Expression of p16 (INK4a), cytokeratin 19, and Ki-67 in canine laryngeal squamous cell carcinoma

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Abstract

The study aimed at morphological and immunohistochemical characteristics of laryngeal squamous cell carcinomas and their metastases in canine lymph nodes and lungs. Tissue sections were stained using classical technique with haematoxylin and eosin. In addition, immunohistochemical studies were performed with p16, cytokeratin, and Ki-67 antibodies. An expression of all examined antigens was detected in laryngeal tumours, while in tumour metastases only expression of p16 protein and cytokeratin was demonstrated. The results pointed to higher proliferative potential of the primary tumour than of their metastases.

Key words: dog, laryngeal squamous cell carcinoma, metastases, p16 (INK4a), cytokeratin, Ki-67.

Introduction

Primary laryngeal tumours seldom develop in companion animals, and if they do, they usually affect older animals or animals of a moderate age (24, 26). Most frequently they are observed in dogs and cats (3, 4). Their aetiology remains to be incompletely clarified; in humans their development is promoted by smoking of tobacco, excessive alcohol consumption, exposure to asbestos and nickel, car exhausts, deficiencies of vitamins A and C, deficiency of iron, chronic inflammatory conditions, and human papilloma virus (41). In animals, the studies are continued on correlation between inhalation of tobacco smoke and development of tumours in respiratory pathways (7, 24). The larynx may be affected by both benign and malignant tumours; in dogs development of squamous cell carcinoma is detected most frequently, while cats are mostly affected by lymphomas (5, 24, 26, 32). Individual categories of laryngeal tumours developing in animals are listed in Table 1 (24).

Squamous cell carcinoma (carcinoma plano-epitheliale) represents a tumour, which is the most frequently encountered in animals of moderate or advanced age, irrespectively of their gender (37). In humans, it may be preceded by leukoplakia, pachyderma, or papillomas in adult individuals (41). Predisposed dog breeds are boxers, schnauzers, and white poodles.

The possibility and rate of metastases development depend on primary tumour location; in cases of internal organ neoplasms, the metastasis development is definitely accelerated (38). Upon an aggressive course of neoplasia, metastases can be noted in both draining lymph nodes and in distant organs (36), which rarely occur in humans (41). The neoplasm is usually a rapidly growing tumour, frequently forming fine papillae, potentially with erosions or ulcerations on their surface (14). Under microscope tumour cells manifest an extensive polymorphism and they infiltrate the surrounding tissue in the form of nests and streaks (5). Individual cells contain a round or oval cell nucleus with centrally located, clearly marked nucleolus and most frequently an abundant cytoplasm (22). Depending on the grade of tumour malignancy, a

<table>
<thead>
<tr>
<th>Benign</th>
<th>Malignant</th>
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<tr>
<td>rhabdomyoma</td>
<td>squamous cell carcinoma</td>
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<tr>
<td>oncocytoma</td>
<td>adenocarcinoma</td>
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<tr>
<td>leiomyoma</td>
<td>fibrosarcoma</td>
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<td>lipoma</td>
<td>osteosarcoma</td>
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<tr>
<td>osteochondroma</td>
<td>chondrosarcoma</td>
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<td>rhadomyosarcoma</td>
<td>lymphoma</td>
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<tr>
<td>mastocytoma</td>
<td>malignant melanoma</td>
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Table 1. Categories of laryngeal tumours
variable number of mitotic figures, frequently abnormal can be noted. Moreover, the tumour structure may contain infiltrates of inflammatory cells, such as lymphocytes, histiocytes, neutrophils, plasmocytes, and macrophages (6, 21). In humans, in addition, overexpression of EGFR, accumulation of p53, mutation of TP53 gene, amplification of HER-2, and loss of 3 p and 9 p, as well as one or a few additional chromosomes have been detected (41).

The present study aimed at morphological and immuno-histochemical characteristics of canine laryngeal squamous cell carcinoma and their metastases in lymph nodes and lungs.

