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Canine Neuroendocrine Tumors of the Pancreas: A Study Using Image Analysis Techniques for the Discrimination of Metastatic Versus Nonmetastatic Tumors

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Abstract. Canine pancreatic neuroendocrine tumors were studied using different image analysis techniques (nuclear image histometry, analysis of argyrophilic proteins of nucleolar organizer regions, determination of the mouse anti-Ki 67 antigen proliferation index, and DNA densitometry) to correlate their biological behavior with objective phenotypic markers. The methods were compared to determine the best method for distinguishing between metastatic and nonmetastatic tumors. Discrimination between the two types of tumor was possible using nuclear image histometry in combination with morphometric analysis of argyrophilic proteins of nucleolar organizer regions. In contrast, the mouse anti-Ki 67 antigen proliferation index, DNA measurement, and immunohistochemical parameters revealed no significant difference between the two types of tumors.

Key words: AgNOR analysis; biologic behavior; DNA parameters; dogs; islet cell tumor; morphometry; neuroendocrine tumor of the pancreas; nuclear image histometry; prognosis; proliferation index; tumor behavior.

Tumors of the pancreatic islet cells should be called neuroendocrine tumors of the pancreas (NETPs) on the basis of a common phenotype characterized by expression of neuroendocrine marker proteins.[8] These tumors have been described in human beings and various other species. They frequently occur in older dogs and rats and less often in other animals.[20] Pancreatic islet hormones, including insulin, glucagon, pancreatic polypeptide, and somatostatin, as well as hormones not found in endocrine cells in the adult mammalian pancreas, such as gastrin or calcitonin, can be demonstrated in different combinations in these tumors.[49] Clinical symptoms depend on the hormones secreted. NETPs are often associated with hyperinsulinism and consequential hypoglycemia, causing predominantly neurological symptoms.[9,12,33] In general, the biological behavior of NETPs is difficult to predict on the basis of their histological features. Accepted histological criteria for malignancy, such as nuclear pleomorphism or infiltration of adjacent tissue, are unreliable in the evaluation of NETPs in human beings[32] as well as in dogs.[30] Only rarely (e.g., with poor differentiation of lymph node metastasis) can these tumors be classified as malignant.[32] Several studies have been undertaken to discriminate tumors with the potential to metastasize from nonmetastatic tumors. In human beings, metastatic tumors have been described to have significantly larger diameters than localized tumors, but there are exceptions.[21,31] Tumor cell nuclear cytoplasmic ratio and nuclear density (number of nuclei per square millimeter) have also been identified as discriminators between metastatic and nonmetastatic tumors.[31] DNA measurement is of little value in predicting the biological behavior of these tumors in human beings.[1,11,14,15,47]

In this study, metastatic and nonmetastatic tumors in dogs were compared by different image analysis techniques to find objective phenotypic markers of malignancy.

Material and Methods

Patients and material

Eighteen cases of canine neuroendocrine tumor of the pancreas from archived material (1981–1993) of the Institute of Veterinary Pathology were studied. The average age of the animals was 10 years at the time of diagnosis. Samples were from five female and 13 male dogs. After euthanasia, complete necropsies were performed on all 18 dogs, and tissue samples were obtained from the pancreatic tumor, regional and mesenteric lymph nodes, liver, spleen, lung, stomach, small intestine, and kidneys. Light microscopy revealed metastases in lymph nodes, liver, spleen, or lung in 10 dogs (pM^+ cases, classified according to the *TNM Classification of Malignant Tumors*[23]), whereas no metastases (pM0 cases) were found in eight dogs.

All tissues were fixed in 7% buffered formalin and embedded in paraffin. Sections were cut at a thickness of 5 μm and stained with hematoxylin and eosin and Congo red for examination by light microscopy. Histological classification of growth pattern (lobular, trabecular, or tubular) was performed according to the method of Bestetti and Rossi.[6]

Additional samples were fixed in 4% buffered paraformaldehyde and embedded in plastic as described by Hermanns et al.[24] Staining with hematoxylin and eosin and Giemsa was performed on 2-μm-thick sections.

