USEFUL IMMUNOHISTOCHEMICAL INDICATORS IN CANINE MAST CELL TUMOURS

Malgorzata KANDEFER-GOLA*, Marcin NOWAK, Janusz A. MADEJ, Stanisław DZIMIRA, Rafal CIAPUTA and Izabela JANUS

1Department of Pathology, Faculty of Veterinary Medicine, Wrocław University of Environmental and Life Sciences, ul. C. K. Norwida 31, 50-375 Wrocław, Poland

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Morphological and immunohistochemical analysis of 45 canine mast cell tumours was performed to determine whether the proteins examined are useful for a more precise description of tumour morphology and a more reliable determination of the prognosis in patients. Tissue sections were stained according to the standard haematoxylin and eosin (HE) technique and with toluidine blue to demonstrate cytoplasmic granules. Immunohistochemical studies were performed, using the cell markers CD117 (c-kit), p16 and von Willebrand factor (FVIII). In CD117 three different staining patterns were observed: (1) membranous reaction, (2) intense staining of cytoplasm, and (3) a diffuse, delicate cytoplasmic reaction. Von Willebrand antibody was evaluated on the basis of the number of blood vessels stained. p16 expression was evaluated by scoring positive nuclear reaction. Positive expression was demonstrated for all examined antigens, but their level of expression differed depending on the grades of tumour malignancy. Statistical analysis of the results documented a pronounced positive correlation between the markers studied and the grade of tumour malignancy (P < 0.001). It was shown that each of the cell markers examined represents a useful prognostic indicator for patients with mast cell tumours. The calculated correlation coefficients demonstrate a strong association between the expressions of CD117, FVIII and p16, and the histological malignancy of a tumour.

Key words: Mast cell tumour, dog, p16, CD117, FVIII

Mast cell tumours or mastocytomas (MCT) belong to the most frequently diagnosed skin tumours in dogs (Ciaputa et al., 2013). They develop at any age and are independent of the dog’s gender. According to Macy (1985), the predisposed breeds include Boxers, Bull Terriers, Labrador Retrievers, Pugs and Boston Terriers. Clinically, mastocytomas exhibit a variable biological behaviour: either individual, benign lesions occasionally infiltrating deeper tissues or multiple, malignant tumours spread throughout the body are noted (Patnaik et al., 1984; Zemke et al., 2001; Hargis and Ginn, 2012). Most frequently, the tumours

*Corresponding author; E-mail: malgorzata.kandefer-gola@up.wroc.pl;
Phone: 0048 (71) 320-5256; Fax: 0048 (71) 320-5414
are accompanied by dermal erythema, pruritus and oedema (the so-called Darier’s sign), imitating inflammatory reaction, which frequently leads to misdiagnosis and erroneously implemented treatment. The phenomenon is linked to mast cell degranulation and release of inflammatory mediators to surrounding tissues (Hargis and Ginn, 2012).

Microscopic evaluation most frequently employs the histological grade of tumour malignancy described by Patnaik et al. in 1984 (Zemke et al., 2001). It is based on the evaluation of tumour invasiveness, the morphology of mast cells and the mitotic index (Table 1). Mast cells manifest a tendency to form larger clumps, and with an increasing grade of histological malignancy they begin to infiltrate deeper tissues. The sublayer of the tumour involves a fibrous or fibrovascular tissue with a tendency for hyalinisation and degeneration of collagen fibres, frequently with infiltration by eosinophils.

**Table 1**

Grades of histological malignancy in mast cell tumours according to Patnaik et al. (1984)

<table>
<thead>
<tr>
<th>Grade of malignancy</th>
<th>Cell morphology</th>
<th>Mitotic index</th>
<th>Surrounding tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Shape</td>
<td>Cytoplasm</td>
<td>Cell nucleus</td>
</tr>
<tr>
<td>I</td>
<td>round</td>
<td>high amount of cytoplasm with numerous granules</td>
<td>round with condensed chromatin</td>
</tr>
<tr>
<td>II</td>
<td>round to oval, occasionally giant or spindle-shaped</td>
<td>moderate amount of cytoplasm with delicate granules, occasionally scanty cytoplasm with large, hyperchromatic granules</td>
<td>round, with loose karyoplasm and single nucleolus, less frequently double nuclei</td>
</tr>
<tr>
<td>III</td>
<td>moderate size, round, oval or spindle-shaped, many giant cells</td>
<td>poorly distinguished cytoplasm containing poorly visible or invisible granules</td>
<td>round, vesicular cell nucleus with a single or multiple nucleoli, frequent bi- or multinuclear cells</td>
</tr>
</tbody>
</table>

