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Expression of hypoxia-inducible factor-1 α and vascular density in mammary adenomas and adenocarcinomas in bitches

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Abstract

Background: The study aimed at examining hypoxia-inducible factor (HIF)1 α expression in adenocarcinomas and adenomas in bitches in regard to tumour malignancy grade, proliferation, apoptosis and vascularisation. Therefore, paraffin sections of 15 adenomas and 64 adenocarcinomas sampled from 79 dogs aged 6 to 16 years were analysed.

Results: A significantly higher HIF-1 α expression was noted in adenocarcinomas in comparison to adenomas ($P < 0.0004$). Moreover, HIF-1 α expression in adenocarcinomas correlated positively with tumour malignancy grade ($r = 0.59$, $P < 0.05$), Ki-67 antigen expression ($r = 0.43$; $P < 0.0005$), TUNEL-positive cells ($r = 0.62$, $P < 0.0001$) and tumour vascularity measured by quantification of vessels characterized by the expression of von Willebrand Factor ($r = 0.57$, $P < 0.05$).

Conclusion: Results of this study indicate a similar biological role of HIF-1 α in dogs and in humans, which may confirm suitability of the animal model in investigations on progression of tumours in humans.

Keywords: HIF-1, Adenocarcinoma, Adenoma, Mammary gland, Dog

Background

Neoplastic cells and cells of malignant tumours at their preliminary stage of development are supplied, in particular, with oxygen and metabolic products *via* diffusion. This secures conditions for their growth and attainment of a tumour diameter not around 2 mm. Subsequent growth of the tumour exceeding this size requires additional supply provided by blood vessels. Therefore, hypoxia develops within neoplastic tissue and tumour cells begin to manifest an increased demand for glucose and an accelerated glycolysis, in such conditions securing the principal source of ATP [1]. Glycolysis was shown to progress very efficiently in tumours growing in hypoxic conditions since they express hypoxia-inducible factor (HIF-1). This factor is responsible for an increased expression of several proteins, including glycolytic enzymes such as hexokinase-1 and -3, phosphofructokinase L, aldolase A and C, phosphoglycerate kinase-1, enolase-1, lactate dehydrogenase and the so called glucose transporters GLUT-1 and GLUT-3 [1]. Intensity of glucose uptake by tumour cells was found to

manifest positive correlation with their aggressiveness [1]. Moreover, HIF-1 stimulates tumour growth by activation of the *VEGF* gene transcription, which codes for vascular endothelial growth factor, the principal inducer of angiogenesis. In the absence of neovascularization tumour growth would be inhibited or even the tumour would show regression. Anti-neoplastic therapy takes advantage of this phenomenon by inhibiting angiogenesis in a tumour e.g. using monoclonal VEGF-specific antibodies [1,2]. It should be added that HIF-1 stimulates also transcription of *IGF2* gene, coding for insulin-like growth factor 2 (IGF2), which facilitates survival of tumour cells also in an environment with a diminished oxygen content [1,3].

HIF-1 is a heterodimer, consisting of HIF-1 α and HIF-1 β subunits. The HIF-1 β subunit undergoes a constitutive expression while the expression of HIF-1 α is low in most cells in normoxia conditions. Inhibition of HIF-1 α expression results from activity of oxygen-dependent hydroxylases which enzymatically modify HIF-1 α chain enabling its binding with von Hippel-Lindau tumour suppressor protein (VHL) [4]. In turn, VHL acts as a recognition factor for ubiquitin-protein ligase E3, which directs HIF-1 α to degradation in proteasomes [5,6]. In normoxia conditions, half-life of HIF-1 α protein is very short but it

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becomes markedly elongated in hypoxia [7]. Stimulation of HIF-1 α synthesis utilizes the signalling pathway leading to a tyrosine kinase receptor, such as HER2 (Human Epidermal Growth Factor Receptor 2), with mediation of phosphatidylinositol-3-kinase (PI3K), serine/threonine kinases (AKT) and mammalian target for rapamycin (mTOR) [8]. The signalling pathway is inhibited by PTEN protein (phosphatase and tensin homologue deleted on chromosome ten), which dephosphorylates the product of PI3K reaction [1]. Therefore, HIF-1 may be regarded as a factor, which allows the cells to adapt to low tissue levels of oxygen.

