QUANTITATIVE AND QUALITATIVE ANALYSIS OF AgNOR IN BENIGN AND MALIGNANT CANINE MAMMARY GLAND TUMORS

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In this retrospective study, quantitative and qualitative analyses of argyrophil nucleolar organizer regions (AgNORs) in 54 malignant and 18 benign canine mammary gland tumors were made. Statistical analysis showed a significant difference in the mean number of AgNORs per cell between benign and malignant tumors (p<0.01). There was no significant difference in the mean number of AgNORs per cell between complex carcinomas, simple carcinomas and carcinomas in mixed tumors (p>0.05), as well as between adenomas and benign mixed tumors (p>0.05). Six different patterns of AgNOR distribution were observed. Types I, II, III and in only two cases, type IV, were observed in benign tumors, while all six types were observed in malignant tumors. The predominant types in malignant tumors were III, IV, V and VI. Cell types II, III and IV were predominant in malignant tumors with 5.5-7 AgNORs per cell, while cell types V and VI were dominant in tumors with more than 7 AgNORs per cell.

Key words: dog, mammary gland, tumors, AgNOR

INTRODUCTION

During the last several years canine mammary gland tumors have raised great interest both in human and veterinary pathology. Due to their short life span, the relatively rapid progression of the disease and a large number of potential foci (ten mammary glands), dogs are becoming an ideal animal model for studying breast tumors in women (Hellmen, 1996; Magaš, 2002).

Thus, the frequency of canine mammary gland tumors immediately follows skin tumors, being at the same time the most common neoplasm in bitches. According to the reports of the majority of authors, these tumors comprise about 25-50% of all canine tumors with an approximately equal malignant and benign neoplasm incidence (Cotchin, 1958; Bostock, 1986; Moulton, 1990; Benjamin *et al*., 1999). Carcinomas comprise about 80-90% of all malignant mammary gland tumors (Fidler and Brodey, 1967; Moulton *et al*., 1970; Moulton *et al*., 1986), sarcomas 5-10%, while carcinosarcomas are rare malignant mammary gland tumors (Misdorp *et al*., 1971; Benjamin *et al*., 1999). Regarding the frequency of different benign mammary gland tumors, adenoma is the most common benign neoplasm

354   **Acta Veterinaria (Beograd), Vol. 53. No. 5-6, 353-360, 2003.**
                   Jelesijević T *et al*. Quantitative and qualitative analysis of AgNOR in benign and
                                         malignant canine mammary gland tumors

comprising more than 60% of all benign neoplasms, followed by benign mixed tumors (30%) and duct papillomas (10%) (Benjamin *et al*., 1999).

The exceptionally diverse histological appearance and the presence of a continuum between benign and malignant tumors, as well as ambiguities related to tumor cell origin and the roles of different growth factors and hormones represent major problems for correct interpretation of canine mammary gland tumors. Due to these extremely complex problems none of the existing histological classifications can entirely fulfil the needs of pathologists, clinicians and pet owners (Jelesijević, 2001). Therefore, great efforts are being made to identify a standard histological classification of mammary gland tumors as well as reliable diagnostic and prognostic methods (Hampe and Misdorp, 1974; Bostock *et al*., 1992; Hellmén, 1996; Benjamin *et al*., 1999; Misdorp *et al*., 1999)

Staining of nucleolar organizer regions (NORs) with silver colloid is indicated in the literature as a highly reliable histochemical prognostic method (Giri *et al*., 1989; Kiupel *et al*. 1998). NORs represent chromosomal parts rich in rDNA that participate in the formation of a nucleolus (Goessens, 1984). Actively transcribing NORs are argyrophil (AgNORs) and can be visualised by applying silver colloid. Eight autosomes Nos. 5, 8, 14, 16, 19, 21, 32, 37 and the Y chromosome showed active NORs in dogs (Howard-Peebles and Howell, 1983; Shibasaki *et al*., 1990).

Numerous studies on different neoplasms in animals and humans indicated that a lower average AgNOR count and higher diameter of AgNORs are associated with a more favourable prognosis and longer survival time (Crocker and Nar, 1987; Crocker and Egan, 1988; Bostock *et al*., 1989; Bostock *et al*., 1992; Kravis *et al*., 1996; Chu *et al*., 2001). Investigation of canine T and B malignant lymphomas showed that the dogs with tumors with a lower average AgNOR count, a higher maximal as well as average AgNOR area, a lower AgNOR – nucleus ratio and a shorter average distance between two AgNORs had a more favourable prognosis (Kiupel *et al*., 1998; Kiupel *et al*., 1999).