The aim of this study was to determine the expression of cell markers, including c-kit (CD117), von Willebrand factor (FVIII) and p16, in mast cell tumours of the canine skin. The results have allowed us to determine the morphol-
logy of tumours more precisely and to evaluate if the proteins examined are helpful in predicting the prognosis. Especially, the findings disclosed the density and size of blood vessels, which create a network sufficient for the nutrition and oxygenation of the tumour. Furthermore, angiogenesis indicates the growth ability, invasiveness and metastatic potential of tumours. The CD117 staining pattern and the number of labelled nuclei in p16 staining are highly correlated with MCT malignancy. Higher MCT grade and blood vessel density indicate increased malignancy and poorer prognosis. A further objective was to study whether the histological malignancy of tumours observed in haematoxylin and eosin (HE) stained specimens was correlated with the expression of p16 and CD117 proteins and the number of blood vessels in the tumours.

Materials and methods

The research was conducted on 45 MCT collected in 2011 at the Department of Pathology, Wrocław University of Environmental and Life Sciences (Wrocław, Poland). The tumours had been excised from dogs of different breeds, sex and age. The studies showed that MCT morbidity reached its peak between 7 and 9 years of age (27 cases), although neoplastic lesions could be detected in animals of various ages (ranging from 2.5 to 14 years), in both sexes (20 female and 25 male dogs were examined). In this study, the highest number of cases were found in American Staffordshire Terriers (n = 8), followed by Labrador Retrievers and mongrel dogs (n = 6 of each), Boxers (n = 5), Dachshunds, Golden Retrievers and Bernese Mountain dogs (n = 3 cases in each breed), while the remaining breeds were represented by single cases.

Tumour fragments were fixed in 7% buffered formalin for 24 h, then embedded in paraffin blocks. Three to four µm thick paraffin sections were stained with haematoxylin and eosin (HE) and with toluidine blue.

Immunohistochemical studies were performed in 4-µm-thick paraffin sections placed on silanised slides. Subsequently the sections were cleared in xylene and passed through a series of alcohols of a decreasing concentration to water. Antigen retrieval was achieved by cooking the slides in citrate buffer (pH 6.0) at 97 °C for 20 min. Endogenous peroxidase was blocked in 3% hydrogen peroxide solution. Subsequently the sections were overlaid with primary antibodies specific for CD117 (Polyclonal Rabbit Anti-Human CD117, e-kit, catalogue no. A4502, diluted 1:100, Dako®), for von Willebrand factor (FVIII) (Polyclonal Rabbit Anti-Human von Willebrand Factor, catalogue No. A0082, diluted 1:200, Dako®) and for p16 (Monoclonal Rabbit Anti-Human Anti-p16/INK4a Antibody, clone EP435Y, diluted 1:200, Merck Millipore). All the specimens were incubated at room temperature for 20 min, then washed in EnVision™ FLEX Wash Buffer (20×). After rinsing them, the sections were overlaid with EnVision™
FLEX/HR visualisation system and incubated at room temperature for 20 min. The immunohistochemical reactions were developed using a solution of 3,3-diaminobenzidine tetrahydrochloride (DAB), EnVision™ FLEX DAB + Chromogen (Dako®). Finally, the sections were rinsed in distilled water, the cell nuclei were counterstained with haematoxylin and the specimens were dehydrated in a series of alcohols. For every marker studied a positive and a negative control was included. The positive control involved samples of healthy skin and employing all the reagents, while the negative control was prepared with PBS instead of antibodies.

