

Expression of Breast Cancer Resistance Protein (BCRP-1) in Canine Mammary Adenocarcinomas and Adenomas

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Abstract. *Background:* Among the recognized factors which induce multi-drug resistance, an increased activity of proteins belonging to the ATP-binding cassette family, including breast cancer resistance protein 1 (BCRP-1), is regarded as the most important. Localization and intensity of BCRP-1 expression was evaluated in mammary adenocarcinomas and adenomas in dogs. The obtained results were compared to the grade of malignancy (G) of the tumours. *Materials and Methods:* Materials for the study were sampled in the course of surgery from 54 dogs, of various breeds, aged 6 to 16 years (36 cases of mammary adenocarcinoma and 18 cases of mammary adenoma). The tumours were histopathologically verified and immunohistochemical reactions were performed to evaluate expression of BCRP-1. The microscopic patterns were photographed and subjected to computer-assisted analysis taking advantage of MultiScanBase Ver. 14.02 software. *Results:* Expression of BCRP-1 was detected in over 85% of adenocarcinomas and almost 28% of adenomas. Samples of tumours with a higher grade of malignancy demonstrated an increased expression of BCRP-1. The two variables manifested a moderate positive correlation ($r=0.35$; $p<0.05$). *Conclusion:* The results point to a role of BCRP-1 protein in biology of tumour cells in dogs.

Development of resistance to an administered drug represents one of principal reasons for failure in chemotherapy of tumours. The situation is additionally

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complicated by the fact that the process often has a multidrug resistance (MDR) character, in which decreased sensitivity of tumours cells to multiple types of cytostatic drugs is observed. Among the now recognised factors which induce MDR, an increased activity of proteins belonging to ATP-binding cassette (ABC) transporters is regarded as the most important. Using ATP, these proteins eliminate administered chemotherapeutic agents from cells and in this way prevent development of their effective, *i.e.* lethal, concentrations in tumour cells (1-3). Clonal selection taking place in the course of chemotherapy should also be kept in mind. This phenomenon involves natural selection in the course of treatment of tumour cell clones most resistant to the cytostatic drug, *i.e.* the clones manifesting an enhanced expression of ABC transporters (1). Among the more than 48 ABC transporters detected so far now in humans, at least 8 proteins of this type are capable of transporting cytostatic drugs and being involved in MDR. Among these, the group includes the so-called breast cancer resistance protein, BCRP-1. The term stems from a breast cancer cell line, MCF-7, from which this protein was isolated for the first time (4, 5). The protein also used to be termed the placenta-specific ABC transporter (ABCP), the mitoxantrone-resistance protein (MXR), and ABCG2 protein (6, 7).

Under physiological conditions a pronounced expression of BCRP-1 is noted in epithelia of the small intestine, colon, ovarian cells, hepatocytes, kidney cells and placental syncytiotrophoblast. The organ distribution of the protein may suggest its involvement in protection of the respective organs against xenobiotics, while its high expression in placental syncytiotrophoblast may point to its participation in mechanisms protecting the foetus and in formation of the mother-foetus barrier (6, 8-13).

Our study aimed at examining expression of BCRP-1 protein and determination of its intensity in the most common mammary tumours of bitches, *i.e.* adenomas and adenocarcinomas. Moreover, in the case of adenocarcinomas, an attempt was made to compare the results with the grade of

Table I. *Semiquantitative 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	

tumour malignancy (G). Additionally, we aimed at comparing the obtained results with respective data related to mammary tumours in humans. Any parallelism in this respect might open the potential for using mammary tumours in dogs as an experimental model for studies on tumour cell biology in humans.

Materials and Methods

Materials for the study were sampled during surgery in 54 dogs of various breeds, aged 6 to 16 years. The tumours were verified as adenomas (18 cases) and adenocarcinomas (36 cases) by histopathology.

