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Immunohistochemical expression of proliferation markers in canine osteosarcoma

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Abstract

Canine osteosarcoma is an extremely malignant bone tumor that often arises in the bones of the limbs. It is a highly metastatic disease distinguished by proliferative bone lesions and a tendency for pulmonary metastasis. Overexpression of proliferative proteins are associated with bad prognosis in human osteosarcoma. Here, we tested the expression of the different proliferative proteins (p53, p16, vimentin, and mdm2) in nine archival samples with canine osteosarcoma. Paraffin-embedded tissue sections were confirmed by histopathology and stained by immunohistochemistry for p53, p16, vimentin and mdm2. Positive expression of these proteins was evaluated as the ratio of positive cancer cells and the intensity staining was assessed in several areas. Histopathologically, 95% of samples were grade II and III. All high-grade osteosarcomas were particularly cellular. The cancer cells were generally large spindle-shaped and large nucleus with distribution of osteoid between the cancer cells. Immunohistochemical detection of p53, p16, vimentin and mdm2 was 89%, 56%, 78%, and 89% of samples respectively. The staining intensity for p53, p16, vimentin and mdm2 was particularly nuclear in 81%, 66%, 78%, and 79% of the cancer cells respectively. Our present work suggests that p53, p16, vimentin, and mdm2 were detected in grade III canine osteosarcomas samples. In addition, these proliferative markers are the significant biomarkers in canine osteosarcomas and can be used as a predictor for diagnostic and prognostic value and allowing cancer differentiation. This primary data supports that both canine and human osteosarcomas share same molecular characters which are approved by expression of proliferative genes.

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Introduction

Osteosarcoma represents one of the prime bone cancers initiated from bone mesenchymal cells and it is the most common bone tumor in human and dogs. Canine osteosarcoma considered as a suitable ordinary animal model to analysis human osteosarcoma (1,2). Canine osteosarcoma is a closely similar to those seen in its human counterparts and both show poor prognosis (3). Moreover, Canine osteosarcoma occurs approximately 10 times more than in humans (2). Both canine and human osteosarcoma share same molecular characters, which are approved by expression of a core set of proliferative genes (4). Targeting specific proliferative protein markers in both dogs and human osteosarcoma might improve not only therapeutic trials, but also could help the comparative pathological investigations to clear up the doubts of this disease (5). Thus, in this work we intended to investigate and analyse the expression of specific proliferative protein markers in naturally occurring unbiased canine osteosarcoma of mixed breed dogs in our region, that diagnosed by certified pathologists at the pathology department, college of

veterinary medicine. These markers are usually used in human tissues for the classification of human osteosarcoma, so we would like to find out if these markers reflect any reactions with the mixed-breed canine osteosarcoma cases in our area. One of the highest normally mutated proliferative genes in human osteosarcoma is TP53, which controls tumorigenesis via regulating specific genes that play a crucial role in different cellular events (6). However, Abnormal expression of p53 protein has been recognized in a different number of canine tumors and gene mutations identified as well (7). Regarding p16, about 60% of aggressive osteosarcomas showed expression of p16 proliferative protein, which shows an essential part in the deregulating of not only retinoblastoma protein, but also p53 pathways (8). In mice with OS immunohistochemistry (IHC) reactions revealed a low expression of p16 in OS tissues (9). Another proliferative marker is Vimentin, which is overexpressed in many epithelial tumors such as gastrointestinal tumors, breast tumor, malignant melanoma, prostate cancer, and a further form of tumors. It is usually overexpressed with cancers associated with elevated tumor growth and invasions and aggressiveness (10). Nevertheless, the main function of vimentin in cancer development is still obscure. Mdm2 (Mouse double minute 2) is a proliferative protein has an important function in oncogenic activity, classified as a p53 interacting protein, which represses p53 transcriptional activity (11). Appreciating this activity in human cancers might improve methods of detecting and evaluating prognosis. Targeting mdm2 or their regulatory proteins may have a promising therapeutic attempt to cure certain types of cancers (12).

While proliferative proteins have been reviewed in human osteosarcomas, findings assessing different proliferative protein expression in canine osteosarcomas are limited. The purpose of this work is to assess the immunohistochemical expression of different proliferation markers (p53, p16, Vimentin and mdm2) in archived spontaneous canine osteosarcomas samples in our area.

Materials and Methods

Tissue samples

This study was constructed on stored tissue sampled from canine osteosarcomas. These samples involved 9 confirmed canine osteosarcoma cases acquired from February 2018 to March 2020 by the Department of Pathology, College of Veterinary Medicine. In the current study, all canine samples were accepted by the ethics committee at the University of Mosul and conformed with ethical approval number (Approval no. UM.VET.2021.011). Samples of canine cancer tissues were surgically excised at the Mosul University, College of Veterinary Medicine, Veterinary Teaching Hospital.