Several proposals have been made in order to standardize the AgNOR counting technique (Crocker *et al*., 1989; Rüschoff *et al*., 1994; Aubele *et al*., 1994). Although, the technique has been standardized, this still represents a weak point of the method. Unfortunately, not only the counting technique but also "external" factors such as; fixation time (Biesterfeld *et al*., 1994), thickness of the sections, temperature, pre-treatment procedures and staining time, have a great influence on AgNOR count (Kiupel *et al*., 1999). Therefore, the prognostic value of the distribution of AgNORs is still being investigated. This parameter is less susceptible to subjective estimation than an individual count of AgNORs, which should help in the comparison of results between different studies (Kiupel *et al*., 1998). Recently, an image analysis technique for interpretation of AgNORs has been favoured by some authors (Kiupel *et al*., 1998; Juntes and Pogačnik 2000), whereas others consider direct counting more accurate (Bostock *et al*., 1992; Bratulić *et al*., 1996). As every histopathological section has a specific thickness, more reliable data concerning AgNOR counts can be obtained by focusing on each AgNOR that is not in the same plane as the other AgNORs within the same nucleolus (Bratulić *et al*., 1996). Such accurate counting of each AgNOR is not possible to achieve by image analysis software. Also, due to its price, image analysis software

Acta Veterinaria (Beograd), Vol. 53. No. 5-6, 353-360, 2003.   355
Jelesijević T *et al*. Quantitative and qualitative analysis of AgNOR in benign and
malignant canine mammary gland tumors

is not affordable for every institution, which probably represents the main disadvantage.

MATERIAL AND METHODS

Seventy-four tissue samples of canine mammary gland tumors were examined in this study. The tumors were classified according to the WHO classification (Misdorp *et al*., 1999). The samples were fixed in 10% neutral buffered formalin and embedded in paraffin. Following the usual rehydration procedure, silver colloid staining was performed on 3 μm thick sections. The working solution was composed of two volumes of 33% $AgNO_{3 (aq)}$ and one volume of 2% gelatine in 1% formic acid, was added and the sections kept in a dark chamber at 25 ^{o}C for 40 minutes. The silver surplus was removed by rinsing in 2% $Na_2S_2O_{3(aq)}$. Contrast staining was performed using nuclear fast red, neutral red and hematoxylin. AgNORs were counted under oil immersion, and each clearly demarcated AgNOR was considered as a unit structure.

Statistical analysis included Student's t-test and analysis of variance. The obtained data were described using the following statistical parameters: arithmetic mean (X), standard deviation (SD), standard error (Sy) and interval of variation (Iv).

RESULTS

After analysis of tumors stained with silver colloid the average number of AgNORs was calculated per cell. Malignant tumors had an average of 7.18 AgNORs while benign tumors had 3.53 AgNORs/cell (Table 1).

Table 1. AgNOR count, for malignant and benign mammary gland tumors

Type of tumor	Number of samples	X	SD	Sy	Iv
Malignant tumors	54	7.18	1.12	0.30	5.75-9.88
Benign tumors	18	3.52	0.77	0.18	1.91-5.05

Student's t-test showed a statistically significant difference in the average number of AgNORs/cell between malignant and benign canine mammary gland tumors ($p < 0.01$).

In cases of adenomas (Figure 1) and benign mixed tumors, an average of 3.37 and 3.81 AgNORs/cell were counted, respectively. Duct papillomas had an average number of 3.41 AgNORs/cell, but, due to the relatively low number of this type of benign neoplasm, they were not included in the statistical analysis inside the group of benign neoplasms (Table 2).
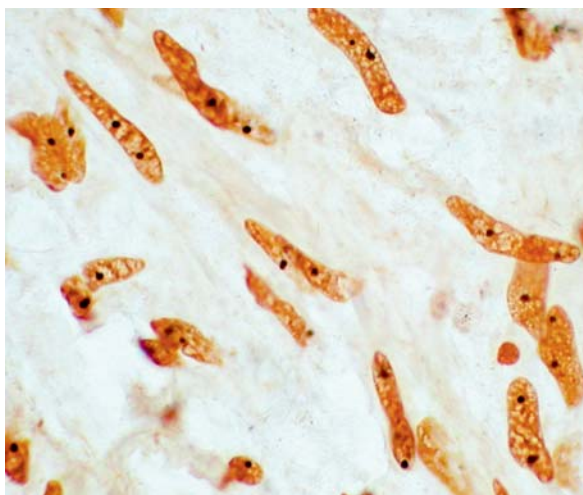
Figure1. Adenoma, myoepithelial type (x1000, silver colloid)

Table 2. AgNOR count within the group of benign mammary gland tumors

Histological type of benign neoplasm	Number of samples	X	SD	Sy	Iv
Adenoma	9	3,37	0,94	0,31	1,91-5,05
Benign mixed tumor	6	3,81	0,61	0,25	2,86-4,71
Duct papilloma	3	3,41	-	-	-

Student's t-test did not show a statistically significant difference in average number of AgNORs/cell between adenomas and benign mixed tumors ($p > 0.05$).

