Immunohistochemical expression of osteopontin in canine and feline tumors

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SUMMARY

Osteopontin (OPN) is a glycoprotein expressed by various tissues and cells. It is also implicated in tumor progression. The protein can mediate cell adhesion and is strongly associated with transformation and tumorigenesis. Overexpression of OPN influences invasion and metastasis of different human tumors, and OPN expression may be use as a possible prognostic marker. It has been detected in a growing number of human tumor types, by immunohistochemistry on tumor tissue sections. The objective of this study was to assess the immunohistochemical expression of OPN in different canine and feline tumors and to examine any possible relation with malignancy. To achieve these aim 40 different kinds of canine and feline tumors were evaluated. OPN was either not expressed or at low levels in benign tumors, but strongly expressed in malignant tumors. This study showed that OPN may be associated with malignancy of cat and dog tumors.

Keywords: Osteopontin (OPN), immunohistochemistry, dog, cat, tumor

Introduction

Osteopontin (OPN) is an acidic, secreted protein classified as a member of the SIBLING (Small Integrin-Binding, N-Linked Glycoprotein) family [10]. It is an adhesive, matricellular glycoprotein, whose expression is elevated in many types of cancer in human and has been shown to facilitate tumorigenesis in vivo [7]. OPN has long been implicated in the process of tumorigenesis. It was originally identified as a secreted marker for transformed cells, because the level of OPN mRNA is elevated in a wide variety of transformed cells in mice [24].

In recent years, substantial progress has been made in the detection and diagnosis of early stage of cancers in humans. However, there are still no molecular indicators that distinguish highly aggressive tumors from moderately aggressive and non-aggressive ones. But a few markers that predict invasiveness have been established [12, 14, 18, 26, 31, 35]. New tumor markers and markers of tumor progression are needed for improved staging and for better assessment of treatments of many cancers [1, 2]. OPN may be one candidate marker for the progression of various malign tumors. In cancer, this molecule can support cell invasion and anchorage independence, thus enhancing tumor progression and metastasis formation [12, 14, 18, 19, 26, 30, 35].

Numerous studies are available about OPN expression in human tumors especially in early reports. For example, Zhou et al. [36] reported that although OPN expression increased in metastatic melanomas compared to nonmetastatic melanoma, they found no correlation between OPN expression and tumor thickness, metastasis or patient survival. However, in contrast to this study, a recent study investigating OPN in primary melanoma found an association between increasing OPN staining and tumor thickness, a higher invasive level and mitotic index. They reported that higher OPN in tumors correlated with decreased recurrence-free and disease-specific survival [21]. Only one report is available on OPN expression in canine tumors. Klopfleisch et al. [16] reported that there is an increased OPN level in canine mammary carcinomas compared with adenomas by PCR, but levels were not found to be statistically significant.

The objective was to investigate the immunohistochemical expression of OPN and evaluate a possible relationship with tumor malignancy in canine and feline tumors.

Material and Methods

In this study, a total of 40 (26 malign and 14 benign) canine and feline tumors (32 dogs and 8 cats) collected from the archive of the department of pathology were used. They were composed of 15 malign mammary tumors, 5 benign mammary tumors, 9 benign soft tissue tumors, 5 malign soft tissue tumors, 2 malign skin tumors, 3 malign bone tumors.
and 1 hematopoietic tissue tumor. Diagnoses of the tumors and malignancy criterion were made according to the WHO [32] classification. Three different blind pathologist were evaluated the malignancy of the tumors. For gross findings, archive notes and pictures were used.