In the detection of CD117 three patterns of colour reaction were distinguished: the first pattern involved a pronounced membranous reaction and in some cells a slight reaction in the cytoplasm, the second pattern was characterised by intense but focal cytoplasmic staining, while the third pattern represented a diffuse, delicate staining of the entire cytoplasm. Each slide was evaluated on the basis of the highest pattern observed in 100 neoplastic cells at the magnification of ×400 (Kiupel et al., 2004). During the statistical analysis the 1st, 2nd and 3rd pattern staining were used as numerical score.

By immunostaining with von Willebrand factor the stained endothelial cells of blood vessels were examined. Initially, the areas most intensely supplied with blood vessels (so-called ‘hot spots’) were identified. For each slide, 5 hot spot areas at the magnification of ×200 were chosen. The mean value of the vessel count in those five fields was retained as the final value. The statistical analysis was performed on the number of blood vessels.

Expression of p16 was evaluated using a semiquantitative scale, scoring the positive nuclear reaction (0–5%: no reaction [−], 6–25%: weak reaction [+], 26–50%: moderate reaction [++] , > 50%: intense reaction [+++]). In each slide 5 randomly selected areas were chosen in which labelled cell nuclei were counted. During the examination, nuclear and infrequently cytoplasmic reactions were observed (×400).

Microphotographs of all examined neoplastic lesions were taken using an Olympus BX53 optical microscope (Olympus, Japan), coupled to a ColorView IIIu digital camera (Olympus, Japan).

The results were subjected to statistical analysis using Statistica PL software (StatSoft Polska) including the determination of Spearman’s correlation coefficient (rho).

**Results**

On the basis of HE and toluidine blue staining, the tumours were grouped in line with the scale of histological malignancy according to Patnaik et al. (1984): 11 tumours represented an MCT of grade I, 21 of grade II and 13 of grade III (Fig. 1).
In grade I tumours CD117 demonstrated first-pattern staining in 100% of the lesions. On the average, two blood vessels were noted per visual field, including 18.1% slides with a single blood vessel, 45.5% slides with two blood vessels and 36.4% slides with three blood vessels. Staining for the proliferation marker p16 demonstrated weak [+] reaction in 72.7% of the cases, and a moderate reaction [++] in 27.3%. In the group studied, no pronounced reaction for p16 protein [+++] was noted. Mostly a delicate cytoplasmic reaction was observed, with only a few nuclei being stained.

In case of grade II tumours 47.6% of the lesions manifested the second pattern of staining for CD117, while 38.1% exhibited the third and 14.3% the first pattern of staining. On the average, four blood vessels were detected per visual field, including 57.1% slides with 4, 28.6% with 3 and 14.3% with 5 blood vessels. In studies for the detection of p16, 66.7% cases manifested a moderate reaction [++], 23.8% cases represented a weak reaction [+] and only in 9.5%
cases was there a pronounced reaction [+++]. Usually about 35% of the nuclei were labelled brown, and staining of cytoplasmic location was infrequently observed.

In all (100%) grade III cases the third-pattern staining of CD117 was noted. On the average, five blood vessels were detected per visual field, including 61.5% cases with 5, 23.1% cases with 6 and 15.4% cases with 4 blood vessels. All the slides demonstrated expression of p16 protein at a high level [+++], and the visual field was dominated by labelled nuclei. The expression of cell markers in different grades of MCT is illustrated in Fig. 2.

**Fig. 2.** Staining pattern of examined antibodies in all grades of mast cell tumour. (a–c) CD117 expression pattern was different, depending on malignancy: a. Grade I. Pronounced membranous reaction is visible in nearly all cells. × 400; b. Grade II. Intense, focal staining of cytoplasm. × 200; c. Grade III. Delicate, diffuse staining of cytoplasm. × 400. (d–f) von Willebrand factor expression pattern was similar in all cases, the most important difference being in the number of disclosed blood vessels: d. Grade I. Two blood vessels are visible. × 200; e. Grade II. Five blood vessels are visible. × 200; f. Grade III. Seven blood vessels are visible. × 200. (g–i) p16 expression, stained nuclei were taken into account: g. Grade I. Only few cells presented a nuclear staining pattern, mostly the cytoplasm was labelled. × 400; h. Grade II. Immunostained nuclei, cytoplasmic reaction was weaker than in grade I. × 400; i. Grade III. In most of the cells nuclear reaction was observed. × 400
Statistical analysis of the results demonstrated a statistically significant (P < 0.001) positive correlation between the grade of tumour malignancy determined by HE staining and the expression of CD117, FVIII and p16 proteins, as well as between individual protein pairs. The correlation coefficient (rho, according to Spearman) manifested values > 0.7 for each pair of staining: HE–CD117: 0.85; HE–FVIII: 0.9; HE–p16: 0.84; CD117–FVIII: 0.89; CD117–p16: 0.72, FVIII–p16: 0.78. Thus, the results demonstrate a pronounced relationship between the cell markers studied.