Histopathologic analysis

Tissue samples were processed and fixed in formalin (10%). Then, the fixed tissues were embedded in paraffin wax blocks using routine methods. A 4-µm section of cancer tissue from each animal was stained mounted on Poly-L-lysine slides for haematoxylin and eosin (H&E) for microscopic evaluation. The stained slides were assessed for the tumor histology and grade by two pathologists (11).

Immunocytochemistry

Tissue Sections were dewaxed in xylene, rehydrated in a sequence of ethanol and then washed in phosphate-buffered saline. After that, hydrogen peroxide-methanol solution (3%) was added to suppress the endogenous peroxidase activity for 30 min. Then, the tissue sections were blocked for 60 min at 25°C. After that, the sections were incubated with primary antibodies at 4°C for overnight. The primary antibodies used were p53 mice monoclonal (dilution 1:200; Wuhan Biotech, China), p16 mice monoclonal (dilution 1:200; Elabscience, USA), vimentin mice monoclonal (dilution 1:200; Elabscience, USA) and (mdm2 mice monoclonal, dilution 1:200, Wuhan Biotech, China). After three times washing, the sections were incubated with poly-HRP Goat Anti-Mice IgG (dilution 1:200, Wuhan Biotech, China) for 60 min at 37°C. After three times washing, the sections were detected with an avidin-biotin complex. The sections were stained with haematoxylin for 60 second, washed in distal water, dehydrated and cover-slipped. Sections were seen using a microscope and photographed using digital camera (13).

Immunohistochemical scoring

Sections were seen using microscope at a high-power magnification (200µm) to assess the immunoreactivity, and then by a less-power magnification (50µm) to identify the places of antibody reaction. Positive expression of p53, p16, vimentin, and mdm2 were evaluated as the average of positive cancer cells and the intensity staining was assessed in three fields. Just nucleus brownish staining for all 4 proteins were considered as a positive expression. Assessment of p53, p16, vimentin, and mdm2 immunostaining was accomplished using a point weighted score system (14). Initially, the proportion of positive cancer cells were recorded using a 4-point scale: (0) for less than 10%, (1) for <25%, (2) for <50%, and (3) for >50%. Then, the staining power of the nucleus was marked using a 4-point scale: negative (0), weak (1), medium (2), and strong scale (3) (15).

Results

The average age of the dogs at the moment of biopsy collections was 8 years. The osteosarcomas were collected from different breeds of dogs and the sites were mainly

distributed in femur (3), humerus (3), radius (2) and Mandible (1). Histopathologically, most of the samples were graded II and III. All grade III canine osteosarcomas were particularly cellular, the cancer cells were generally large spindle-shaped, high nucleus to cytoplasm ratio, multiple nucleoli, hyperchromasia, and numerous mitoses. In addition, Osteosarcomas demonstrate osteoid production between the cancer cells and considered as osteoblastic osteosarcomas. The osteoid is filled as a light pink between the cancer cells (Figure 1).

Immunohistochemical findings showed that p53, p16, vimentin, and mdm2 proteins were positively stained in the nucleus. For p53 protein, the mean percentage expression rates of positive cancer cells in 9 canine osteosarcomas samples were 89% (8/9) (Figure 2). The positive expression rates of p16 in all osteosarcomas samples were 56% (5/9) (Figure 3). vimentin expression was positive in 78% of samples (7/9); the most characteristic dissemination pattern was nuclear expression (Figure 4). For mdm2 protein, the percentage expression rates of positive cancer cells in 9 canine osteosarcomas samples were 89% (8/9) stained positively; nuclear staining was the most common (Figure 5).

Regarding the intensity of proteins expression, immunohistochemical analysis of p53 and mdm2 staining in grade III canine osteosarcomas showed diffusely strongly positive nuclear staining in 81 and 79%, moderately diffuse nuclear staining in 19 and 21% respectively, and weakly cytoplasmic staining (Figure 5). Regarding the intensity of p16 and vimentin expression in grade III canine osteosarcomas, the results revealed a sporadic staining pattern with a combination of negative and positive staining zones. In addition, there was focally strong positive nuclear staining intensity in 66 and 78%, moderately diffuse nuclear staining in 44 and 22% respectively, and negative cytoplasmic staining (Figures 3 and 4).