Average numbers of AgNORs/cell within the group of malignant tumors were: 7.29 for simple carcinomas (Figure 2), 7.12 for complex carcinomas and 7.04 for carcinoma in mixed tumors (Table 3).

Table 3. AgNOR count within the group of malignant mammary gland tumors

Histological type of malignant neoplasm	Number of samples	X	SD	Sy	Iv
Simple carcinoma	23	7.29	1.11	0.22	5.80-9.82
Complex carcinoma	20	7.12	0.98	0.42	5.85-9.32
Carcinoma in mixed tumor	11	7.04	1.38	0.23	5.75-9.88

Analysis of variance did not show a statistically significant difference in average number of AgNORs/cell between any of the three types of malignant tumors ($p > 0.05$).

Acta Veterinaria (Beograd), Vol. 53. No. 5-6, 353-360, 2003.
Jelesijević T *et al*. Quantitative and qualitative analysis of AgNOR in benign and malignant canine mammary gland tumors
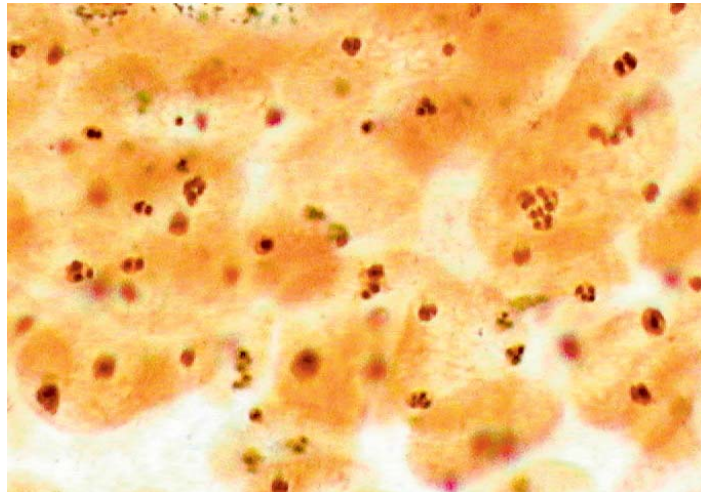
357

Figure 2. Simple carcinoma (x1000, silver colloid)

Based on the type of AgNOR pattern distribution, all the cells in the studied material were classified into six types. A certain number of cells showed the presence of two or even three different AgNOR patterns at the same time. Type I is characterized by the presence of one either centrally or peripherally located relatively large nucleolus with a large black coloured AgNOR in it (Figure3/I). In type II cells, 3-6 moderately sized round-shaped AgNOR structures were observed (Figure 3/II). In type III cells, the nucleoli usually contained 3-7 dot like AgNORs, while
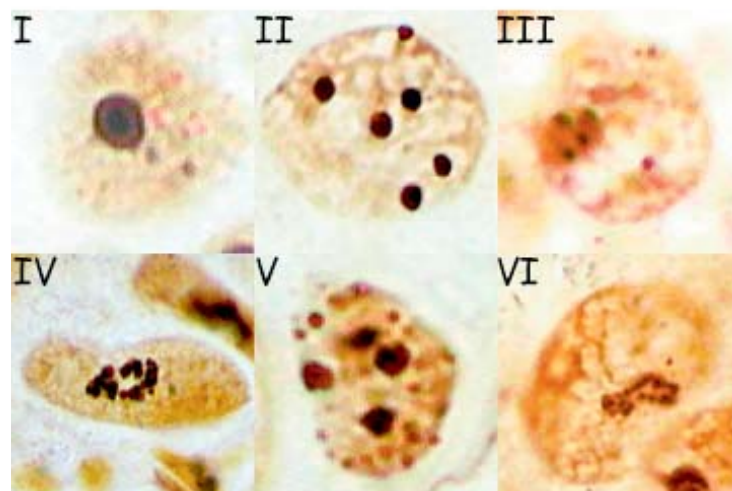

Figure 3. AgNOR pattern distribution

358 **Acta Veterinaria (Beograd), Vol. 53. No. 5-6, 353-360, 2003.**
Jelesijević T *et al*. Quantitative and qualitative analysis of AgNOR in benign and
malignant canine mammary gland tumors

in the rest of the nucleoplasm AgNORs like those in cells of type II and V could be observed (Figure 3/III). Type IV cells were characterized by the presence of an irregularly shaped nucleolus, containing several (3-6) aggregated/clustered AgNORs, while in the rest of the nucleoplasm AgNORs like those in cells of type II and V were frequently observed (Figure 3/IV). Type V cells were distinguished by 5-20 diffusely distributed dot like AgNORs (Figure 3/V). Type VI cells were characterized by a relatively large, elongated, sickle- or irregularly shaped nucleolus containing up to 20 dot like non-clustered/aggregated AgNORs (Figure 3/VI).