**Discussion**

Canine mastocytoma represents a tumour that remains to be poorly understood despite its very high incidence. This reflects its variable biological behaviour and incompletely recognised, probably multifactorial aetiology.

The factor, which is responsible for the proliferation, differentiation and degranulation of mast cells, involves the kit ligand and is termed also as mast cell growth factor (MCF) or stem cell factor (SCF). The ligand, located on mast cells, binds to the transmembrane receptor of KIT tyrosine kinase (classified as CD117) (Webster et al., 2006; Passantino et al., 2008). This involves a protein coded by the c-kit proto-oncogen. The role of c-kit mutation has been emphasised in the aetiology of mastocytoma. As a result of such mutations the KIT receptor becomes stably stimulated even in the absence of the ligand (Kiupel et al., 2004; Morini et al., 2004). Most frequently, mutations of the duplication type (ITD – internal tandem duplication) and deletions of exon 11 are detected (Zemke et al., 2001; Zemke et al., 2002; Webster et al., 2006), but they are present in only 30–50% of dogs carrying grade II or III mastocytoma (Downing et al., 2002; Zemke et al., 2002). These results indicate that, apart from the mutated c-kit protein, probably also other factors play a significant role in the induction of mast cell tumours; however, the mutations are definitely linked to the histological grade of tumour malignancy.

Our investigations have corroborated the results obtained by the groups of Kiupel, Webster, Zemke and Passantino, which strictly linked the obtained pattern of CD117 staining with the histological malignancy of mastocytoma (Zemke et al., 2001; Kiupel et al., 2004; Webster et al., 2006; Passantino et al., 2008). Moreover, our results have confirmed the observations of Kiupel et al. (2004) and Webster et al. (2006) that the type of reaction pattern makes it possible to predict the probability of tumour progression. Low-grade histological tumour malignancy is mainly linked to the membranous reaction and insignificant staining of the cytoplasm, while high-grade malignancy is usually associated with a diffuse cytoplasmic reaction. Therefore, despite the apparently low or intermediate histological malignancy estimated by HE staining (MCT of grade I or II), occasionally quite distinct results can be obtained in anti-CD117 staining. A pro-
nounced positive correlation with the grade of histological malignancy has been demonstrated (estimated in HE staining at the level of 0.85) which confirms the important role of the c-kit protein. In studies on grades I and III, 100% of slides showed the first and the third pattern of staining, respectively. For tumours of grade II malignancy, the results were more divergent: most of the slides manifested the second pattern of staining although reactions of both the first and the third types were detected as well. This is thought to be linked to morphological heterogeneity of the tumours. In the studies of Northrup et al. (2005) and Kiupel et al. (2011) it was shown that subjective evaluation by a pathomorphologist also plays an important role. The highly pronounced correlation between the expressions of CD117 and p16 (0.72) points to a strong relationship between the proteins in MCT. This confirms the earlier drawn conclusions on the strict dependence between the CD117 staining pattern, the higher histological malignancy of the mast cell tumour and a less favourable prognosis.