For histopathology, histochemistry and immunohistochemistry, paraffin blocks were cut at a thickness of approximately 5 μm. All slides were stained routinely with hematoxylin and eosin (HE). For fat tissues, oil red O method was used to cryostat section. The Van Gieson method and immunohistochemistry were used for the evaluation of soft tissue tumors. Then tumor samples were stained with Ki67, proliferating cell nuclear antigen (PCNA), desmin, vimentin, pancytokeratin, smooth muscle actin (SMA), S100, glial fibrillary acidic protein (GFAP), carcinoembryonic protein (CEA) and alpha fetoprotein (AFP) for the identification of origin and determination of malignancy. After this evaluation, sections were stained immunohistochemically in order to demonstrate OPN activity. Commercial kits provided from, Abbiotech, San Diego, CA were used for immunohistochemical examination of OPN [Rabbit Polyclonal Osteopontin, 250801, 1:200 dilution], Ki67 [Rabbit Polyclonal Ki-67 Antibody, 250733, 1:100 dilution]; PCNA [Rabbit Polyclonal PCNA Antibody, 250812, 1:100 dilution]; desmin [Mouse Monoclonal Desmin Antibody, 251743, 1:100 dilution]; Vimentin [Mouse Monoclonal Vimentin Antibody, 251809, 1:100 dilution]; Pancytokeratin [Mouse Monoclonal Pancytokeratin Antibody, 251788, 1:100 dilution], SMA [Mouse Monoclonal SMA, 251813, 1:200 dilution]; S100 [Mouse Monoclonal S-100 Antibody, 251795, 1:100 dilution], GFAP [Rabbit Polyclonal GFAP Antibody, 250661, 1:200 dilution]; CEA [Rabbit Polyclonal CEA Antibody, 250598, 1:200 dilution]; and AFP [Mouse Monoclonal Alpha-Fetoprotein Antibody, 251700, 1:100 dilution]; NSE [Rabbit Polyclonal NSE Antibody 251399, 1:100 dilution], using a routine streptavidine-biotin peroxidase technique.

To evaluate the severity of the immunohistochemical reaction of tumor cells with markers, semiquantitative analysis was performed using an arbitrary visual scale with a grading score ranging from (-) to (+++) as follows: (-) = negative, (+) = focal weak staining, (++) = diffuse weak staining, (+++) = diffuse strong staining. To evaluate the percentage of immunopositive cells, 100 cells were calculated in 10 different microscopic high-powered fields of each slide, and then were examined under the 40x objective of a trinocular microscope (Nikon E600) and microphotography apparatus.

Statistical analysis was done with SPSS (Statistical Package for Social Sciences) 13.0 software (SPSS Inc, Chicago, Ill, USA). Results are expressed as mean ± SD. In the statistical evaluations, 1-way analysis of variance test was used to observe any differences between immunopositive cells of malignant and benign tumors control group. Duncan multiple comparison method was used. In addition, logistic regression, and Pearson's correlation tests were used for association between parameters. P values <0.05 were accepted as statistically significant.

**Results**

**MACROSCOPICAL FINDINGS**

In this study 20 mammary tumors, 14 soft tissue tumors, 3 bone tumors, 2 skin tumors and 1 hematopoietic tissue tumor were examined. Species and diagnosed tumors are shown in Tables I-II. Ages of the cats with tumors ranged from 5-12 years and for dogs 7-17 years. Most of the dog tumors were observed in small breeds. Grossly tumor masses were between 1x1.5 cm and 15x17 cm in diameter (Fig.1A-1D). Generally, malignant tumors were bigger than the benign ones and they usually diagnosed in older animals. Mammary tumors were the most common tumors in both cats and dogs.

Breast tumors in cats were observed between 5-12 years of age, the age of the dogs ranged from 7-17. Most of the animals were tumor small breed dogs and owned cats. Macroscopically tumor masses were varied from 1x1.5 cm, up to 15x17 cm in diameter. Generally, big tumors were....

### Table I: Immunoreactions scores of the cat tumors with examined markers

<table>
<thead>
<tr>
<th>Case</th>
<th>Diagnosis</th>
<th>OPN</th>
<th>Ki67</th>
<th>PCNA</th>
<th>Des</th>
<th>Vim</th>
<th>PCK</th>
<th>SMA</th>
<th>NSE</th>
<th>S100</th>
<th>GFAP</th>
<th>CEA</th>
<th>AFP</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Mammary AC</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>_</td>
<td>+</td>
<td>++</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>++</td>
</tr>
<tr>
<td>2</td>
<td>Lymphoma</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
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<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>3</td>
<td>Vaccine sarcoma</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>4</td>
<td>Malignant melanoma</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>_</td>
<td>+</td>
<td>_</td>
<td>_</td>
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<td>Mammary AC</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>_</td>
<td>+</td>
<td>++</td>
<td>_</td>
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<td>++</td>
</tr>
<tr>
<td>6</td>
<td>Fibrosarcoma</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>_</td>
<td>+</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>7</td>
<td>Mammary AC</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>_</td>
<td>+</td>
<td>_</td>
<td>_</td>
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<tr>
<td>8</td>
<td>Mammary adenoma</td>
<td>+</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>+</td>
<td>+</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>++</td>
</tr>
</tbody>
</table>