**Material and Methods**

The material included five cases of laryngeal tumours detected upon autopsy. In three cases, the tumour was accompanied by metastases to regional submandibular lymph nodes, and in one case additionally to the lungs. In two cases no metastases were evident upon macroscopic examination. Tissue samples were fixed in 7% buffered formalin for 24 h and processed to form paraffin blocks; 3-4 µm sections were routinely stained with H&E.

Immunohistochemical examination was performed in paraffin sections on silanised microscopic slides (Dako®), dewaxed in xylene, and passed through alcohol solutions with decreasing concentration, and finally in water. p16 and cytokeratin antigens were retrieved in EnVision™ FLEX Target Retrieval Solution, high pH (50x) (Dako®), and heated in a water bath at 96°C for 20 min. Ki-67 antigen was retrieved in the same manner as p16 antigen. Endogenous peroxidase was blocked in EnVision™ FLEX Peroxidase-Blocking Reagent for 10 min. Subsequently, the sections were overlaid with primary antibodies: p16 – monoclonal antibody produced in mouse, Clone DCS-50 (Sigma-Aldrich®) diluted 1:100; cytokeratin (CK) - monoclonal human antibody produced in mouse, Clone RCK108 (Dako®) diluted 1:50; Ki-67 - human antibody produced in mouse, Clone MIB-1 (Dako®) diluted 1:50. Negative and positive controls were included. As positive control, normal, non-tumour tissue treated with the same antibody reagents like in neoplastic tissue, was used. In negative control, antibodies were omitted.

Expression of Ki-67 was graded using a semiquantitative scale for evaluation of percentage of positive cells (0–5% - no reaction (-), 6%–25% scanty reaction (+), 26%–50%, moderate reaction (++), >50% intense reaction (+++). Upon evaluation of p16 and cytokeratin, the scale of Remmele (10, 40) was used, the technique taking into account both percentage of positive cells (A) and intensity of reaction colour (B), while the final score represented a score of the two parameters, ranging between 0 and 12 points (no reaction – 0 pts (-); scanty reaction 1-2 pts (+), moderate reaction 3-4 pts (++), intense reaction 6-12 pts (+++) (Table 2).

<table>
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<th>A</th>
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<tr>
<td>0 pts – absence of cells with positive reaction</td>
<td>0 pts – no reaction colour</td>
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<tr>
<td>1 pts – up to 10% positive cells</td>
<td>1 pt – weak reaction colour</td>
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<tr>
<td>2 pts – 11%–50% positive cells</td>
<td>2 pts – moderately intense reaction colour</td>
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<tr>
<td>3 pts – 51%–80% positive cells</td>
<td>3 pts – intense reaction colour</td>
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<td>4 pts – &gt;80% positive cells</td>
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Microphotographs of all the examined neoplastic lesions were taken using Olympus BX53 optical microscope (Olympus, Japan), coupled with ColorView IIIu digital camera (Olympus, Japan).

**Results**

In all examined cases, laryngeal tumours formed a diffuse, irregular, grey-pink proliferative lesions, embracing laryngeal surface (Fig. 1).

Regional lymph nodes were intact or slightly enlarged due to a secondary inflammatory process or, in the cases of metastases (three cases), significantly enlarged, cavernous, with obliterated structure (Fig. 2).

In the case of sample with pulmonary metastases (a single case) cross-section of lobes displayed variable size, cream-pinkish nodules of a uniform cross-section (Fig. 3).

Histopathological examination of laryngeal samples demonstrated neoplastic proliferation, classified according to WHO recommendations as squamous cell carcinoma, infiltrating the region of laryngeal cartilages (Fig. 4). Neoplastic cells, arranged in nests separated by strands of connective tissue, manifested a significant polymorphism. Cells with large nuclei, with clearly marked nucleoli and visible figures of mitotic division, prevailed. Haemorrhages with subsequent accumulation of haemosiderin granules were also noted. Moreover, an infiltration of inflammatory cells, composed predominantly of lymphocytes, and foci of necrosis were also present.

Sections of neoplastically altered lymph nodes manifested diffuse regions of necrosis, lymphocytic inflammatory foci, and diffuse infiltrates of neoplastic cells (Fig. 5).