Immunohistochemical staining procedures and quantitative evaluation

Immunohistochemical staining was performed on paraffin-embedded material using the indirect immunoperoxidase method. The antibodies used were guinea pig–anti-porcine insulin (Dako, Glostrup, Denmark; 1:2,500 dilution), rabbit–anti-porcine glucagon (Dako; 1:500 dilution), rabbit–anti-human somatostatin (Dako; 1:400 dilution), rabbit–anti-human pancreatic polypeptide (Dako; 1:1,500 dilution), rabbit–anti-human neuron-specific enolase (Dako; 1:100 dilution), mouse anti-Ki 67 antigen (MIB-1) (Dianova, Hamburg, Germany; 1:30 dilution). As secondary antibodies, goat–anti-mouse immunoglobulin G (IgG) was used for MIB-1 staining (Dianova; 1:200 dilution), rabbit–anti-guinea pig IgG was used for insulin immunohistochemistry (Dako; 1:50 dilution), and goat–anti-rabbit IgG (Dako; dilutions ranging from 1:50 to 1:200) was used for all other procedures. Diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical Company, Deisenhofen, Germany) served as the chromogen for all procedures. For determination of the MIB-1 proliferation index, methyl green (Dianova) was used as a nuclear counterstain, and for all other procedures, Mayer's hematoxylin served as the nuclear stain. For a reagent control, an additional slide was treated with nonimmune dog serum instead of the primary antibody.

Immunohistochemistry was evaluated in 10 high-power fields (40× objective) using the CAS 200 system (Becton Dickinson, Elmhurst, IL, USA) to determine the percentage of positive cells, as described by Van Diest et al.[10] Immunoreactivity was graded on a scale of 1^+ to 4^+, with 1^+ = 1–20% positive cells, 2^+ = 21–49% positive cells, 3^+ = 50–79% positive cells, and 4^+ = 80–100% positive cells.

Histometric staining procedures

The Feulgen staining reaction (hydrolysis 5 N HCl, 1 hour at room temperature; Schiff's reagent 1 hour) and staining for argyrophilic proteins of nucleolar organizer regions (AgNORs) were performed on 5-μm-thick sections, as described by Aubele et al.[3] After the Feulgen reaction, the sections were placed in a 2:1 solution of 50% silver nitrate and 2% gelatin in 1% aqueous formic acid for silver staining. After removal of the silver colloid the slides were dehydrated and mounted.

Morphometric and DNA measurement

Nuclear image histometry and DNA assessment of Feulgen-stained nuclei (well-isolated, nonoverlapping nuclei of 100 tumor cells and 20 lymphocytes as an internal control) of the paraffin-embedded sections were performed using the Axiomat microscope system (Zeiss, Oberkochen, Germany) at a 100× objective (numerical aperture [n.a.] 1.3) and a narrow band pass filter of 547 nm. The nucleus was evaluated as a digitized image (128^2 pixels per image, gray value resolution = 256 channels) to calculate tumor size and to correlate image elements. Each cell image was processed, and more than 150 features were extracted from the nucleus, such as morphometric (e.g., area and shape factor) and textural chromatin pattern features[42] (e.g., co-occurrence and run length) and distribution of eu- and heterochromatin regions, using Interactive Data Language software (RSI, Boulder, CO, USA). Using nuclear profile area, nuclear mean density, and correction factor F of section thickness,[19] the integrated optical density (IOD) was calculated. The mean IOD value of lymphocytes of each specimen was used for DNA scaling of tumor cells. Histogram features were evaluated using ACAS software (Ahrens system, Hamburg, Germany). The DNA profiles were divided into diploid (1.8c–2.3c stemline) and aneuploid (>2.3c stemline); parameters such as S phase, 2c deviation index, and 2.5c exceeding rates were also calculated.

