to the cell membrane. (B) The control negative reaction is also shown. (C) Grade 1 MCTs were positive for E-cadherin in mast cell membranes at the edges of the tumor. (D) However, the inner portion of well-differentiated MCTs (Grade 1) was positive for E-cadherin immunostaining in the cytoplasm of mast cells. (E) We observed almost the same number of cells with positive cytoplasmic immunostaining in Grade 2 MCTs and Grade 1 MCTs. A decrease in the intensity of immunostaining was observed in Grade 2 tumors compared with Grade 1 MCTs. (F) Grade 3 MCTs showed a significant reduction in the number of cells with positive cytoplasmic immunostaining for E-cadherin compared with well differentiated tumors (Grade 1 MCTs). With the use of anti-E-cadherin clone NCH-38, we observed that the expression of E-cadherin was predominantly cytoplasmic in MCTs of all three grades, and the intensity of the expression decreased as MCT advanced to a higher grade.

terstained with Mayer's hematoxylin. The sections were observed and photo-graphed (400× magnification) in 10 random fields of high cell density and a total of 1300 cells were counted on each slide. The staining was scored as the presence or absence of positive cells. The subcellular positivity of the immunostaining in the membrane, cytoplasm and/or nuclei was noted.

Statistical analysis

For histological analysis, the data were expressed as the mean ± standard deviation (SD). A non-parametric analysis among groups was performed using the Kruskal–Wallis test (Prism version 5, Graphpad Software). Survival curves were generated by the Kaplan–Meyer method and survival rates were compared using the log-rank test. All statistical analyses were performed using SPSS 11.5 statistical software. Values of $P < 0.05$ were considered statistically significant.

Results

Clinical history

There was no significant effect of gender or age on tumor grade. A total of seven dog breeds were included. Twelve of 18 dogs in this study had died at the time data analysis was performed (nine due to MCT and three of causes unrelated to MCT). All animals diagnosed with Grade 3 MCT died due to the disease. At the end of the study, three animals diagnosed with Grade 1 MCT and three dogs diagnosed with Grade 2 MCT were still alive. The data are summarized in Table 1. The survival time ranged from 2–26 months for the animals with MCT 1; from 9–22 months for the animals with Grade 2 MCT, and from 1–7 months for animals with Grade 3 MCT. Statistical analysis of survival time is presented in Fig. 1. There was a recurrence of disease in 33.3% (2/6) of Grade 1 MCT cases and in 33.3% (2/6) of Grade 2 MCT cases. All (6/6) Grade 3 MCT recurred.

In total, 40 tumors at different sites were observed, approximately half of which were single neoplasms and the other half were multiple tumors with 2–9 nodules. In dogs with multiple MCTs (i.e. neither metastases nor local tumor recurrence), survival data were analyzed considering each tumor as a separate event and by analyzing the data with only the first tumor that had been diagnosed. The most common anatomical location was the chest 27.5% (11/40), followed by the pelvic limbs 17.5% (7/40), lateral abdomen 17.5% (7/40), head/neck 10% (4/40), ear canal 7.5% (3/40), posterior thigh 7.5% (3/40), lateral thigh 5% (2/40), scrotum 5% (2/40) and digits 2.5% (1/40; Table 1).

Expression and localization of E-cadherin

Anti-human E-cadherin clone NCH38 antibody detected the expression of E-cadherin in the membrane and cytoplasm of the canine MCT cells (Fig. 2). The comparative analysis of E-cadherin immunostaining by clone NCH38 revealed significantly more positive cells with cytoplasmic labeling in Grade 1 MCT than in 3 MCT ($P = 0.021$; Fig. 3). There were also more positive cells in Grade 2 than in Grade 3 MCT ($P = 0.021$; Fig. 3).