were diagnosed as malignant. Five of the canine mammary tumors consisted cartilage and bone formation. Breast adenocarcinomas were lobular structure and mostly whitish-yellow color at macroscopical examination. The majority of the malignant breast tumors had necrotic cavity in the middle part of the mass. These necrotic areas were usually reddish-brown, sometimes whitish-yellow colored liquid containing cystic formation were commonly observed. Bleeding was observed in malignant breast tumors.

Soft tissue tumors localized under the skin, vagina, bladder, and ranging from 1x2 cm up to 4x6,4cm in diameter. The cut surface of the masses was generally soft and pink in color except malignant melanoma that was blackish. Fat, connective and muscular tissue tumors localized in the urogenital system, while histiocytoma, plasmacytoma and melanomas were located on the face and neck.

In the skin tumors, one squamous cell and 1 basal cell carcinoma were present. Skin tumors localized at the face and ears and diameters ranging from 1x2 up to 2x5 cm. The masses were whitish-pink in color and slight hard, of the surface of the tumors were generally ulcerated. Metastases were detected in some skin tumors to the regional lymph nodes. There were three cases of osteosarcoma (2 dogs and 1 cat) in our materials. In two tumors from dogs were observed lung and kidney metastasis.

**HISTOPATHOLOGICAL FINDINGS**

Histopathologically, the most prominent findings were severe hyperemia and which were seen in both malign and benign tumors. Hemorrhages were more common in surgically removed tumors. In mammary mixed tumors, cartilage and bone metaplasia were characteristic (Fig. 2A). Benign tumors kept the normal appearance of cells while marked pleomorphic features, numerous mitotic figures, papillary or cystic glands, necrosis and inflammatory reactions were seen in malignant tumors (Fig. 2B). Connective and myoepithelial tissue proliferations were also seen together with epithelial proliferation. In some malignant cases lymph node and lung metastases were diagnosed. Metastatic masses included numerous mitotic figures and anaplasia; more so than the than primary foci. No metastases were seen in benign cases.
Fourteen soft tissue tumors were diagnosed histopathologically, histochemically and immunohistochemically according to cellular morphology (Figs. 2C-D). One feline vaccine sarcoma was examined in this study. Histopathologically, yellowish-brown pigments with numerous macrophage infiltrations were observed, with proliferated connective tissue and abundant mitotic figures.

Diagnosis of skin tumors was made according to characteristic histopathological appearance. For example, keratin pearls were typically observed in squamous cell carcinomas. An inflammatory reaction was seen in both squamous and basal cell carcinomas. Dermal involvement by epidermal cells was observed in both tumors. During the histopathological examination of osteosarcomas, high mitotic activity and disturbed bone tissue were commonly observed. There was less calcification or mineralization in metastatic masses than primary foci. Microscopically, hemorrhages and clusters of big lymphoblastic cells were common in a spleen with lymphoma. Generally metastases were the most prominent malignancy criterion. Mitotic activity, anaplasia or pleomorphism was also used for evaluation.

**IMMUNOHISTOCHEMICAL FINDINGS**

In this study, tumor tissues were immunostained with desmin, vimentin, pancytokeratin, SMA, GFAP, NSE, CAE and AFP in order to detect the origin of the cells. Ki67, PCNA and S100 were used to detect malignancy stages. Ki67 and PCNA reactions were especially used to evaluate the malignancy criteria. Marked expression was observed in malignant tumors in these markers. More than 25 malignant and 15 benign tumors were immunostained with OPN antiserum and examined any differences in OPN expression in malign and benign ones were correlated the OPN expression of the tumor cells. The reaction between the tumors and markers is given in tables I and II. Statistical analysis for immunopositive cell numbers of dogs and cats benign and malignant tumors are shown in table III. This study shows that generally more than one cell type proliferated in mammary tumors. Especially in mixed tumors of dogs, numerous cells exhibited PCNA and Ki67 positive immunoreactions, and expressions were observed in epithelial, fibrous and even nervous cells.