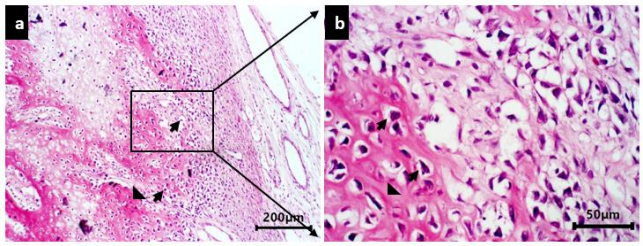

Figure 1: Histological appearance of canine osteosarcomas. a. Sheets of pleomorphic cancer cells (arrows) with regions of malignant osteoid production (arrowhead). H&E. 200µm. b. Cancer cells with varied morphology and hyperchromatic nuclei (arrows). In addition, distribution of a very noticeable eosinophilic, homogenous, glassy osteoid material which characteristic of the Osteosarcoma (arrowhead). H&E. 50µm.

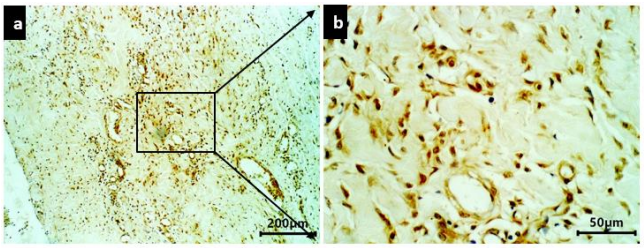

Figure 2: Nuclear expression of p53 in canine osteosarcomas. (IHC. 200µm. (a) and IHC. 50µm. (b)).

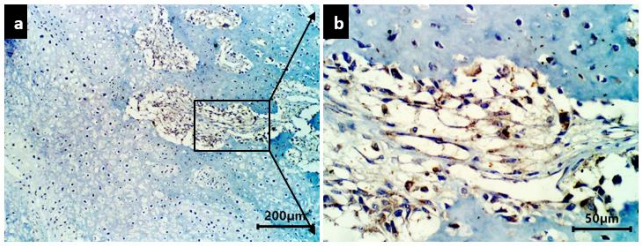

Figure 3: Nuclear expression of p16 in canine osteosarcomas. (IHC. 200µm. (a) and IHC. 50µm. (b)).

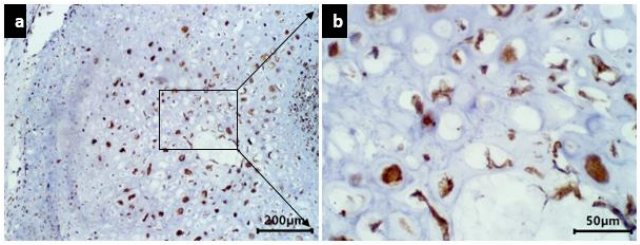

Figure 4: Nuclear expression of vimentin in canine osteosarcomas. (IHC. 200µm. (a) and IHC. 50µm. (b)).

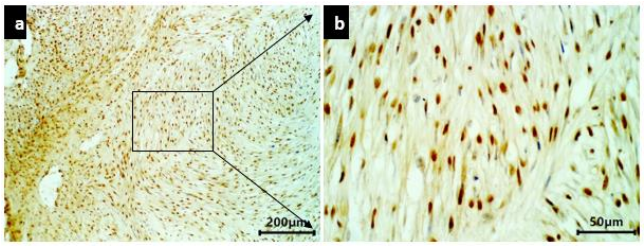

Figure 5: Nuclear expression of mdm2 in canine osteosarcomas. (IHC. 200µm. (a) and IHC. 50µm. (b)).

Discussion

In the current decades a complete study of the genetic variations and systematic progressions in human osteosarcoma has been recognized by the transcription investigation of malignant samples (16,17). In dogs,

osteosarcoma continues to be difficult to cure and gives a bad prognosis due to malignancy and aggressive nature of the cancer. Recent molecular studies of osteosarcoma in humans and dogs exposed multiple nucleotide polymorphisms linked the development of osteosarcoma (18). We have aimed to know the proliferation markers of osteosarcoma in dogs and to link these outcomes to those found from osteosarcoma from human.

In this study we examined four proliferation markers that have been already exhibited in human osteosarcoma. Actually, the importance of p53 (6), p16 (8), vimentin (19), and mdm2 (20) is well documented in human osteosarcoma. Nevertheless, little is identified around the proliferation markers expression in canine osteosarcoma.

P53 protein was the most commonly expressed in around 89% of canine osteosarcoma samples.

Interestingly, Kawaguchi and colleagues also exhibited a human osteosarcoma with high p53 staining as seen in canine osteosarcoma (21). In normal cells, p53 expresses in low quantity (22). In canine osteosarcoma, the expression of this marker proposes unusual p53 action. In this work, the expression of this marker is same to prior works against canine osteosarcoma samples (23). Furthermore, Strong nuclear expression was shown in 81% of the cells. The nuclear location of this protein could be the cause of increased proliferation of osteoblast cancer cells.