Immunohistochemistry using anti-human E-cadherin clone HECD-1 antibody detected the expression of E-cadherin in the cell membrane, cytoplasm and nucleus of canine MCT (Fig. 4). The membrane immunostaining was observed only in Grade 1 MCTs, while cytoplasmic and nuclear immunostaining was observed in all three grades of MCT. There were fewer positive-staining cells in the highest grade MCT. Comparative analysis of immunostaining for E-cadherin clone HECD-1 revealed significantly more cytoplasmic labeling in Grade 1 MCT compared with Grade 3 MCT ($P = 0.028$). There were significantly more positive cells for Grades 1 than for Grade 3 MCT ($P < 0.001$). However, when we compared immunostaining within tumors of the same grade, there was no difference between the cytoplasmic and nuclear markings in Grade 1 MCT ($P = 0.07$), Grade 2 MCT ($P = 0.12$), or Grade 3 MCT ($P = 0.43$).
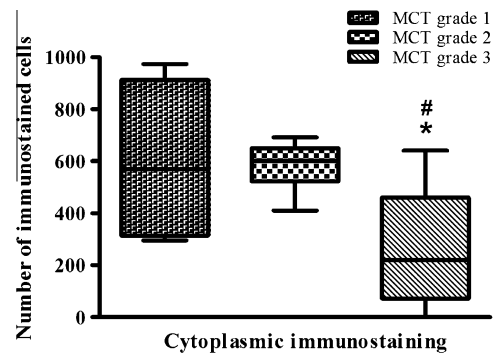

Fig. 3. Comparative analysis of cytoplasmic immunostaining of E-cadherin (NCH-38) in all MCT grades. Error bars represent standard deviations. [#] Indicates $P = 0.021$ vs. MCT Grade 1. [*] Indicates $P = 0.021$ vs. MCT Grade 2.

The results showed that E-cadherin is differentially expressed in canine cutaneous MCTs (Fig. 5).

Discussion

Despite the fact that cutaneous MCTs are one of the most frequent and thus one of the most commonly studied neoplasms in dogs, there are few studies characterizing the expression profiling of cancer-related molecules in all the three grades MCT. There is evidence that normal mast cells are attracted to the tumor site, which could promote tumor growth through the release of inflammatory cytokines (Conti et al., 2007). Once mast cells are attracted to tumors by chemo-attractants, such as stem cell factor (SCF), they are triggered to secrete molecules that act as growth factors, stimulating tumor growth, angiogenesis, and metastasis. Mast cells thus promote the tumor microenvironment remodeling and tumor growth, increasing the secretion of inflammatory chemicals and the activity of NF-kappaB, which augments the suppression of T-cells and natural killer cells in tumors (Huang et al., 2008).

In this study, the most commonly affected breed was the Boxer, which is consistent with studies in the literature reporting a hereditary predisposition for animals related to the Bulldog, such as Boxers (O'Keefe, 1990; Simoes et al., 1994; Lemarié et al., 1995; Fox, 1998; London and Seguin, 2003; Misdorp, 2004; Costa et al., 2007; Strefezzi et al., 2009; Costa-Casa Grande et al., 2008). Golden Retrievers are also reported to be over-represented (Bostock and Dye, 1973). It has been observed that animals >9 years are more likely to be affected with tumor recurrence and death, and these results agree with the findings reported by Cahalane et al. (2004). Our results regarding anatomical site of MCTs also confirm previously published data showing that the most common sites are the chest and perineum, followed by the distal parts of the limbs and the head and neck (O'Keefe, 1990; Fox, 1998).

All of the animals diagnosed with Grade 3 MCTs died as a consequence of tumor development. Tumor grade was not associated with duration of tumor growth and survival time after tumor recurrence ranged from 2–5 months regardless of the grade tumor. The survival time of dogs with multiple MCTs was not significantly different to dogs with solitary tumors. Multiple tumors were more prevalent in this study than previously reported in the literature (3–14% from Macy, 1985; O'Keefe, 1990; Simoes et al., 1994).