Cell types I, II, III and in only two cases cell type IV were observed in benign tumors. Although cell types I and II were also observed in malignant tumors, cell types III, IV, V and VI were the predominant cell population. Cells types II, III and IV were predominant in malignant tumors with 5.5-7 AgNORs per cell, while cell types V and VI were dominant in tumors with more than 7 AgNORs per cell.

DISCUSSION

Statistical studies suggest that canine malignant mammary tumors can be classified in order of increasing malignancy. Non-infiltrating carcinomas are at the very beginning, followed by complex carcinomas and simple carcinomas. Simple carcinomas have an increasing malignancy scale of their own – tubulopapillary carcinomas, solid carcinomas and anaplastic carcinomas, but the most malignant tumors of the canine mammary gland are sarcomas (Misdorp *et al*., 1999; Benjamin *et al*., 1999). As the average number of AgNORs/cell indicates the malignancy grade it could be expected that simple carcinomas have a higher AgNOR count than complex carcinomas or carcinomas in mixed tumors. Average AgNOR counts among carcinomas in this study were higher in simple carcinomas than in complex carcinomas they had a higher average AgNOR count per cell than did carcinomas in mixed tumors. However, the differences were not shown to be statistically significant. These results are similar to other studies regarding average AgNOR count and canine mammary gland tumors (Bostock *et al*., 1992; Harari *et al*., 1995; Bratulić *et al*., 1996; Juntes and Pogačnik, 2000). No significant difference between adenomas and benign mixed tumors were observed on comparing the same parameter by Student's t-test.

In one study on canine malignant lymphomas a significant correlation between AgNOR distribution and prognosis was observed (Kiupel *et al*., 1998). The AgNOR pattern distribution described in the mentioned study was considered in our work on AgNOR pattern distribution in the examined canine mammary gland tumors. Based on subjective estimation an inverse correlation between AgNOR count and size of AgNORs was noted.

According to the results of our study, it can be concluded that the presence of cell types V and VI is strongly suggestive of a malignant tumor of the canine mammary gland.

This study showed that besides the average AgNOR count, AgNOR pattern distribution could be a reliable parameter of malignancy. As the latter parameter is less susceptible to subjective estimation than the average AgNOR count, it is possible to compare results between different studies. Further investigations are nec-

Acta Veterinaria (Beograd), Vol. 53. No. 5-6, 353-360, 2003.
Jelesijević T *et al*. Quantitative and qualitative analysis of AgNOR in benign and
malignant canine mammary gland tumors

359

essary to establish a possible correlation between AgNOR pattern and survival
time of bitches with mammary gland tumors.

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360

Acta Veterinaria (Beograd), Vol. 53. No. 5-6, 353-360, 2003.
Jelesijević T *et al*. Quantitative and qualitative analysis of AgNOR in benign and
malignant canine mammary gland tumors

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KVANTITATIVNA I KVALITATIVNA ANALIZA AgNOR KOD BENIGNIH I MALIGNIH TUMORA MLEČNE ŽLEZDE KUJA

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SADRŽAJ

U ovoj retrospektivnoj studiji izvršena je kvalitativna i kvantitativna analiza arigrofilnih organizatora nukleolusa (AgNORs), 54 maligna i 18 benignih tumora mlečne žlezde pasa. Uočene su statistički značajne razlike u prosečnom broju AgNORs po ćeliji između benignih i malignih tumora (p<0.01). Nisu dokazane statistički značajne razlike u prosečnom broju AgNORs po ćeliji između kompleksnih karcinoma, prostih karcinoma i karcinoma u mešovitom tumoru (p>0.05). Zapaženo je šest različitih tipova distribucije AgNORs. Tipovi I, II, III i samo u dva slučaja tip IV su uočeni kod benignih tumora, dok su kod malignih tumora uočeno svih šest tipova. Dominantni tipovi kod malignih tumora su III, IV, V and VI. Ćelije tipa II, III i IV dominiraju kod tumora kji imaju prosečno 5.5-7 AgNORs po ćeliji, dok tipovi V i VI su dominantni kod tumora koji imaju prosečno više od 7 AgNORs po ćeliji.