OPN was strongly expressed in malignant tumors and a correlation was observed with the Ki67 reaction,
immunohistochemically. In contrast to malignant tumors, benign tumors showed minimal to no expression of OPN. Especially in malignant tumors, heterogeneous appearance of OPN expression was seen in different areas and expressed in different severity of OPN in same tumor. While a strong reaction was observed in some areas; slight expression was seen in the other areas within the same tumor mass. Similar findings were also seen in the Ki67 reaction of tumors. In mammary tumors, mitotically active tumor cells, both epithelial and mesenchymal origins were markedly expressed OPN.

In osteosarcomas, OPN activity was not influenced by decalcification. OPN expression was marked in proliferated cells. Interstitial areas tested negative for OPN in osteosarcomas. In skin tumors, squamous cell carcinomas

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>OPN</th>
<th>Ki67</th>
<th>PCNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malign mammary tumors</td>
<td>14</td>
<td>50.73±9.18</td>
<td>26.00±4.25</td>
<td>44.00±5.39</td>
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<tr>
<td>Benign mammary tumors</td>
<td>6</td>
<td>5.80±2.16</td>
<td>5.20±2.49</td>
<td>8.00±2.73</td>
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<tr>
<td>Malign soft tissue tumors**</td>
<td>5</td>
<td>61.00±12.98</td>
<td>25.40±4.03</td>
<td>46.00±6.24</td>
</tr>
<tr>
<td>Benign soft tissue tumors***</td>
<td>9</td>
<td>8.00±4.52</td>
<td>7.11±3.05</td>
<td>6.00±2.64</td>
</tr>
<tr>
<td>Malign skin tumors ****</td>
<td>2</td>
<td>69.00±19.79</td>
<td>22.00±11.31</td>
<td>40.00±4.2</td>
</tr>
<tr>
<td>Malign bone tumors*****</td>
<td>3</td>
<td>60.33±11.50</td>
<td>24.33±5.03</td>
<td>41.66±2.51</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

*: Differences statistically significant. The differences between the means of groups carrying different letters in the same column are statistically significant.

**: Vaccine sarcoma, malignant melanoma, fibrosarcoma and malignant histiocytoma

***: Leiomyoma, lipoma, fibroma, histiocytoma and fibrolipoma.

****: Squamous cell carcinoma and basal cell carcinoma.

*****: Osteosarcoma.

Table III: Statistical analysis results of the percentage of immunopositive cells of malign and benign dog and cat tumors.
more strongly expressed OPN than basal cell carcinomas (Figs. 3A-D).

Discussion

Although elevated levels of OPN expression have been observed in several types of human tumors, its expression in cat and dog tumors has not been reported in detail [2,8,12,14,18,23,26,35,36]. Only one report is available on OPN expression in canine mammary tumors it reported little increase of OPN level between adenomas and carcinomas by PCR [17]. In contrast, in this study, we observed a marked increased in OPN expression in different type of malign canine and feline tumors, including mammary tumors compared to benign tumors by immunohistochemical method. Marked OPN immunoreaction was seen in most cells in malignant tumor, while weak and a few immunopositive cells were seen in benign ones. To the best of our knowledge, this is the first study that comprehensively showed the localization of OPN expression in canine and feline tumors by immunohistochemical method.

OPN was identified as a tumor-associated protein in transformed cells in cultures [24, 25] and has been shown to be present in some human tumor samples [4]. In human breast cancer, OPN has been shown to contribute functionally to the malign behavior of cells [29]. This study showed that a similar relation can be seen in canine and feline mammary tumors. In this study, we observed a heterogenic distribution at the OPN expression in different areas of same tumor. Immunohistochemical localization of the OPN was parallel to the expression of Ki-67 and PCNA. Microscopically marked OPN expression was observed in tumor areas composed of highly proliferated cells. Possible causes of the Klopfleisch et al. [17] results may be related to this heterogenic distribution of OPN expression in the canine mammary tumors.
Numerous studies reported that OPN overexpression is also related with breast cancer evolution and metastasis in humans; therefore, there is a potential use for OPN in monitoring the disease status of patients with breast cancer [11]. Most of our study material was composed of mammary tumors and OPN was more strongly expressed in malignant mammary tumors than in benign ones. This result indicated that OPN may be used for a prognostic aim in canine and feline mammary tumors. But further studies are needed to explain the relation of OPN level and malignancy criteria in mammary tumors.