AgNOR analysis

AgNOR evaluation was carried out using the Samba 2000 system (Alcatel, TITN Answare, Meylan, France). The measurements were performed with a 40× objective (n.a. = 0.65) and a narrow band filter 546 nm in wavelength. The resulting pixel distance was 0.165 mm. For each case one slide was evaluated by random selection of several areas within the tumor (central as well as peripheral or invasive tumor areas). At least 100 isolated tumor cell nuclei were scanned per slide. The nuclei and AgNOR dots were segmented automatically by a thresholding method with some degree of interactive control to cut conglomerates or to reject artifacts and poorly focused nuclei. Only AgNOR parameters evaluated by stereological methods were calculated: RAN, relative AgNOR area = total AgNOR area/total nuclear area; NA, number of AgNORs/total nuclear area; and SV, total sum of perimeter/total nuclear area.

Measurement of MIB-1 proliferation index

The MIB-1 proliferation index was determined using the CAS 200 system and the Quantitative Proliferation Index software program. At least 1,000 nuclei in each tumor were scanned (40× objective) in randomly chosen fields.[17] The two components of the cell nucleus (DAB immunoperoxidase reaction product and methyl green counterstain) were sensed separately by the two image channels of the CAS 200 camera at 500 nm and 620 nm, respectively. Measurements were made of the total area of the nuclei from the 500-nm image and the total area of the antibody-tagged nuclei in the 620-nm image. After correction for average nuclear area (either methyl-green-stained or antibody-tagged, respectively), the ratio of these measurements, expressed as a percentage, was the resulting proliferation index.

Statistics

Evaluation was performed using SAS software (SAS Institute, Inc., Cary, NC, USA) and the BMDP statistical package (Statistical Software, Inc., Los Angeles, CA, USA). Stepwise linear discriminant analysis or logistic regression analysis was used to classify data according to a two-class-case analysis method (a posteriori probability analysis[7] [APOP]). For this procedure up to five features were selected on the basis of F statistics.