In our study, Grade 1 MCTs were not different in terms of tumor development, progression, recurrence and death rate compared to Grade 2 MCT. However, when Grades 1 and 2 MCT were compared to Grade 3 MCT, there was a significant difference between the groups, and Grade 3 disease carried a poor prognosis. These results confirm the histological findings observed in a study performed in 95 canine cutaneous MCTs reported by Kiupel et al. (2011), which
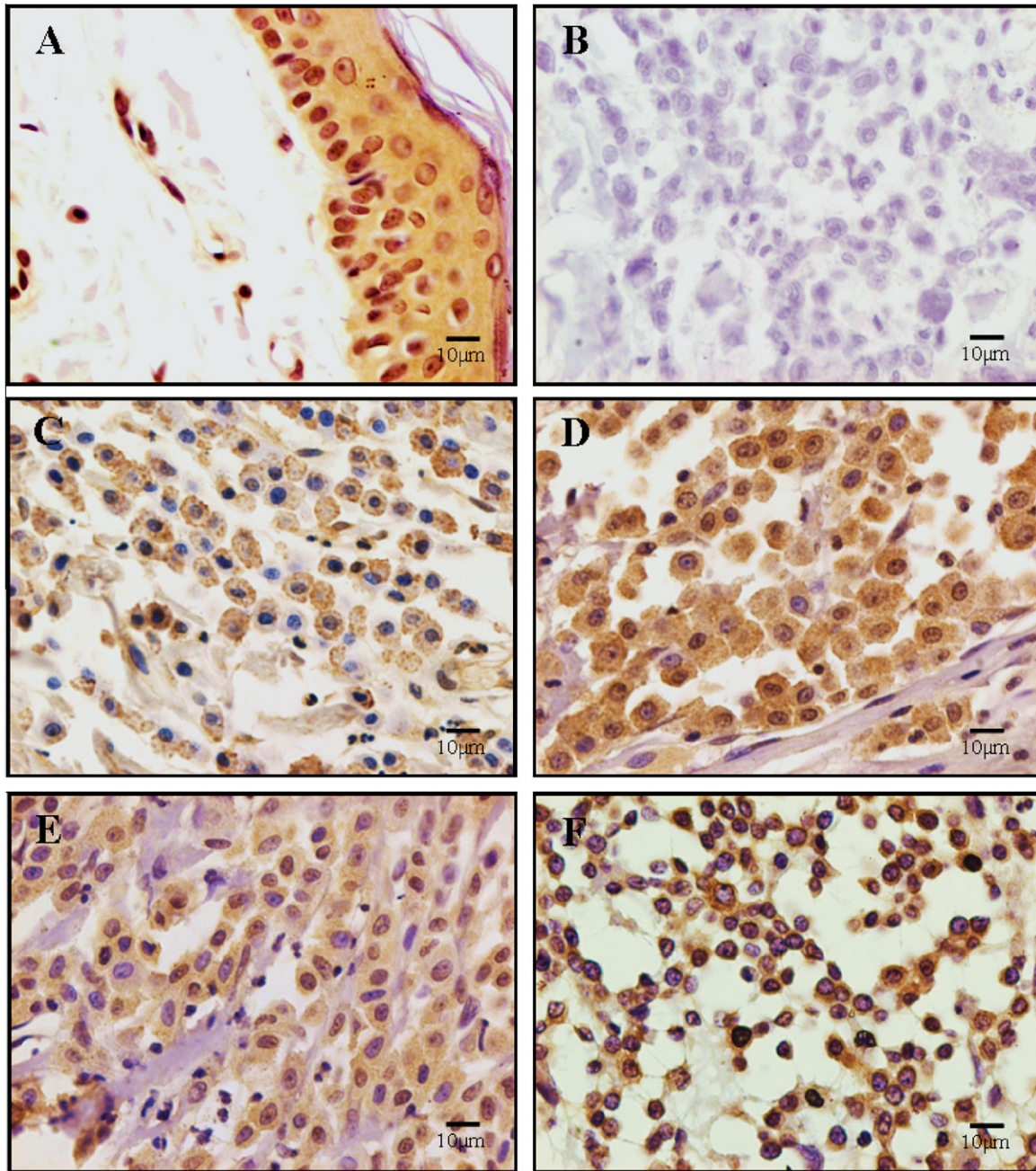
Fig. 4. (A) Photomicrograph of E-cadherin immunostaining (HECD-1) in all MCT grades. There was a positive cytoplasmic and nuclear immunostaining of epithelial cells and positivity was not observed in the membrane. (B) The negative control reaction. (C) Positive immunostaining for E-cadherin was observed in the membrane and nucleus of some well-differentiated mast cells on the edges of Grade 1 tumor. (D) In the inner portion of the same Grade 1 tumor, positive immunostaining was observed in the cytoplasm and nucleus in most cells. (E) We observed almost the same number of cells with positive immunostaining for E-cadherin in Grade 2 MCTs and Grade 1 MCT. However, a decrease in the intensity of immunostaining was observed in Grade 2 tumors. (F) Grade 3 MCTs showed a significant reduction in the number of cells with positive cytoplasmic and nuclear immunostaining for E-cadherin compared with well differentiated tumors (MCTs Grade 1).

suggested that only two grades should be used to enhance agreement between pathologists and provide a more meaningful prognosis.

