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SHORT COMMUNICATION

Immunohistochemical and Histological Features of a Spontaneous Leydig Cell Tumour in a Rat

Rafał Ciaputa[1]*, Adam Szymerowski[2], Izabela Janus[1], Przemysław Prządka[3], Małgorzata Kandefer-Gola[1] and Marcin Nowak[1]

[1]Department of Pathology, Division of Pathomorphology and Veterinary Forensics, Faculty of Veterinary Medicine, Wroclaw University of Environmental and Life Sciences, C.K. Norwida 31, 50-375 Wroclaw, Poland
[2]Student Research Group of Anathomopathologists, Department of Pathology, Division of Pathomorphology and Veterinary Forensic University of Environmental and Life Sciences, Wroclaw, Poland
[3]Department and Clinic of Surgery, University of Environmental and Life Sciences, Poland
*Corresponding author: rafal.ciaputa@upwr.edu.pl

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A B S T R A C T

Neoplastic testicular lesions are diagnosed increasingly frequently in dogs and humans. In rats, spontaneous testicular tumours, particularly Leydig cell tumours, are very rare. The aim of the study was to carry out a histological and immunohistochemical analysis of a Leydig cell tumour in a rat as well as to present the similarities and differences in the expression of the proteins used to diagnose this type of tumour in dogs and men. Following the histopathological and immunohistochemical analysis (including antibodies against vimentin, calretinin, inhibin-α, β catenin and E-cadherin), significant similarities were found in the level of expression of the studied cell markers of the rat, dog and male leydigoma. This indicates their usefulness as diagnostic markers of testicle tumours both in humans and animals.

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INTRODUCTION

In both humans and dogs, testicular tumours are a group of malignant neoplastic lesions of the male reproductive tract that are diagnosed increasingly frequently. According to the WHO classification, Leydig cell tumours in dogs are some of the more frequent reproductive tract tumours (Ciaputa *et al*., 2012; Ciaputa *et al*., 2014; Ciaputa *et al*., 2015). In rats, spontaneous neoplastic testicular lesions are very rare, although they were reported in various laboratory rats strains (Teerds *et al.*, 1991; Rao *et al.*, 1992; Bomhard and Rinke, 1994).

The Leydig cell tumour derives from a neoplastic proliferation of interstitial cells that are mainly responsible for androgen secretion. Macroscopically, there is a clear demarcation between the tumours and the surrounding testicular interstitium, and the tumours form small, single or multiple soft, yellowish brown foci with areas of haemorrhage. Their histopathological features include the presence of spindle cells which are round, polygonal (Grieco *et al*., 2008; Ciaputa *et al*., 2012) or, less frequently, elongated. The cytoplasm of neoplastic Leydig cells may contain vacuoles. The nucleus is small, round and may contain single nucleoli (Peters *et al*., 2001; Ciaputa *et al*., 2012).

The aim of the study was to carry out a detailed histological and immunohistochemical analysis of a Leydig cell testicular tumour in a pet rat as well as to compare the similarities and differences in the expression of the proteins used to diagnose this tumour in rats, dogs and humans. Demonstrating a similarity in the expression of the studied proteins in leydigomas in men, dogs and rats may enable the use of rats as models of testicular oncogenesis.

MATERIALS AND METHODS

The study sample was collected intraoperatively from a 1.5 year old male laboratory rat kept at home**.** The excised tumour was fixed in 7% buffered formalin for 24 hours, then embedded in paraffin blocks and cut into 4µm sections. The sections were stained with hematoxylin and eosin and assessed using the WHO testicular tumour classification (Kennedy *et al*., 1998). The studied canine testicular tumours were obtained from samples archived by the Department of Pathology, Division of Pathomor-

phology and Veterinary Forensics of the Wroclaw University of Environmental and Life Sciences, while the samples of male testicular tumours were obtained from an archive of the Hist-Med s.c. N.Z. M. Kosiński P. Prajs Pathomorphology Unit on ul. Kamieńskiego 73a in Wrocław. The immunohisto-chemical analysis was carried out to compare the expression of the studied proteins in the rat testicular tumour with 10 canine tumours and five male tumours.