In samples of pulmonary tissue, well delineated from pulmonary tissue, focal metastases were observed, presenting neoplastic structure of squamous cell carcinoma type. Moreover, the surrounding tissue contained slight amounts of oedematous fluid, accompanied by haemostasis, haemorrhages, and granules of haemosiderin (Fig. 6).
Expression of neoplastic markers in individual organs was detailed in Table 3. In analysis of the intensity of Ki-67 protein expression, a relatively pronounced nuclear reaction at the level of (+++) was detected in the primary tumour (Fig. 7), while in metastases expression of the marker was lower.

In tests using p16-specific antibodies a very strong (+++, 8 in Remmele’s scale) cytoplasmic reaction was detected in all neoplastic lesions in larynx, lymph nodes and lungs (Fig. 8).
Discussion

In animals laryngeal tumours develop infrequently and they are estimated to be detected in around 0.02% of all bioptic and histopathological studies (9). Most of the studies published until now have involved individual cases of affected animals. Squamous cell carcinoma represents one of the most frequent dermal tumours of epithelial origin, while in internal organs it is relatively rare (42). In most cases it develops in tonsils, stomach, and urinary bladder (38). Cases of squamous cell carcinoma in the larynx were described in dogs, cats, horses, and the black bear (16, 26, 35). The syndrome of non-specific signs, such as coughing, aphonia, lack of appetite, or apathy, frequently bypassing the accessory studies during the diagnostic process, cause difficulties in establishing the correct diagnosis. In the studies diffuse, massive lesions involving the entire laryngeal surface have been observed. This may point to highly aggressive nature of the neoplastic tumour, and to difficulties in diagnosis and treatment at early stages of the tumour development.

As mentioned above, the rate of metastasis development depends on primary location of the tumour (38). While dealing with dermal squamous cell carcinoma, it should be remembered that the lesion expresses local invasiveness and metastatic potential primarily to local lymph nodes. It is not until, the disease process is advanced that secondary neoplastic lesions can be detected in more distant organs. In the case of squamous cell carcinoma of inner organs, including larynx, the course of neoplastic process is definitely more aggressive. The tumour manifests an intense growth, a pronounced invasive character, and a high rate of metastasis development in surrounding tissues, local lymph nodes, and the more distant organs (22). In the study, metastatic lesions have been detected in both local lymph nodes and lungs. All examined tumours demonstrated infiltration of laryngeal cartilage and high mitotic index. Furthermore, no significant differences in the immunoexpression level of primary tumour and metastases were observed. This confirmed our assumptions concerning a highly invasive character of the neoplastic process in all cases.

At present, immunohistochemical examination becomes an indispensable element of histopathological investigations. Using various markers, it is possible to confirm the diagnosis by verifying the efficacy of the conducted treatment or to define prognosis. In veterinary medicine, such tests are not routinely performed, mainly due to high costs of the antibodies and equipment required for the immunohistochemical laboratory. An additional problem involves application of antibodies specific for human tissues and the relatively insufficient available and verified data on cross-reactions with animal tissues. In the investigations, we have applied antibodies used in diagnosis of laryngeal tumours in humans, i.e.
antibodies specific for p16, cytokeratin, and Ki-67. Their reactivity with canine tissues has been confirmed in earlier studies (18, 25, 27–30).