Neoangiogenesis has a key importance in neoplastic processes. Blood vessels of the tumour manifest a number of abnormalities in both structure and function. The basement membrane and the pericytes may form a few loosely linked layers, in which cells can have an irregular shape. The leaky endothelium gives rise to haemorrhages and facilitates the passage of tumour cells into the circulation (Crivellato et al., 2008). Mast cells show a tendency to accumulate in places of new blood vessel formation. The released mediators control the proliferation and function of endothelial cells (Metcalfe et al., 1997; Crivellato et al., 2008). According to Crivellato et al. (2008), these mediators include VEGF (vascular endothelial growth factor), FGF-2 (fibroblast growth factor-2), TGF-β (transforming growth factor-β), TGF-α (tumour necrosis factor-α), IL-8, MMP-2 (matrix metalloproteinase-2), MMP-9 (matrix metalloproteinase-9), NGF (nerve growth factor), PDGF (platelet-derived growth factor), angiopoietin 1, leptin, adrenomedullin, tryptase, chymase, histamine and heparin. In addition, mediators are produced which aim at the degradation of extracellular matrix (ECM), which facilitates the development of metastases and promotes the angiogenetic process (Baram et al., 2001). Numerous studies have demonstrated that the higher density of blood vessels within MCT is connected with a poorer prognosis (Theoharides and Conti, 2004; Crivellato et al., 2008; Strefezzi et al., 2009).

The present study has corroborated the observations of Ranieri et al. (2003), Preziosi et al. (2004) and Strefezzi et al. (2009) that the number of blood vessels represents an important aspect of malignancy: an increasing grade of histological malignancy of the tumour was accompanied by a growing density of vascular network. Moreover, the statistical analysis of results obtained in the present study has demonstrated a pronounced positive correlation at the level of 0.9 between the grade of malignancy and the expression of FVIII. This confirms that in tumours of a higher malignancy grade the cells are better supplied with blood. Therefore, it can be concluded that such a supply is linked with evident progres-
sion of the tumour. Statistical analysis has demonstrated a positive correlation at the level of 0.89 between the expressions of FVIII and CD117. The results corroborate the pronounced relationship between the density of blood supply and the pattern of CD117 staining and, thus, the histological grade of tumour malignancy.

The p16 protein, which belongs to the inhibitors of cyclin-dependent kinases, controls the passage of cells from G₁ into S phase and is considered to represent a factor inhibiting the growth of neoplastic cells. Physiologically, expression of the marker is high in currently non-dividing cells and low in intensely dividing cells. Moreover, the protein acts as a controller of tumour cell apoptotic process, in which a decreased expression of p16 induces an increased resistance of cells to apoptosis, which may promote their immortality (Rocco and Sidransky, 2001; Witkiewicz et al., 2011). DeInnocentes et al. (2009) and Witkiewicz et al. (2011) observed an increased expression of p16 in senescent cells, cells with damaged DNA and cells under oncogenic stress. High level of the protein was shown also in certain neoplastic lesions, first of all in those induced by oncogenic viruses as well as in ovarian cancer and in adenocarcinomas of the milk gland (DeInnocentes et al., 2009; Phillips et al., 2009; Witkiewicz et al., 2011). According to DeInnocentes et al. (2009), this is probably linked to genetic mutations of deletion type in the \( p16 \) gene. Despite several biochemical and genetic studies, the role of the markers remains to be fully clarified while its increased or decreased expression is probably linked to multiple factors (Rocco and Sidransky, 2001).

In our own studies we have demonstrated a very pronounced reaction \([+++]\) for p16 in all examined grade II I cases and a weak reaction in 72.7% of grade I cases, in which less than 25% of the cells proved to be positive. Moreover, the statistical analyses have demonstrated a pronounced correlation at the level of 0.84 between the expression of p16 protein and the histological malignancy of tumours evaluated in HE-stained slides. This has allowed us to confirm the suggestion that in mast cell tumours the expression of this marker increases with the grade of histological malignancy. The correlation coefficient between the expression of p16 protein and that of FVIII was found to be 0.78. The results indicate a strong interrelationship between the two markers studied: accordingly, an increased expression of the p16 protein is associated with both a higher degree of histological malignancy found by HE staining and a higher density of the blood vessel network.

In summary, we can state that each of the cell markers examined represents a useful prognostic indicator for patients with MCT. The calculated correlation coefficients confirm a strong link between the expressions of CD117, FVIII and p16 and the histological malignancy of a tumour. An increased expression of the cell markers studied points to a higher likelihood of progression of dermal mastocytoma in dogs.
References