Formalin-fixed, paraffin-embedded tissue was freshly cut (4 µm). The sections were mounted on Superfrost slides (Menzel Gläser, Germany), deparaffinized with xylene and gradually hydrated. Activity of endogenous peroxidase was blocked by 5 min incubation with 3% H₂O₂. Detection of BCRP-1 expression was preceded by 15 min exposure of the sections in a microwave oven to boiling Antigen Retrieval Solution (DakoCytomation, Denmark) at 250 W. For demonstration of BCRP-1 expression in the paraffin sections, monoclonal mouse antibodies were used (clone BXP-21, 1:50; Chemicon/Millipore, Billerica, MA, USA). The antibodies were diluted in the Antibody Diluent, Background Reducing (DakoCytomation). The sections were incubated with the antibody for 1 h at room temperature. Subsequently, incubations were performed with biotinylated antibodies (15 min, room temperature) and with streptavidin-biotinylated peroxidase complex (15 min, room temperature) (LSAB2, HRP, DakoCytomation). Diaminobenzidine (DAB; DakoCytomation) was used as a chromogen (7 min, room temperature). All the sections were counterstained with Meyer's haematoxylin. In every case, controls were included in which specific antibody was substituted by the Primary Negative Control (DakoCytomation).

Microphotographs of all studied tumours were subjected to computer-assisted image analysis *via* a computer coupled to an Axiophot optical microscope (Carl Zeiss, Germany). The set had the potential to record images and to perform their digital analysis. The measurements took advantage of MultiScanBase V 14.02 software (Computer Scanning Systems, Warszawa, Poland).

Microscope examination allowed for sorting of the tumours into three groups of a variable malignancy grade. The grade was established using the scale of Bloom-Richardson in modification of Elstona and Ellis. The method for the evaluation 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 a magnification of x400 (0-7, 8-16, ≥17). The sum of the points provided potential to distinguish 3 grades of malignancy (G) among the tumours: 0-5, G1; 6-7, G2; 8-9, G3.

Expression of BCRP-1 was appraised using the modified semi-quantitative immunoreactive score (IRS) scale according to Remmele (Table I) (14). The method takes into account both the proportion of positively stained cells and the intensity of the reaction colour, 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 points (+), moderate reaction = 3-4 points (++) , intense reaction = 6-12 points (+++)). The results were subjected to statistical analysis using Statistica PL software (STATSoft, Krakow, Poland) employing Mann-Whitney test and Spearman's correlation analysis.

Results

The expression of BCRP-1 protein was demonstrated both in mammary adenomas and adenocarcinomas of bitches (Figures 1, 2). In all the cases with a positive reaction, the membraneous-cytoplasmic expression of BCRP-1 was detected. BCRP-1 expression was noted in over 85% of adenocarcinomas and almost 28% of adenomas. Evident differences were observed in the protein expression intensity in both groups of tumours. In cases of adenocarcinoma, over 69% of the tumours manifested BCRP-1 expression evaluated at +, 8% at ++ and almost 3% at +++. In cases of adenoma expression of the protein never exceeded the level of +. It should be noted that in over 19% of adenocarcinomas and over 72% of adenomas, no expression of BCRP-1 was observed.

The distribution of BCRP-1 expression as related to tumour malignancy grade (G) was also interesting (Figures 3, 4). In adenocarcinomas qualified as G1, over 25% of tumours manifested no expression of BCRP-1 while 75% manifested expression at the level of +. In cases of G2 adenocarcinoma, lack of BCRP-1 expression was noted in over 6% of tumours, the expression at + was seen in over 81% , and at ++ in over 12% of the tumours. G3 adenocarcinomas exhibited BCRP-1 expression at + level in 50% cases, at ++ in over 16% , and at +++ also in over 16% of the cases. It should be added that only the G3 group of tumours manifested expression of the studied protein at +++ level; in G1 and G2 groups, no such a strong expression of BCRP-1 was observed. Moreover,