Our study aimed at demonstration of HIF-1 α protein expression and determination of its intensity in the most frequently manifested malignant and benign mammary tumours of epithelial origin (adenocarcinomas and adenomas) in bitches. Moreover, an attempt was made to correlate the obtained results with expression levels of the Ki-67 proliferation antigen and with blood vessel density of the tumours.

Methods

The research we performed was approved and financed by the National Science Center of Poland. As this research was performed on archival material routinely collected during surgical-treatment procedures and no additional harm was done to the animals due to the experiments, we did not require an additional ethics approval for our research. All the experiments were performed on disposable material which were not utilized for future scientific experiments. Only paraffin-embedded tissues were used for the study.

Tissue material and immunohistochemistry (IHC)

Material for the study was sampled during surgery in 79 female dogs of various breeds, aged 6 to 16 years. The tumours were verified by histopathological examination of the HE sections and represented adenomas (15 cases) and adenocarcinomas (64 cases).

Formalin-fixed, paraffin-embedded tissue was freshly cut (4 μ m). The sections were mounted on Superfrost Plus slides (Menzel Gläser, Braunschweig Germany) and subsequently deparaffinised by boiling in Antigen Retrieval Solution (High pH = 9 for HIF-1 α , Low pH = 6 for Ki-67; DakoCytomation, Glostrup, Denmark) using PT Link Rinse Station (DakoCytomation). Then, the sections were incubated (20 min; room temperature, RT) in Link48 automated staining platform (DakoCytomation) utilizing murine primary monoclonal antibodies diluted in the Background Reducing Antibody Diluent (DakoCytomation) and directed against HIF-1 α (1:600; Novus Biologicals, Littleton, USA), von Willbrand Factor (vWF; 1:800; DakoCytomation) or Ki-67 (ready-to-use, DakoCytomation). The visualization of the studied

antigens was performed using EnVision FLEX (DakoCytomation), according to the manufacturer's instructions. All the sections were counterstained with Meyer's hematoxylin. In all the cases, controls were included, in which specific antibody was substituted by the Primary Negative Control (DakoCytomation).

Apoptosis detection was performed utilizing the ApopTag[®] Peroxidase In Situ Apoptosis Detection Kit (Millipore, Billerica, USA). Paraffin sections were dewaxed in xylene, rehydrated in alcohol and rinsed in distilled water and 1xPBS, pH 7.4. Then, the sections were incubated in Proteinase K (DakoCytomation) for 5 min in RT and rinsed in 1xPBS. Endogenous peroxidase was blocked by 5 min incubation in 3% H₂O₂/1xPBS. Subsequently, the sections were incubated with Equilibration Buffer for 10 min in RT, with subsequent incubation with TdT Enzyme and Reaction Buffer at 37°C for 1 h. The reaction was stopped after 10 min incubation in the Stop Buffer and rinsed in 1xPBS. Then, anti-dioxygenin peroxidase-conjugated antibodies were applied for 30 min at RT. Following that, the sections were incubated for 10 min with diaminobenzidine (DAB; DakoCytomation) to visualize the TUNEL-positive cell nuclei. Finally, the sections were counterstained with Mayer's hematoxylin and, after dehydration in alcohols, mounted in SUB-X Mounting Medium (both DakoCytomation).

Quantification of IHC reactions

Microphotographs of all the studied tumours were subjected to computer-assisted image analysis *via* a computer coupled to an Olympus BX53 optical microscope (Olympus, Japan). The set had the potential to record images and to perform their digital analysis. The measurements took advantage of Cell^A software (Olympus Soft Imaging Solution GmbH, Germany).

Microscope examination allowed determination of the malignancy grade of the adenocarcinomas. The grade was established using the scale of Bloom-Richardson in modification of Elston and Ellis [9]. The evaluation method of the malignancy grade included three parameters scored in the scale from 0 to 3 points: formation of tubules (evident, moderate, slight), polymorphism of cell nuclei (slight, moderate, marked), number of mitotic figures per 10 microscope fields at the magnification of \times 400 (0–7, 8–16, \geq 17). The sum of the points provided potential to distinguish three malignancy grades (G) among the tumours: 0–5 pts. – G1, 6–7 pts. – G2, 8–9 pts. – G3.