Calretinin and E-caherin were retrieved from the tissues fixed in formalin using an EnVision™ FLEX Target Retrieval Solution, High pH (50x) DAKO® kit no. K8004, where the samples were heated in a 96ºC water bath for 20 minutes. The Ki-67 antigen was detected using the EnVision™ FLEX Target Retrieval Solution, Low pH (50x) DAKO® cat no. K8005 and heating the samples in a water bath at 96ºC for 20 minutes. The endogenous peroxidase was blocked using an EnVision™ FLEX Peroxidase-Blocking Reagent for 10 minutes. Then, primary DAKO® Monoclonal Mouse Anti-Human Calretinin Clone DAK-Calret 1 antibodies (1/100 dilution), Monoclonal Mouse Anti-Human E-Cadherin, Clone NCH-38 antibodies (1/50 dilution), Monoclonal Mouse Anti-Human β-Catenin – clone β-Catenin-1 antibodies (1/100 dilution), Monoclonal Mouse Anti-Human Inhibin-α Clone R1 antibodies(1/100 dilution), Monoclonal Mouse Anti-Human Ki-67 Antigen Clone MIB-1 antibodies (diluted to 1/100) and Monoclonal Mouse Anti-Vimentin, Clone V9 antibodies (1/100 dilution) were mounted on the sections. They were then incubated for 20 minutes at room temperature. Next, the sections were rinsed with the EnVision™ FLEX Wash Buffer (20x), the EnVision™ FLEX /HR SM802 visualisation system was mounted on them, and they were incubated at room temperature for 20 minutes. The immunocytochemical reaction was elicited with a 3,3diaminobenzidine tetrahydrochloride (DAB) solution, EnVision™ FLEX DAB+ Chromogen DAKO. The sections were then rinsed in distilled water, the nucleus was stained with hematoxylin, dehydrated in graded alcohol series and mounted in balm. The photographs of the analysed tumours were subjected to computer-aided image analysis using a computer coupled with an Olympus BX53 (Olympus, Japan) optical microscope and an Olympus ColorViewIIIu (Olympus, Japan) digital camera. The measurements were taken using the cell^A software (Olympus Soft Imaging Solution GmbH, Germany).

The expression of calretinin, E-cadherin, β-catenin, inhibin-α and vimentin was appraised using the modified semiquantitative immunoreactive score (IRS) according to Remmele (Brouckaert *et al.*, 2013). The method takes into account both the proportion of the positively stained cells and the intensity of the reaction colour, while its final results represent the product of both parameters, with values ranging from 0 to 12 points (no reaction = 0 points (-); weak reaction = 1-2 points (+), moderate reaction = 3-5 points (++), intense reaction = 6-12 points (+++)). The expression of Ki-67 was evaluated quantitatively by estimating the percentage of positive cells (0-5% = no reaction (-), 6-25% = weak reaction (+), 26-50% = moderate reaction (++), above 50% = intense reaction (+++)).

RESULTS AND DISCUSSION

Macroscopically, the tumour was greyish-yellow and the nuclei were clearly separated from the testicular parenchyma, while small hemorrhagic foci were visible in cross-section. The histological analysis revealed the presence of numerous round or polygonal cells with abundant, vacuolised cytoplasm. The neoplastic cell nuclei were the size of two erythrocytes, or less commonly three, pushed peripherally or squeezed toward the cell membrane. Numerous extravasations and cysts filled with a protein fluid were also found. There were few mitotic figures (i.e. one or two) per high power field (Fig. 1A,B,C).

The immunohistochemical analysis revealed that the expression of vimentin in the rat (R) was intense +++. It was also intense (+++) in 70% and moderate in 30% (++) of the studied dogs (D). In all the studied male samples (M), the expression of vimentin was also intense (+++). The expression of calretinin in the studied rat, as well as in canine and human specimens was intense (+++). In the rat, the expression of inhibin-α was intense (+++). In dogs, this expression was intense in 60% of the canine samplesand moderate ++ in 40% of them. In all the studied male samples, the expression of inhibin-α was intense (+++). The expression of β catenin and E-cadherin in all the studied samples was intense (+++). No Ki-67 expression was observed in the rat samples, while 80% of the canine samples showed intense Ki-67 expression (+++) and 20% of the samples showed moderate expression (++). In men, the Ki-67 expression was intense in all the studied tumours (Fig. 2 A,B,C,D,E).

Tumours of testis are the most frequently diagnosed neoplastic diseases in men. Similar problems are observed in dogs. A study carried out between 2009 and 2011 in Lower Silesia found that 6% of 1743 dogs had testicular tumours, while a study between 2012 and 2013 found that 6.1% of 4174 dogs had testicular tumours (Ciaputa *et al*., 2013; Ciaputa *et al*., 2017). Finding a similarity in the histologic structure of the described spontaneous Leydig-cell tumour in a rat may broaden our understanding of the duration and speed of carcinogenesis. In men and dogs, leydigomas are usually observed in older-aged individuals. They are relatively less common in men than dogs (Ciaputa *et al*., 2012; Ciaputa *et al*., 2014; Ciaputa *et al*., 2015). Studies carried out on various laboratory rats strains also show a high incidence of Leydig-cell tumours in old animals (Teerds *et al.*, 1991; Rao *et al.*, 1992; Bomhard and Rinke, 1994). It seems that the studied rat had the qualities of an aging organism.