E-cadherin is a cell adhesion protein complex widely described in the literature as a tumor suppressing agent, and loss of E-cadherin function contributes to lack of cellular differentiation and growth of the primary lesion by several potential mechanisms (Shiozaki et al., 1996; Brunetti et al., 2003; De Matos et al., 2007; Gama et al., 2007). Tegoshi et al. (2000) demonstrated that E-cadherin was present in the cell membrane of normal mast cells. In addition, an in vitro experimental study using anti-E-cadherin

antibodies indicated that it is functional and crucial for colony formation in those cells. The expression of E-cadherin was also detected in the human mast cell line HMC-1. It is possible that E-cadherin is involved in interactions between mast cells and epithelial cells, while other functions of its expression in mast cells remain to be elucidated (Nishida et al., 2003).

E-cadherin is found in the plasmatic membrane of normal or well-differentiated canine mast cells. Our study demonstrated a decrease of E-cadherin expression and subcellular localization of cellular protein in more aggressive grades of canine MCTs, accompanied by reduced staining in the plasma membrane and de-
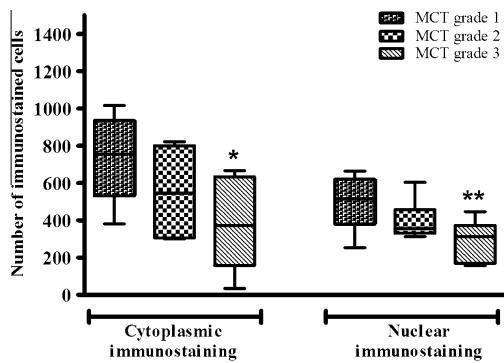
Fig. 5. Comparative analysis of cytoplasmic immunostaining vs. nuclear immuno-staining of E-cadherin (HECD-1) in all grades MCT. Error bars represent standard deviations. * Indicates $P = 0.028$ vs. Grade 1 MCT. ** Indicates $P < 0.001$ vs. Grade 1 MCT.

creased expression in the cytoplasm and/or nucleus as the tumor took on a more aggressive phenotype. These results were found for both of the antibodies used in this study (NCH-38 and HECD-1 clones). The results indicated a change in the subcellular localization of E-cadherin and decreased expression in higher grade MCTs, corroborating the results reported by Salahshor et al. (2008) and Chetty et al. (2008), which showed a frequent accumulation of E-cadherin in the nucleus in the more aggressive stages of esophageal squamous carcinoma and pancreatic tumors. The mechanisms that lead to the loss of E-cadherin expression in the cell membrane are still unclear. The disruption of cell-to-cell adhesion might be mediated by several factors, such as digestion by proteases (Lochter et al., 1997; Steinhusen et al., 2001), transcriptional repression (Batlle et al., 2000; Cano et al., 2000; Yap et al., 2007), endocytosis (Fujita et al., 2002), mutations in the E-cadherin gene (Oda et al., 1994; Saito et al., 2001) and methylation of E-cadherin promoter (Yoshiura et al., 1995).

The goal of any molecular research in cancer is to develop better tools for disease control. Further studies in primary cell cultures are first required to better understand the molecular mechanisms involved in decreased expression of E-cadherin in MCTs, and to investigate possible means to reverse this.

Conclusions

This study demonstrated a decrease in the expression of E-cadherin in the most aggressive (Grade 3) canine cutaneous MCTs. In addition, there was a change in subcellular localization of the adhesion molecule in Grades 2 and 3 MCTs. The results suggest that E-cadherin plays a crucial role in the behavior of MCT cells.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Acknowledgements

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