Expression of HIF-1 α was appraised using the modified semi-quantitative immunoreactive score (IRS) scale according to Remmele (Table 1) [10]. The method takes into account both proportion of positively stained cells and intensity of the colour reaction, while the final score is the product of the parameters, with values ranging from 0 to 12 points (no reaction = 0 points (-); weak reaction = 1–2

Table 1 Semi-quantitative immunoreactive score (IRS) taking into account both the percentage of stained cells (A) and the intensity of reaction product (B) in which the final results correspond to the product of the two variables (AxB)

Point score	A	B
0	No cells with positive reaction	No colour reaction
1	≤ 10% Cells with positive reaction	Low intensity of colour reaction
2	11-50% Cells with positive reaction	Average intensity of colour reaction
3	51-80% Cells with positive reaction	Intense colour reaction
4	> 80% Cells with positive reaction	

points (+), moderate reaction = 3–4 points (++) , intense reaction = 6–12 points (+++).

Microvessel density (MVD) of vWF-positive vessels was quantified under × 200 magnification in five intratumoural areas of the lesion and the final score was determined as a mean of the five quantified areas.

The Ki-67 antigen expression and TUNEL stained sections were scored under × 400 magnification in five areas, in which number of positive cells presenting brown reaction colour were counted. The final score represented the percentage of positive tumour cells to all tumour cells in the examined sections.

Statistical analysis

The results were subjected to statistical analysis using Prism 5.0 (GraphPad, La Jolla, USA) software, employing Mann–Whitney test and Spearman's correlation analysis.