We were able to find similarities in the expression of chosen cell markers in the rat, canine and male leydigoma. The presence of the expression of vimentin in the tumours confirmed their mesenchymal origin (Ciaputa *et al*., 2014). Calretinin was used to show secretory activity. It is thought that a strong expression of calretinin in steroid secreting cells may indicate tumour secretory activity (Ciaputa *et al*., 2014). Indicating the expression of inhibin-α as a marker of the tumour growth potential, which is an inhibitor of this process, also seems justified (Ciaputa *et al*., 2014). The study also included an analysis of proteins responsible for intercellular adhesion, such as E-cadherin and β-catenin. The analysis of their expression

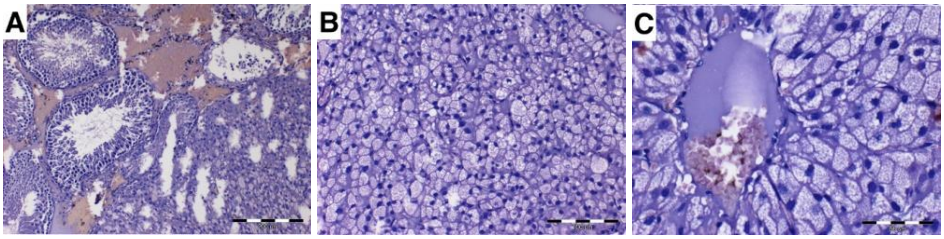

Fig. 1 A,B,C: Leydig cell tumour in a rat. Numerous round or polygonal neoplastic Leydig cells with abundant, vacuolised cytoplasm (A,B,C). The cell nuclei are enlarged and localised peripherally (A,B,C). Haemorrhages (A) and cysts filled with a protein fluid (C) can be seen between the neoplastic cells. (H&E, A. 100x, B. 200x and C 400x).

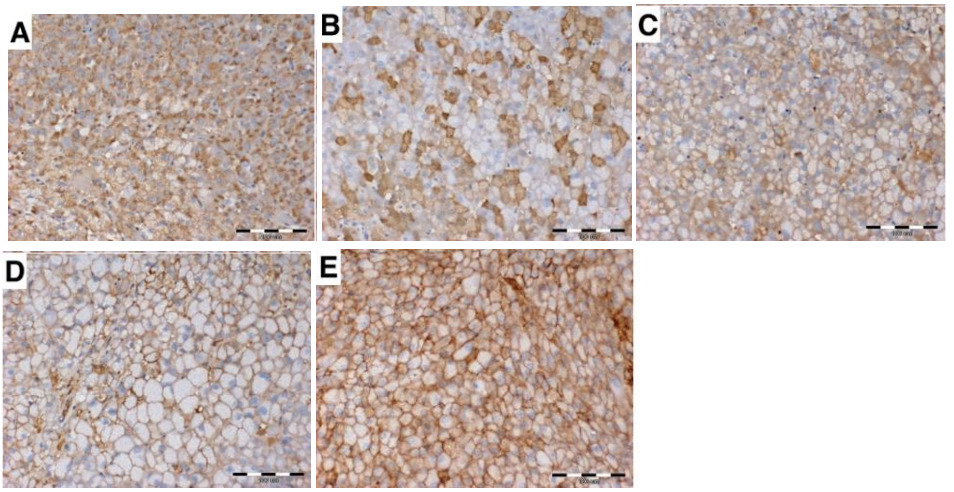

Fig. 2 A: Cytoplasmic expression of vimentin. B: Cytoplasmic expression of calretinin. C: Cytoplasmic expression of inhibin-α. D: Membrane expression of β catenin. E: Membrane expression of E-cadherin. (IHC, A. B. C. D. E. 200x).

is very useful in determining the metastatic potential of a tumour (Ciaputa *et al*., 2014). In combination with the nuclear expression of the Ki-67 proliferative antigen, it is possible to assess the degree of the tumour malignancy (Ciaputa *et al*., 2014). In this study, we did not find an expression of the Ki-67 protein in the rat sample. However, it is not possible to completely exclude the proliferative potential of the studied tumour, as 1-2 mitotic figures were seen in the high power microscopic field. Nevertheless, we found that this marker was not useful in the assessment of the mitotic index of the rat leydigoma. Our own studies, which were carried out on a large group of the most common testicular tumours in dogs and men, have shown diagnostic utility of the above described cell markers (Ciaputa *et al*., 2012; Ciaputa *et al*., 2014; Ciaputa *et al*., 2015).

The presence of these proteins in a rat leydigoma suggests that they are useful as diagnostic markers of testicular tumours in humans and animals.

There is ongoing research into animal cancer models. Rats, which are often used in *in vivo* studies, could be used in studies on oncogenesis or cancer treatment of these types of tumours if there is confirmation of the histologic and immunohistochemical similarities between the rat and human testicular tumours. In addition, the diagnosis of the same type of testicular tumour in the rat, dog and human is interesting. The rat is increasingly treated as a domestic pet rather than a laboratory, therefore reports describing spontaneous tumours in domestic rats are in our opinion valuable even with a small number of cases. Due to increasingly improving pet rat owner care, rats are able to live longer, which may lead to the development of numerous age-related diseases, including neoplastic lesions. To date, these have been rarely diagnosed due to the short life expectancy of this species.

Authors contribution: RC, AS, IJ, MK-G, MN: executed the experiment and analyze results, photographic recording, preparing and elaboration the manuscript. PP: helped in clinical diagnosis and surgical procedure.

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