Classification was performed on the specimen level. By

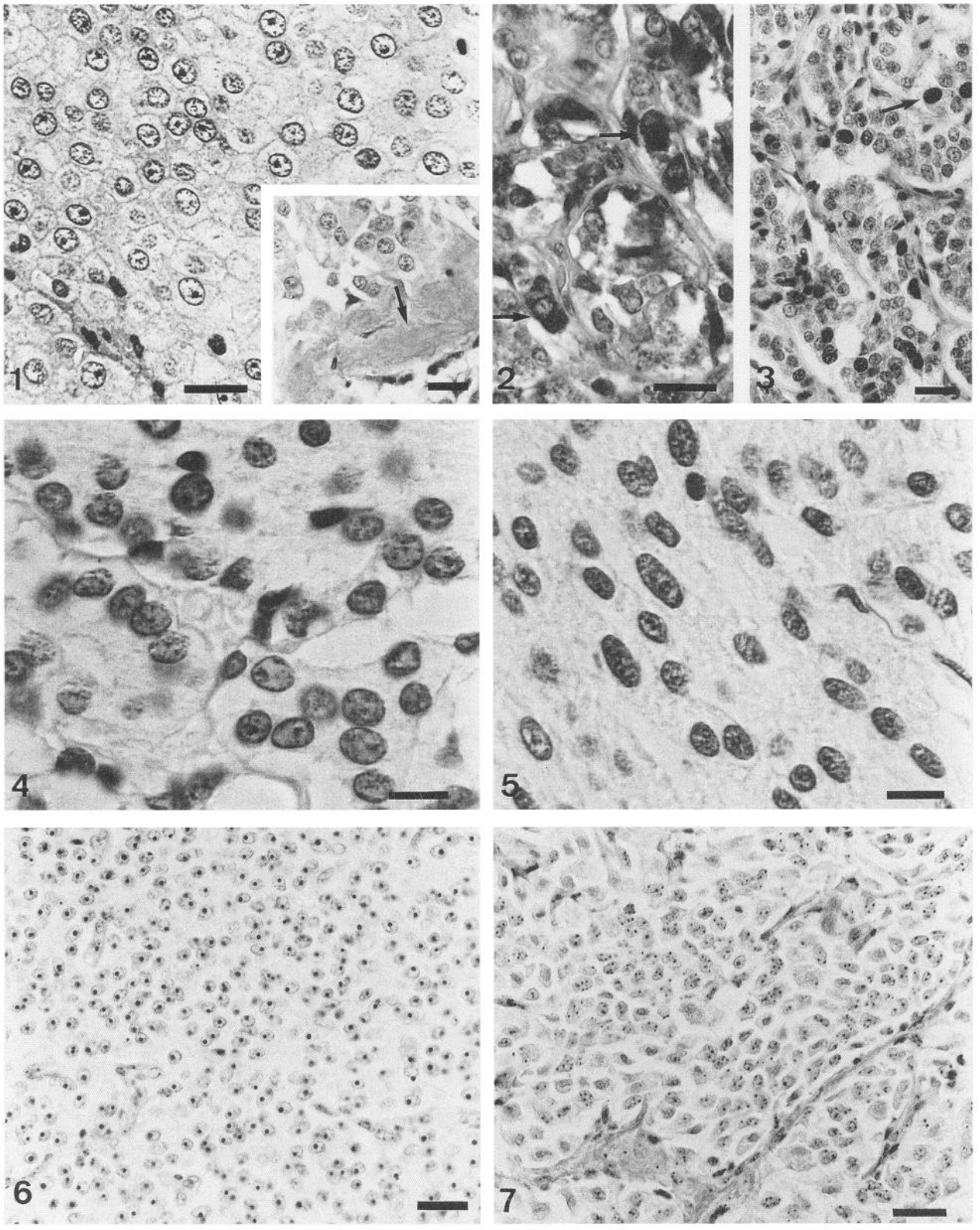

Fig. 1. Neuroendocrine tumor of the pancreas; dog No. 2. Tumor with isomorphic round nuclei, prominent nucleoli, and faintly stained cytoplasma, lobular pattern. HE. Bar = 10 μm. *Inset:* Amyloid deposits (arrow), dog No. 2. Congo stain. Bar = 20 μm.

Fig. 2. Neuroendocrine tumor of the pancreas; dog No. 3. Numerous cells with positive staining for insulin (arrows), trabecular pattern. Indirect immunoperoxidase method, diaminobenzidine tetrahydrochloride chromogen, Mayer's hematoxylin counterstain. Bar = 10 μm.

calculating the mean of cell features of a given specimen, one (case-specific) mean APOP value for the specimen was obtained. Using these APOP values, the specimens were assigned (correctly or falsely) to either the pM0 or the pM^+ group, depending on whether their values were less or greater than 0.5.

Contingency tables were tested by the Pearson chi-squared test. The significance level was $P < 0.05$.

Results

Histology and immunohistology

Isotropic tumors were characterized by a predominance of uniform, somewhat round nuclei with fine chromatin, prominent nucleoli, and granular or faintly stained cytoplasm (Fig. 1). The tumors showed either a uniform histological pattern ($n = 13$) or a mixture of two types ($n = 5$). In the majority of cases the histological pattern was trabecular ($n = 17$), whereas a minority of tumors had either lobular ($n = 5$) or tubular ($n = 1$) structures. Mitoses were rare. Amyloid deposits were found in 17% (3/18) of cases.

The definite diagnosis of neuroendocrine tumor of the pancreas was made by positive immunohistochemical staining for insulin (Fig. 2), glucagon, somatostatin, pancreatic polypeptide (PP), or neuron-specific enolase (NSE). Eighty-nine percent (16/18) of these tumors were positive for insulin, 33% (6/18) showed a positive reaction for somatostatin, 28% (5/18) were positive for glucagon, and 22% (4/18) were positive for PP. One tumor was positive exclusively for PP (4^+), and one tumor was negative for all four islet hormones tested but positive for NSE (3^+). Except for one case (3^+), the percentage of cells positive for insulin exceeded 80% (4^+). The staining reactivity was less intense for glucagon (1^+), somatostatin (1^+), and PP (1^+). Data are summarized in Table 1.

Statistically, the degree of multihormonality detected by immunohistochemical evaluation and type of growth pattern showed no correlation with biological behavior.