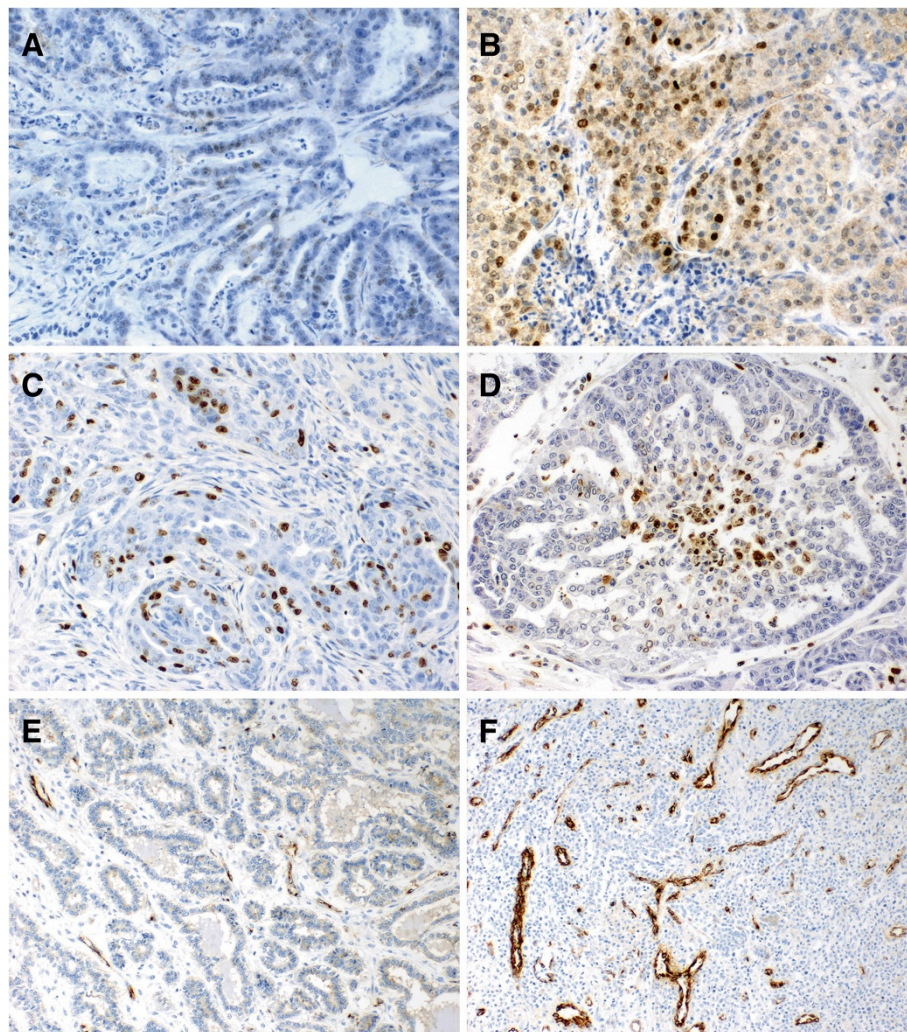
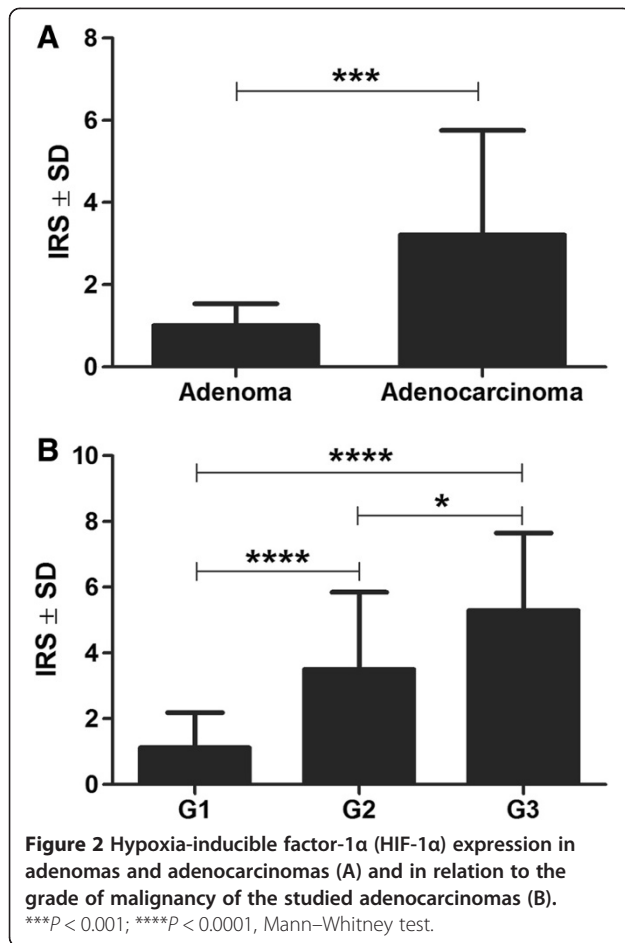


Figure 1 Immunohistochemical expression of HIF-1α in adenoma (A) and adenocarcinoma (B). High expression intensity of Ki-67 antigen (C) and TUNEL in cancer cells (D). Von Willbrand Factor (vWF) expression in endothelial cells noted in adenoma (E) and adenocarcinoma (F). Magnification × 200 (A-D); ×40 (E-F).