In the process of establishing the diagnosis, the first stage involves determination of origin of neoplastic tumour. In unclear situations, expression of some markers, including cytokeratins may be helpful. Cytokeratins (CK) represent one of the most important elements of cytoskeleton in epithelial cells of humans and animals. They belong to the group of cytoplasmic proteins forming i.e. intermediate filaments (8, 19, 43). The proteins may be categorised into the soft and solid forms. In the group of cytokeratins, two subfamilies differing in molecular weight, including cytokeratins I (CK9-CK20) and cytokeratins II (CK1–CK8), can be distinguished (23). Cytokeratins undergo the process of ubiquitination (taking place during proteasome-mediated protein degradation), which results in modification of structure and, indirectly, function of various proteins. Deformed cytokeratins disrupt shape of cytokeratin network in the cytoplasm, thereby its structure becomes more loose and the natural cytoskeleton structure becomes destroyed. Accumulation of the altered proteins increases aggressiveness and invasiveness character of the cells. Furthermore, processes of mitosis and apoptosis become disturbed, which promotes immortality of the altered cells (15). Expression of cytokeratins is used, first of all, for distinguishing neoplastic cells of epithelial origin from undifferentiated or poorly differentiated neoplastic cells stemming from the other germ layers (17). Moreover, cytokeratins were applied in evaluation of the degree to which a tissue is abnormally differentiated (11). Cytokeratin 19 was used in the study due to its versatility and prognostic value in patients with squamous cell carcinoma. In the performed studies on laryngeal carcinomas, a pronounced (+++) cytokeratin expression has been detected in all cases. Positive result of such staining allowed to confirm epithelial origin of primary tumours and metastases.

In the diagnostic process, early detection and diagnosis of a tumour are of a primary significance. The biomarker p16 (INK4a) allows to detect disturbances in the cell cycle, pointing to an abnormal process of cell division. The protein plays a particular role in the negative control of the cell cycle. It represents an inhibitor of cyclin-dependent kinases (CDK1), which decreases the rate of cell cycles through inactivation of specific CDK1, such as kinases of D1, CDK4, and CDK6 type (40). Therefore, CDKs inhibitors are considered to form one of the signal cascade elements, which inhibit or slow down replication in the course of cell differentiation process (31). Inactivation of p16 was noted in neoplastic cell lines, which may suggest its antineoplastic effect (33). On the other hand, several authors have demonstrated high expression of the protein in malignant tumours, such as uterine carcinoma and adenocarcinoma, lymphatic leukaemia, malignant melanoma, and squamous cell carcinoma (1, 39, 44). Moreover, the expression was found to be correlated with the grade of tumour malignancy (1). Effectiveness of the marker in description of behaviour manifested by other tumours (malignant melanoma or gastric carcinoma) found confirmation in earlier studies (18, 25, 34). Until now, no investigations have been performed using p16 (INK4a) protein in lesions of laryngeal squamous cell carcinoma type in dogs. Summing up, positive reaction for p16 (INK4a) provides an information on disturbances in the cell cycle, and, indirectly, on neoplastic tumour growth rate and potential for development of metastases (10). In the study, high levels of expression of p16 (INK4a) protein were detected in all examined laryngeal carcinomas and their metastases. As mentioned earlier, this points to disturbances in cell cycle and, thus, to the rate of cellular divisions and growth of the tumours. Furthermore, in one case the observed expression level was higher in primary tumour than in metastasis.

In histological evaluation the subsequent important element involves assessment of tumour proliferative potential. The rate of cell proliferation may be estimated through expression of various proteins, including Ki-67. Its presence can be detected in all phases of cell cycle except G0 phase. Starting at G1 phase of the cycle, concentration of the protein continues to grow, to reach its maximum level at M phase (20). Expression of the protein is best visible in active cells, ready for proliferation (2). In addition, in humans a positive correlation was detected between cells manifesting expression of Ki-67 and rate of primary tumour growth, and potential to manifest a relapse (12). The correlation was confirmed also in veterinary medicine (27–30). Following analysis of 60 mammary gland tumours in bitches, Gizinski et al. (13) documented relationship between pronounced expression of Ki-67 protein and low level of differentiation of the tumour cells, and presence of metastases. In the studies, using Ki-67 proliferative antigen, its moderate expression (++) was documented in the primary tumour and its low expression in metastases. This indicates a higher proliferative potential of cells in primary tumour, its dynamic growth, which correlated with a poor prognosis for the patient.

Summing up, it may be stated that histopathological analysis and immunohistochemical tests with an appropriately selected panel of antibodies allow for a correct and rapid diagnosis of neoplasms. In several cases they may also enable the clinician to define prognosis, and to select an effective therapy of the defined type of tumour.
References


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