MIB-1 proliferation index and DNA ploidy

On average, the percentage of proliferating cells (positive for mouse anti-Ki 67 antigen [MIB-1], Fig. 3) was higher in the pM^+ group (3.6%) than in the pM0 group (1.3%). The values ranged from 0% to 3.4% in the pM0 group and from 0.4% to 12.9% in the pM^+ group. This difference represents a tendency only and was not statistically significant for discrimination between the two groups.

An aneuploid pattern was seen in only one pM^+ case evaluated by DNA measurement. All other cases showed a diploid pattern. Therefore, DNA ploidy could not be used for discrimination between pM^+ and pM0 cases. For all other DNA parameters, such as S phase and 2c deviation index, no correlation with pM status could be determined. Furthermore, the MIB-1 proliferation index was not correlated with any of these DNA parameters.

Nuclear image histometry and AgNOR analysis

Using nuclear image histometry of the three nuclear features with the highest F values, which provided best results for discrimination between the two groups, pM^+ cases were classified correctly in 80% (8/10) of cases, and pM0 cases were classified correctly in 75% (6/8) of cases ($P < 0.05$). The discriminating features were shape factors, such as MM1 and MM2 (first and second invariant moment of the shape,[26] respectively), reflecting elliptical nuclei. pM0 cases had higher values for these features, meaning that the percentage of nuclei with an elliptical shape was higher than that in pM^+ group (Figs. 4, 5). In addition, the chromatin parameter HAA (relative amount of euchromatin) was important for distinction between pM0 and pM^+ cases (the value was higher in pM0 cases than in pM^+ cases). Using analysis of argyrophilic nucleolar organizing proteins (AgNORs) (Figs. 6, 7), the best discrimination was obtained with the RAN feature (mean RAN for pM0 = 9.8% and mean RAN for pM^+ = 7.5%; correct classification for pM0 cases = 75% (6/8)

←

Fig. 3. Neuroendocrine tumor of the pancreas; dog No. 4. Cells with positive nuclear reaction for mouse anti-Ki 67 antigen (arrows), trabecular pattern. Indirect immunoperoxidase method, diaminobenzidine tetrahydrochloride chromogen, methyl green counterstain. Bar = 20 μm.

Fig. 4. Neuroendocrine tumor of the pancreas; dog No. 10 (pM^+ case). Round nuclei are prominent, and the round shape correlates with metastasis. Feulgen stain. Bar = 10 μm.

Fig. 5. Neuroendocrine tumor of the pancreas; dog No. 3 (pM0 case). Numerous elliptical nuclei can be seen, and the elliptical shape correlates with benign biological behavior. Feulgen stain. Bar = 10 μm.

Fig. 6. Neuroendocrine tumor of the pancreas; dog No. 10 (pM^+ case). The majority of nuclei have one argyrophilic nucleolar organizer protein. Combined Feulgen/silver stain. Bar = 20 μm.

Fig. 7. Neuroendocrine tumor of the pancreas; dog No. 3 (pM0 case). The majority of nuclei have two or three argyrophilic nucleolar organizer proteins. Combined Feulgen/silver stain. Bar = 20 μm.

Table 1. Histological and immunohistological pattern of 18 canine neuroendocrine tumors of the pancreas and their metastasis status.*