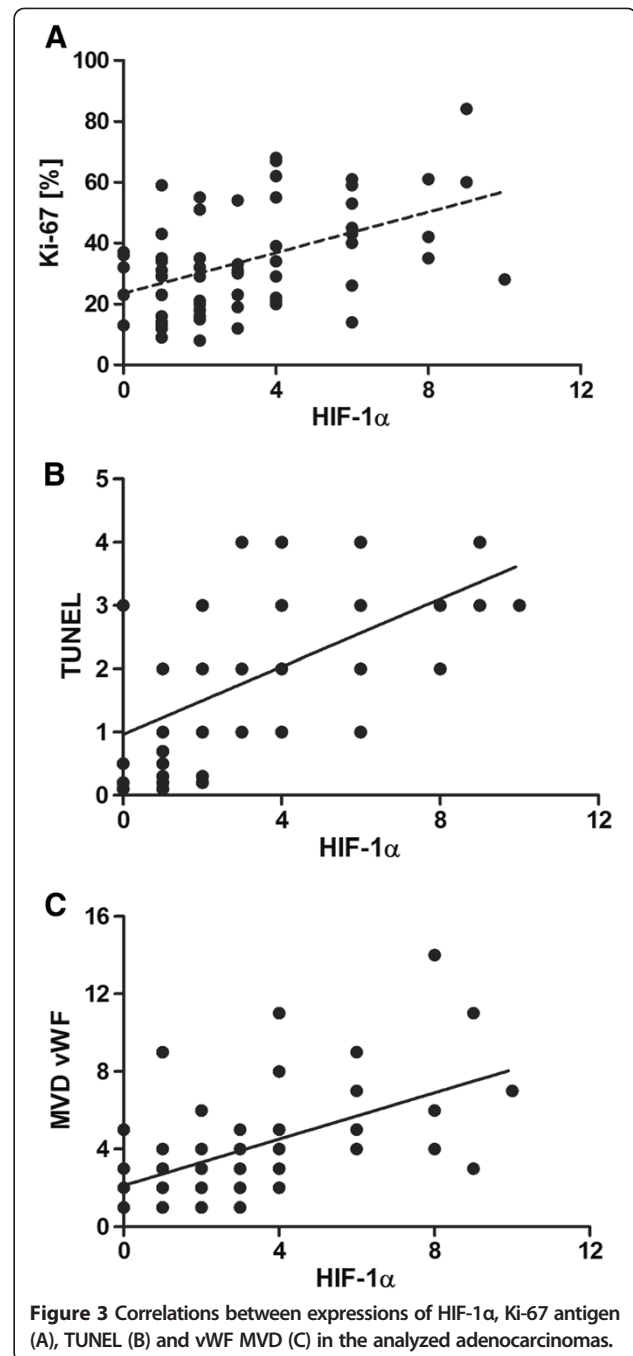
In all the analyses, results were considered statistically significant when $P < 0.05$.

Results

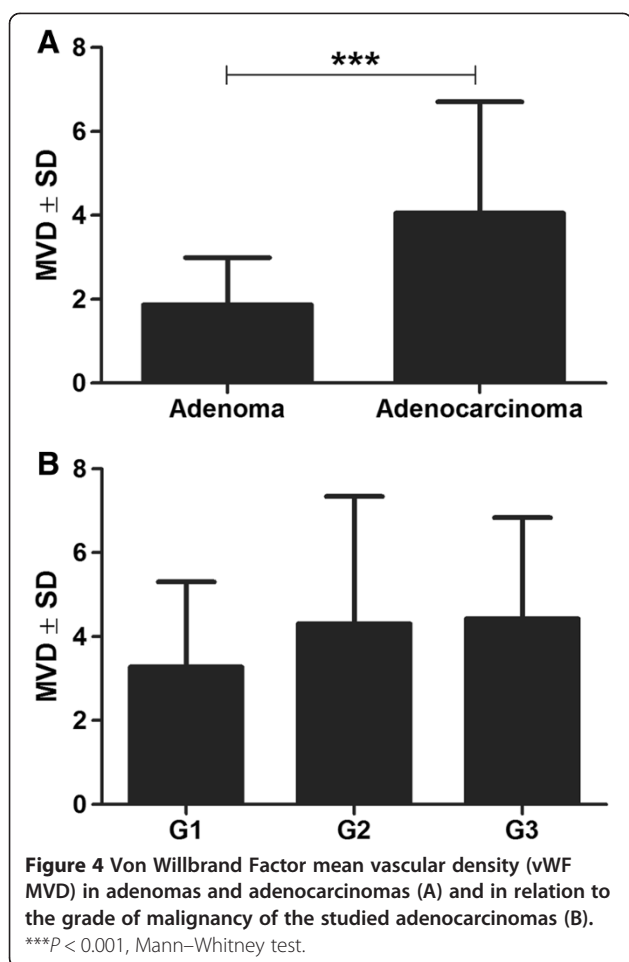
Expression of HIF-1 α protein was demonstrated both in mammary adenomas and adenocarcinomas in bitches (Figures 1A, 1B). In all the cases with positive reaction (90% adenocarcinomas and in 86% adenomas) a nuclear-cytoplasmic HIF-1 α expression was noted. Moreover, in the two groups of tumours evident differences were seen in intensity of the protein expression. In adenocarcinomas, over 39% tumours manifested HIF-1 α expression evaluated at +, over 26% expression evaluated at ++ and 25% at +++.