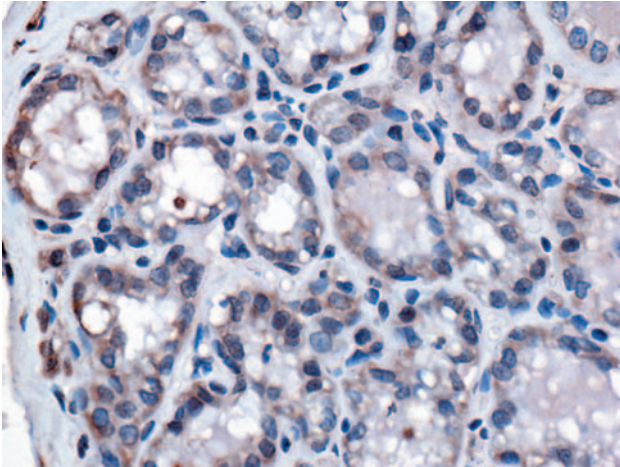


Figure 1. Low expression of BCRP-1 in cells of canine mammary gland adenoma ($\times 400$, counterstained with haematoxylin).

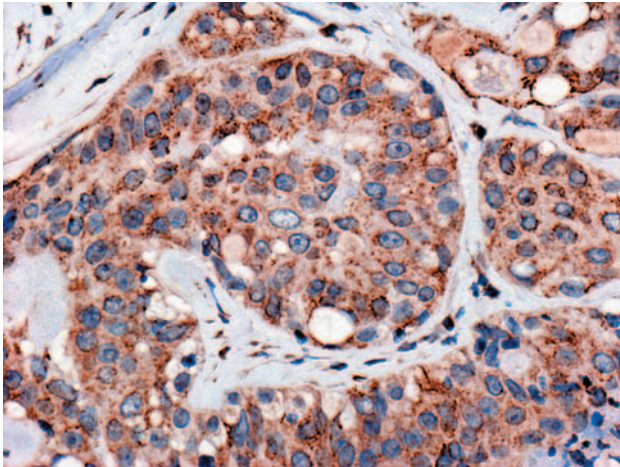


Figure 2. High expression of BCRP-1 in cells of canine mammary gland adenocarcinoma ($\times 400$, counterstained with hematoxylin).

significant differences in BCRP-1 expression were noted which depended on the grade of malignancy (G), with the highest levels of the protein noted in tumours of the highest grades of malignancy (Figure 3).

In turn, statistical analysis using Spearman's correlation demonstrated a significant, moderate positive correlation between BCRP-1 expression and malignancy grades G ($r=0.35$; $p<0.05$) (Figure 5).

Discussion

Due to progress in immunocytochemistry, it has been possible to precisely document the presence of BCRP-1 protein in cell membranes and cytoplasm in many types of

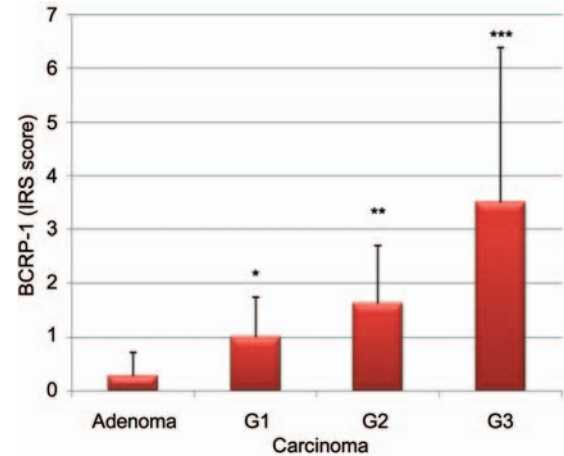


Figure 3. BCRP-1 expression level in studied tumours as related to their grade of malignancy * $p<0.05$ (G1 vs. adenoma); ** $p<0.001$ (G2 vs. adenoma); *** $p<0.05$ (G3 vs. adenoma).

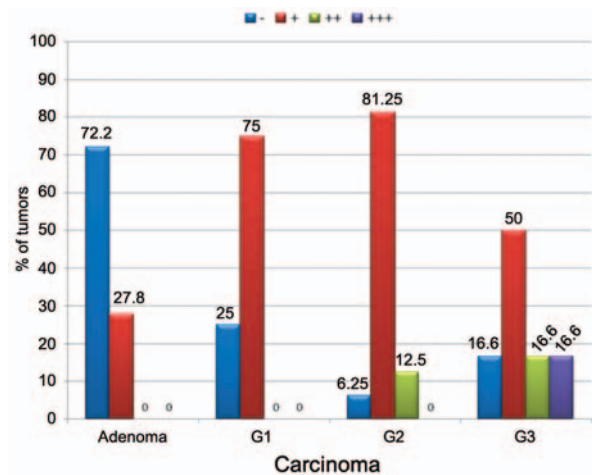


Figure 4. Proportion of tumours which manifested BCRP-1 expression, as related to their grade of malignancy (G).