Dog No.	Breed	Age (years)	Sex	Histological Type†	Insulin‡	Glucagon‡	Somatostatin‡	Pancreatic Polypeptide‡	Amyloid Deposits‡	Metastasis§
1	Bearded Collie	9	F	tr-lob	++++	−	+	−	−	−
2	Boxer	10	M	lob	++++	+	+	−	d	−
3	Boxer	11	M	tr	++++	−	+	−	−	−
4	German Shepherd	8	M	tr	++++	−	−	−	d	−
5	German Shorthair	8	M	tr	++++	−	−	−	−	−
6	Lhasa-Apso	6	M	tr-lob	++++	−	−	−	−	−
7	Springer Spaniel	12	F	tr	++++	−	−	−	−	−
8	Westhighland Terrier	10	M	tr	++++	−	+	−	−	−
9	Boxer	10	M	tr-lob	++++	+	−	−	−	ln
10	Boxer	12	M	tr	++++	−	+	+	d	ln
11	Cocker Spaniel	8	M	tr	+++	+	−	+	−	sp
12¶	German Shepherd	11	M	tr-lob	−	−	−	−	−	ln
13	German Shepherd	18	M	tr-tu	++++	−	−	−	−	li
14	German Shorthair	8	M	tr	++++	+	−	−	−	li, ln
15	Golden Retriever	11	M	tr	−	−	−	++++	−	li
16	Irish Setter	11	F	tr	++++	−	+	+	−	li, ln
17	Mongrel	13	F	tr	++++	+	−	−	−	lu
18	Schnauzer	7	F	tr	++++	−	−	−	−	ln
Σ					16	5	6	4	3	

* Case Nos. 1 through 8 had no metastasis (pM0 group); case Nos. 9 through 18 had metastasis (pM^+ group).
† lob = lobular; tr = trabecular; tu = tubular.
‡ d = detected; + = 1 to 20% of cells were positive; ++ = 21 to 49% of cells were positive; +++ = 50 to 79% of cells were positive; ++++ = 80 to 100% of cells were positive; − = negative.
§ li = liver; ln = lymph node; lu = lung; sp = spleen.
¶ Case No. 12 was positive for neuron-specific enolase.

and correct classification for pM^+ cases = 90% (9/10); $P < 0.05$).

The nuclear feature MM1 and the AgNOR feature RAN applied in combination for the distinction of pM0 from pM^+ cases resulted in a correct classification of 88% (7/8) in the pM0 group and of 90% (9/10) in the pM^+ group ($P < 0.01$). For both features the values were higher in the pM0 than in the pM^+ cases.

Discussion

Digital image analysis provides objective measurements of cell and tissue features that previously were interpreted subjectively.[4] Besides objectivity, image analysis has other advantages over conventional visual assessment, including reproducibility and the ability to detect changes not immediately apparent to the naked eye.[18] In pathology, image histometry has been advocated as a means of grading and predicting the prognosis of tumors such as invasive breast cancer[18] or as a way of gaining insight into nontumorous processes such as gastritis, duodenitis, or glomerulonephropathy.

The results of this study were both compatible and incompatible with the present understanding of tumor biology. Using combined nuclear imaging and AgNOR analysis it was possible to correctly classify metastatic and nonmetastatic tumors 90% of the time.

The best discriminating feature was the shape factor MM1, which indicated a higher percentage of nuclei with an elliptical shape in the pM0 group. We interpret this as a reflection of partial epithelial differentiation due to endodermal (epithelial) histogenesis,[50] which results in a more benign biological behavior. Epithelial differentiation in such neuroendocrine tumors has been proven by their cytokeratin expression.[36]

Similar changes in nuclear shape have been correlated with increasing malignancy in colorectal lesions. It has been shown that there is a significant difference in the percentage of elliptical nuclei in mild and moderate dysplasia, in contrast to the round nuclei in severe dysplasia and carcinomas.[29] Furthermore, in human long-term survivors with renal cell carcinomas, the ellipsoidity of nuclei was significantly greater than in short-term survivors.[48]

The size and number of argyrophilic nucleolar organizing proteins (AgNORs) are associated with ribosomal protein synthesis in general and can be correlated with cell differentiation as well as cell proliferation and malignant transformation.[3] Therefore, it is

surprising that the total AgNOR area was larger in the pM0 than in the pM^+ group, but this has been seen in human beings with neuroendocrine tumors of the pancreas (NETPs) as well.[43] The increased AgNOR area in pM0 cases in our study correlates with an increased euchromatin area (HAA, discriminating feature of nuclear image histometry) in the pM0 group compared with the values of the pM^+ group. These results are again contrary to the concept that transformation of a normal to a neoplastic cell is characterized by an increase of protein synthesis[27] and that an increase of AgNOR content reflects the grade of malignancy in tumors such as melanoma, lymphoma, and breast carcinoma.[3,13]