In adenomas, 86% of the examined tumours manifested expression of the protein which, however, did not exceed the +intensity. It should be noted that over 9% of adenocarcinomas and over 13% of adenomas manifested no HIF-1 α expression. Statistical analysis using Mann-Whitney test demonstrated a significantly higher HIF-1 α expression in adenocarcinomas than in adenomas ($P = 0.0004$) (Figure 2A).

The relationship between distribution of HIF-1 α expression intensity and malignancy grade was also of interest. In



G1 adenocarcinomas almost 28% of the tumours manifested no HIF-1 α expression, 61% of them manifested the expression at + level and 11% at ++ level. In cases of G2 adenocarcinomas, 3% of the tumours manifested no HIF-1 α expression, over 37% showed + expression, over 37% of them demonstrated ++ expression and almost 22% +++ expression. G3 adenocarcinomas exhibited HIF-1 α expression at + level in over 14% of cases, at ++ level in over 28% of cases and at +++ level in over 57% of cases. It should be added that only in the group of G3



adenocarcinomas over 50% of the tumours manifested high expression of the protein (+++) while no such strong HIF-1 α expression could be noted in G1 or G2 tumours.

Spearman's correlation test demonstrated a pronounced positive correlation between HIF-1 α expression and tumour malignancy grade ($r = 0.59$; $P < 0.05$). Mann-Whitney test revealed significant differences in HIF-1 α expression between particular malignancy grades of adenocarcinomas (G1 vs. G2 - $P < 0.0001$; G1 vs. G3 - $P < 0.0001$; G2 vs. G3 - $P = 0.02$) (Figure 2B).

Tumour cell proliferation was determined by assessing the expression of the Ki-67 antigen (Figure 1C). Similarly, positive correlations were disclosed between tumour's malignancy grade and expression of Ki-67 proliferation antigen ($r = 0.61$; $P < 0.0001$), and between expressions of HIF-1 α and Ki-67 ($r = 0.43$; $P < 0.0005$; Figure 3A).

Studies on intensity of apoptosis using the TUNEL approach (Figure 1D) demonstrated that level of apoptosis in canine mammary adenocarcinomas manifested positive correlation with expression of HIF-1 α protein and the correlation demonstrated a high level ($r = 0.62$; $P < 0.0001$; Figure 3B).

An extremely important and interesting aspect of the study was the examination of a correlation between HIF-1 α protein expression and vWF MVD (Figures 1E, 1F). Both in adenomas and in adenocarcinomas the correlation proved to be pronounced and positive. Its level was slightly higher in malignant tumours (adenocarcinomas: $r = 0.57$; $P < 0.05$; Figure 3C) than in benign tumours (adenomas $r = 0.52$; $P < 0.05$). Mann-Whitney test demonstrated significant differences in vWF MVD between individual malignancy grades (G1 vs. G2 - $P = 0.251$; G1 vs. G3 - $P = 0.098$; G2 vs. G3 - $P = 0.586$) - Figure 4.

Discussion

Immunohistochemical studies on human tumours demonstrated pronounced expression of HIF-1 α in many common tumours, which probably represents a consequence of both hypoxia within the tumour and various genetic disturbances in neoplastic cells [11]. Moreover, HIF-1 α expression was shown to manifest positive correlation with expression of VEGF and with density of microvessels in most of tumours of central nervous system [12], ovarian cancer [13], ductal mammary carcinoma [14], colon adenocarcinoma [15], endometrial adenocarcinoma [16], ductal pancreatic adenocarcinoma [17], small-cell pulmonary carcinoma [18]. The relationship seems to be of significance since not only elevated HIF-1 α expression is linked to increased VEGF levels, but absence of HIF-1 α expression in several cases results in a decreased VEGF level and, thus, an inhibited process of neoangiogenesis. The latter observation has been corroborated in studies on embryonal stem cells of mice devoid of HIF-1 α expression, in which VEGF mRNA level was markedly decreased and could not be induced by hypoxia. Similarly in our study we have demonstrated a pronounced positive correlation between expression of HIF-1 α and density of microvessels both in a malignant (adenocarcinoma) and benign (adenoma) tumours, even if in the latter case the correlation has shown a slightly lower level. However, it should be added that some reports documented absence of a significant correlation between expressions of mRNA for HIF-1 α and VEGF in hypophyseal adenomas [19]. Their authors suggested that in such tumours VEGF expression may exhibit no pronounced dependence on expression of HIF-1 α .