P16 protein was noticed in 56% of canine osteosarcoma samples. In human, it was showed that p16 expression linked to the progress of human osteosarcoma (24). It has been confirmed that p16 appears to play a substantial part in cancer progression and cancer cell proliferation (25). Regarding the scoring intensity of p16 expression in grade III canine osteosarcomas, our results revealed a strong positive nuclear staining intensity in 66% of the osteoblast cancer cells. Recently, it has been shown that increase expression of p16 is correlated with most metastatic carcinoma (26). Therefore, p16 can be contemplated as a diagnostic marker in grade III canine osteosarcomas.

Vimentin staining was observed in 78% of positive cancer cells in canine osteosarcomas tissues in the current study, and notably strong positive nuclear staining intensity. Vimentin immunoblotting has previously exposed an expression in the cytoplasm of osteoblastic osteosarcoma cells (27). However, vimentin overexpression was detected in the nucleus. The possible explanation for this finding is that, the nuclear overexpression of vimentin in many cancer types correlates with increased progression, invasion, and bad prognosis (10).

Increase expression of mdm2 has been described in the carcinogenesis and tumor progression in different type of sarcomas (28). It has been indicated that mdm2 protein is correlated with increase osteoblast cancer cells proliferation (29). Here in this work, the mdm2 expression was observed in 8 0f 9 samples 89% of canine osteosarcomas cases. As for the staining intensity of proteins expression, mdm2 was noticed in the nucleus of osteoblast cancer cells in around 80% of the cells. These results appear to suggest that increase mdm2 protein expression shows increase the cancer cell proliferation in canine osteosarcomas.

Our results have shown that immunohistochemical expression patterns of p53, p16, vimentin, and mdm2 are all detect in grade III canine osteosarcomas samples. The increase expression of these proliferation markers reflects the degree of malignancy in canine osteosarcomas. Consequently, early detection of these protein in the canine osteosarcomas may improve the prognosis of the cases and give an effected treatment for the canine osteosarcomas.

Conclusion

p53, p16, vimentin, and mdm2 are a significant biomarker in canine osteosarcomas as they can be used for prognosis of the cases with osteosarcomas. Furthermore, we propose that molecular investigates should be done for the purpose of associate abnormal proteins expression with original gene mutations.

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Conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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الكشف عن التعبير الكيميائي النسجي المناعي لعلامات الانتشار في الغرن العظمي في الكلاب

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الخلاصة

الغرن العظمي في الكلاب هو ورم عظمي خبيث للغاية يحدث غالبا في عظام الأطراف. هذا المرض ينتشر بشكل كبير ويتميز بآفات العظام التكاثرية والميل إلى حدوث ورم خبيث رئوي. يرتبط الإفراط في التعبير عن البروتين التكاثري بالتشخيص السيئ في الغرن العظمي البشري. في البحث الحالي، قمنا باختبار التعبير الكيميائي المناعي للبروتينات التكاثرية المختلفة (p53، p16، vimentin، mdm2) في ٩ عينات محفوظة من الغرن العظمي في الكلاب. تم تأكيد أقسام الأنسجة المضمنة بالبارافين عن طريق علم الأنسجة وتم صبغها بالتلوين الكيميائي النسجي المناعي لـ p53 و p16 و vimentin و mdm2. تم تقييم التعبير الإيجابي لهذه البروتينات على أنه النسبة المئوية المتوسطة للخلايا السرطانية الإيجابية وتم تقييم كثافة التلوين في مجالات مختلفة. من الناحية النسيجية ، كانت ٩٥٪ من العينات من الدرجة الثانية والثالثة. كانت جميع عينات الغرن العظمي ذات المراحل المتقدمة خلوية بشكل خاص. كانت الخلايا السرطانية بشكل عام مغزلية كبير مع نواة كبيرة مع توزيع عظمي بين

الخلايا السرطانية. كان الكشف الكيميائي النسجي المناعي عن p53 و p16 و vimentin و mdm2 و٨٩٪، ٥٦٪ و ٧٨٪ و ٨٩٪ من العينات على التوالي. كانت كثافة تلطيخ p53 و p16 و vimentin و mdm2 نوويا بشكل خاص في ٨١٪ و ٦٦٪ و ٧٨٪ و ٧٩٪ من الخلايا السرطانية على التوالي. يشير عملنا الحالي إلى أن بروتينات p53 و p16 و vimentin و mdm2 تم اكتشافها في عينات الساركوما العظمية في الكلاب من الدرجة الثالثة. بالإضافة إلى ذلك، تعد علامات الانتشار هذه علامة حيوية مهمة في الغرن العظمي في الكلاب ويمكن استخدامها كمؤشر للقيمة التشخيصية والإنذارية والسماح بتمييز السرطان. تدعم هذه البيانات الأولية أن كلا من الغرن العظمي في الكلاب والبشر يشتركان في نفس الخصائص الجزيئية التي تمت الموافقة عليها بالتعبير عن الجينات التكاثرية.