The use of cancer biomarkers to predict future patterns of disease has been an emerging issue, especially as cancer treatments have made such positive strides [3, 12-16, 18-20, 26, 35]. Breakthroughs in the development of reliable cancer biomarkers may be imminent due to advances in genomics and computer technology, which allows the analysis of vast quantities of data [9, 27, 34]. OPN has been studied in human and murine tumors and identified as a tumor-associated protein; this study showed that it can also be used for cat and dog tumors. Our study focuses on the role of OPN in cancer in dogs and cats, and its potential as a biomarker. OPN seems to be more than just a marker of malignancy because this protein may play a functional role in malign-gene expression and cancer cell behavior in cats and dogs. In addition, tumor diagnosis, anti-OPN treatment may be used for cancer in humans and animals.

Numerous in vitro and in vivo studies implicate OPN’s role in tumorigenesis, or more specifically, in tumor promotion [6, 12, 14, 18, 19, 22, 25, 26, 33, 35]. Authors reported that, the lack of induced OPN expression in OPN-null mice significantly suppresses benign squamous papilloma in vivo development, relative to wild-type mice, when subjected to the two-stage (initiation-promotion) mouse skin chemical carcinogenesis model [15]. OPN shows marked expression in squamous cell carcinomas, which have metastatic potential, but minimally expressed in solid basal cell carcinomas previously reported in humans [8]. Recently, marked suppression on papilloma development by lack of OPN expression in mice was reported [15]. Experimental evidence suggests that promoter-induced OPN expression plays a critical role in regulating the rate-limiting step of tumor promotion, possibly by providing the initiated cells a conducive environment in which to prolong their survival and, consequently, facilitate tumor development. In this study we also observed the marked expression of OPN in squamous cell carcinoma more than so basal cell carcinoma. However, only two tumors were studied for this study. More comprehensive studies are needed for better evaluation of this issue.

High levels of OPN in several cancers are indicative of a poor prognosis. Overall and disease-free survivals are inversely related to OPN levels in several cancers in humans [12, 14, 18, 19, 26,35]. There is strong correspondence between high OPN and lower mean survival time in tumors (82%) and plasma (100%) measurements, with large mean differences in survival times, indicating a useful role for OPN in patient stratification. Patient survival is largely determined by tumor aggressiveness. Hence, it is not unexpected that OPN, a prognostic measure for survival, can also be a marker for grade, stage, and early progression [14, 31]. Although this study addressed the relation of OPN and malignancy of canine and feline tumors, studies are needed to determine for example, the blood or tissue OPN level and malignancy or survival time in these species. We have demonstrated that OPN may be used by both tumor markers and progression markers in cats and dogs. The identification of OPN in tumor tissues can be used for diagnosis and clinical outcomes of tumors in these species. Furthermore, these observations have implications for the design of many experiments, on both cell lines and tissues of cats and dogs. Future research needs to assess whether the blood level of OPN, or possible combination with other markers, can further improve its diagnostic value in these species.

As a result, OPN was immunohistochemically identified as the leading candidate for tumor markers in cats and dogs tumors, from our initial results firstly. It is a secreted, integrin-binding protein that has already been reported as a marker of tumor progression in human tumors. The results presented here provide the first data to suggest that OPN may be a marker of cat and dog cancer progression.

Acknowledgement

This study was supported by the Projects Commission of University of Mehmet Akif Ersoy (Project number: 0116-NAP-10).

References

5. - CHAMBERS AF, WILSON SM, KERKVLIET N, O'MALLEY FP, HARRIS JF, CASSON AG: Osteopontin...

33. - WU Y, DENHARDT DT, RITTING ST: Osteopontin is required for full expression of the transformed phenotype by the ras oncogene. *British J Cancer.*, 2000, **83**, 156-163.