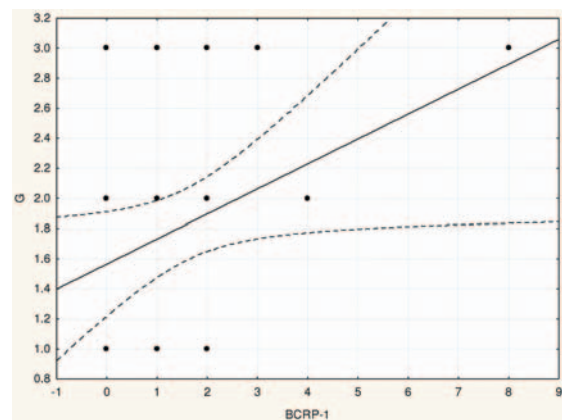


Figure 5. Correlation between expression of BCRP-1 and G in cells of canine mammary adenocarcinoma. Correlation coefficient $r=0.35$; $p<0.05$.

human tumours. In most cases, a much more intense reaction has been noted in cell membranes (8, 15-25). In a study performed by Diestra *et al.* (15) among 150 patients with 21 types of neoplastic tumours, BCRP-1 expression, was classified as moderate to strong in 92 cases, weak in 27 cases, while no expression of the protein was observed in 31 cases. The most pronounced expression of BCRP-1 protein was demonstrated in carcinomas of the alimentary tract (oesophagus, stomach, colon), in melanoma, in small cell pulmonary carcinoma and in endometrial cancer. The authors also examined BCRP-1 expression in breast cancer, detecting a positive reaction in over 55% of the analysed tumours. Similarly, in our studies we detected BCRP-1 expression in the two types of epithelial mammary tumours in bitches, including a clear, more pronounced, reaction in adenocarcinomas than in adenomas. Moreover, expression of the protein was proven to be much more pronounced in canine mammary cancer (over 85% positive cases) than in analogous studies performed in cases of human breast cancer (slightly more than 55% positive results) (15). The lower expression of the protein in breast cancer in women was confirmed in a study by Kanzaki *et al.* (26), who detected low expression of *ABCG2* gene (coding for BCRP-1) in 43 cases of breast cancer. The positive correlation between expression of BCRP-1 and malignancy grade G of the tumours we detected here also deserves attention. We noted that an increase in tumour malignancy was paralleled by an increase in expression of BCRP-1. In the group of adenocarcinomas classified as representing G1 grade, the highest expression of the protein amounted to +, in G2 group to ++, and in G3 group to +++. Such a distribution of protein expression may point to the involvement of the protein in formation of the malignant phenotype in mammary cancer of bitches. The study by Scharemborg *et al.* (27), in which an augmented expression of BCRP-1 was noted in primitive, resting haemopoietic stem cells in humans and mice, should also be mentioned. This may confirm the hypothesis on the involvement of BCRP-1 in blocking of the differentiation of primordial stem cells, in which a decrease in BCRP-1 level would provide a signal for differentiation toward mature cells (1). Thus, it may be assumed that in an analogous manner high expression of BCRP-1 in cells of canine mammary cancer may result in the prevalence of less differentiated tumour cells and, therefore, potentially in greater malignancy. The assumption has been confirmed by our results in the form of positive correlation detected between expression of BCRP-1 and the tumour malignancy grades.

Since veterinary oncology has not yet developed commonly accepted and well-documented chemotherapy procedures designed for tumours of mammary glands, it is difficult to unequivocally suggest the role of BCRP-1 in multidrug resistance in dogs. In addition, the situation is

aggravated by the lack of studies on expression of the protein in canine mammary tumours. Results obtained by us argue for involvement of ABC proteins, including BCRP-1, in the biology of tumour cells in dogs.

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