A very important element of HIF-1 α biological activity is the induction of protein synthesis involved, i.e., in development of metastases, including vimentin, fibronectin, metalloproteinase 2, cathepsin D, urokinase-type plasminogen activator receptor (uPAR). Moreover, HIF-1 α protein can induce a decrease in E-cadherin expression, the protein important for cell adhesion [20]. In studies on *in vitro* invasiveness of human pulmonary adenocarcinoma cells, Shyu et al. [21] demonstrated that in conditions of normoxia, HIF-1 α manifested expression in cells presenting high

invasiveness (CL1-5) while no expression of the factor was noted in cells of a low invasiveness (CL1). In parallel, the authors found that an increased HIF-1 α expression in cells of human pulmonary carcinoma was linked to their increased invasiveness, probably due to an increased expression of uPAR receptor and metalloproteinase 1 and 2 (MMP1 and MMP2). In our study we have analogously found that HIF-1 α expression has manifested positive correlation with certain prognostically unfavourable traits of the tumours, i.e., with malignancy grade and proliferative potential, reflected in expression of the Ki-67 antigen.

Shin et al. [18] found that levels of HIF-1 α and VEGF mRNA expression were higher in tissues of an early or advanced colon carcinoma than in colon adenoma. They suggested that MMP-2, HIF-1 α and VEGF might represent useful parameters for detection of early carcinogenesis and progression of colon carcinoma. In turn, Osada et al. [13] showed that HIF-1 α expression in cell nucleus and HIF-2 α in cytoplasm of neoplastic cells was linked to an unfavourable prognosis in women with ovarian carcinoma. The authors found also that HIF-1 α expression in cell nucleus represented an independent prognostic factor in women with such a cancer. Similar results were obtained by Daponte et al. [22], who noted that survival of females with serous ovarian carcinoma, the cells of which demonstrated strong HIF-1 α expression, was significantly abbreviated as compared to patients in whom neoplastic cells showed low or no HIF-1 α expression. A pronounced expression of HIF-1 α is linked to an increased risk of death at early stages of various types of malignant tumours, including carcinomas of uterine cervix, esophageal, mammary carcinoma and cerebral oligodendroglioma [12,23-25]. Moreover, reports are available which show that, augmented expression of HIF-1 α correlated with a increased apoptosis of neoplastic cells [25,26]. Similarly, in our investigations on canine mammary adenocarcinomas we have found that an increased HIF-1 α expression was paralleled by higher levels of apoptosis among neoplastic cells. This may indicate that certain tumour cell subpopulations are more sensitive to an increasingly intense hypoxia. Moreover, it has been demonstrated that experimentally induced overexpression of HIF-1 α in cells of non-small-cell pulmonary carcinoma (line A549) inhibits tumour development, i.e., through a more pronounced apoptosis [3].

Conclusions

High expression of HIF-1 α in neoplastic cells, expressing their adjustment to hypoxia conditions in the tumour, in the course of why some tumours may provide a useful marker of canine tumour aggressiveness based on observed positive correlations with the tumour malignancy grade and the Ki-67 antigen expression. The HIF-1 α expression in mammary adenomas and adenocarcinomas

of bitches may indicate that the biological role of the protein is similar in tumours of canines and humans. This may confirm suitability of using the animal model in studies on progression of tumours in the man.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MN and JAM initiated and planned the study, MN, JPM and PD completed tissue processing, staining and performed the IHC, JPM and BP performed the statistical analysis, compiled the results and drafted the manuscript. All authors were significantly involved in designing the study, interpreting of data and composing the manuscript. All authors read and approved the final manuscript.

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