On the other hand, abnormal DNA content has been reported in *non*neoplastic conditions such as diabetes mellitus[41] and in nesidiodysplasia.[2] These alterations, together with the increased amount of euchromatin and AgNOR area, are possibly, at least in endocrine organs, related to functional states and not to malignant behavior.[51]

In human beings, DNA ploidy is of no value in the prediction of malignancy in pancreatic endocrine tumors using flow cytometry[1,11,14,16] or combined flow and image DNA analyses.[15] Moreover, these neuroendocrine tumors show "paradoxical DNA aneuploidy"[15] meaning that clinically and histopathologically benign neuroendocrine tumors are characterized by a highly aneuploid DNA distribution pattern, whereas neuroendocrine carcinomas have a diploid DNA ploidy pattern.

In our study, DNA analysis was applied critically with regard to the serious limitations of this technique in sections due to capping of nuclei in 5-μm-thick histological sections.[19] These methodological limitations might explain why DNA parameters (especially the S phase) did not correlate with the mouse anti-Ki 67 antigen (MIB-1) proliferation index. In addition, no DNA parameters used in this study were of value for discrimination between the pM0 and pM^+ groups.

On average, the MIB-1 proliferation index was higher in the pM^+ group than in the pM0 group, but there was an overlap of results with no clear break for a cutoff value. Therefore, the percentage of MIB-1-positive cells could not be used for distinction between pM0 and pM^+ cases in this study. Our results are in accordance with the principle that increased proliferative activity (independent of the method used for determination) is correlated with malignancy of tumors such as breast carcinoma[5] and especially with reports of increased proliferative activity of malignant NETPs in human beings, as determined by flow cytometry,[22] and mitotic counts in invasive NETPs in dogs.[12] In addition, the proliferating cell nuclear antigen (PCNA) index has been shown to be statistically significantly correlated with mitotic and Ki 67 indices, and a PCNA index >5% has been found to be a reliable tool for predicting malignancy in endocrine tumors of the pancreas in human beings.[40]

Our immunohistochemical results were in accord with those of other studies showing multihormonality of NETP in human beings and dogs,[6,20,21,32,37,39] but the degree of multihormonality was not correlated with malignancy in our study. The most likely explanation for the frequent presence of several cell types in a pancreatic endocrine tumor is that they are derived from a multipotential stem cell, which may differentiate in various directions.[38] Similar to what has been found in other studies of neuroendocrine tumors in human beings,[45] growth pattern was not correlated to biological behavior.

In our study amyloid deposits (in 17% [3/18] of cases) were less frequent than in other reports of studies in dogs[39] (32% [10/31]) and human beings[49] (24% [17/72]). Amyloid in NETP consists of islet amyloid polypeptide, which is cosecreted with insulin after glucose stimulation.[34]

The mean age at diagnosis (10 years) and the percentage of pM^+ tumors (55% [10/18]) found in this study are similar to what has been reported in other studies,[9,33,35] all of which reported approximately 45% pM^+ tumors (33/73,[9] 18/40,[33] and 16/35[35]). Therefore, insulin-producing NETPs seem to be malignant more often in dogs than in human beings, with malignancy occurring in only about 10 to 15% of insulin-producing tumors in human beings.[11,46]

A reliable marker for malignancy in NETP in human beings or dogs has not yet been found. Nevertheless, some features, such as the overexpression of the proto-oncogenes Ha-*ras* and Ki-*ras*,[25] have been associated with progression of NETP in human beings. Another possible predictor of NETP progression has been expression of the *rig* gene (rat insulinoma gene[28]), which has been shown to be important for cellular growth and replication and to be activated in human and rat insulinomas.[44]

In summary, the results of this investigation illustrate the difficulty of predicting the biological behavior of NETP on the basis of phenotypic features such as increased nuclear size, pleomorphism, proliferation index, and DNA content. Nevertheless, discrimination of pM^+ tumors from pM0 tumors was possible in a high percentage of cases using nuclear image histometry in combination with AgNOR analysis.

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