Thesis of doctoral (PhD) dissertation

INVESTIGATION OF P-GLYCOPROTEIN EXPRESSION AND FUNCTION IN DOGS WITH LYMPHOMA AND MAST CELL TUMOR

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1. Background and aims of the study

1.1. The clinical presentation of lymphoma and mastocytoma, the significance of therapeutic resistance

Lymphoma and mastocytoma are clinically the most important neoplasms in dogs.

Lymphoma is a tumor of haematopoietic origin, composed of malignant lymphoid cells. It can manifest in different anatomical regions, most commonly lymph nodes, gastrointestinal tract, lymphatic organs of the chest, skin. The multicentric form is the most important in clinical oncology, begins with swelling of the peripheral lymph nodes and is present in about 80% of the patients. During the progression to higher stages (stages IV and V), other organs will be involved such as the spleen, liver and later bone marrow and peripheral blood. The knowledge of the immunophenotype is essential for prognosis and therapeutic planning. The B-cell and T-cell phenotypes are classified primarily from lymph nodes. The lymphoma is treated with multimodal chemotherapy protocols, mainly performed with the so-called CHOP protocol, which is a combination of cyclophosphamide (C), doxorubicin (H; hydroxydaunorubicin), vincristine (O; Oncovin) and

prednisolone (P) drugs. During the 19 weeks of the protocol, the stage of the disease (states of lymph nodes, spleen and liver) and the status of the patient's immune system (blood count, liver and kidney function) are checked every week before the cytotoxic drug administration. Adverse reactions caused by chemotherapy drugs are detected according to the recommendations of the Veterinary Cooperative Oncology Group (VCOG). In addition to the CHOP, other chemotherapy protocols are available, such as the CHOP protocol with L-asparaginase (L-CHOP), the LOPP protocol (L, lomustine; O, oncovin/vincristine; P, procarbazine; P, prednisolone), or a combination of lomustine and L-asparaginase. Survival time with chemotherapy regimens is typically 10-12 months. Progression of the lymphoma leads to the death of patients, the treatment failure is almost always due to chemotherapy resistance.

Mast cell tumor is a proliferation of malignant mast cells, that form solid neoplasm mainly in the skin. In addition to the cutan masifestation, mucosal, visceral and bone marrow forms are known. During progression, regional (lymph node) metastasis (stages III, IV, V) or distant metastasis (stage V) may be detected in the higher

stages. Following diagnosis and staging, surgical resection is recommended whenever possible. If indicated, depending on the histological malignancy (grade) and subsequent metastases, chemotherapeutic agents are used. Systemic drug therapy of mastocytoma can be performed with tyrosine kinase inhibitors (such as masitinib and toceranib), vinblastine and lomustine. As with lymphomas, failure of chemotherapy for mastocytomas is attributed to therapeutic resistance.

When a patient shows multidrug resistance during treatment, the change of drug can not achieve the adequate therapeutic response. Resistance is caused by a combination of numerous pharmacodynamic and pharmacokinetic factors, including ABC-proteins. Among these, the P-glycoprotein (P-gp) is particularly important. The P-gp transmembrane protein is responsible for the export of numerous xenobiotics, including several drugs used in the treatment of the tumors under discussion; vincristine, vinblastine, doxorubicin, prednisolone. The expression and activation of P-gp is partly intrinsic and partly enhanced by environmental effects (e.g. hypoxia, inflammation, carcinogens, cytotoxic compounds), leading to the failure of drug therapy and shorten survival times.

1.2. Aims of the study

1) Determination of P-gp expression and activity in samples from patients before definitive tumor chemotherapy. We wanted to investigate whether our methods could be used to demonstrate increased P-gp expression and increased activation.

2) Comparison of two testing methods (immunohistochemistry and flow cytometry)

3) Examination of survival times

-Overall survival time (OST)

-Relapse free period (RFP)

4) Predict patients survival times based on P-gp expression and activity results. We wanted to know if increased P-gp expression and activity could lead to shorter survival times.

2. Materials and methods

2.1. Patients of the study

There were 95 dogs with lymphoma included in the study, 79 were B-cell and 16 were T-cell type. Most dogs (76) were treated by CHOP protocol. In addition, typically after relapse, 13 patients received L-CHOP protocols and 6 patients received lomustine treatment. In the B-cell group, 26 patients received chemotherapy after relapse. The appearance of side effects led to dose reduction or forced postponement of treatment in several cases. The cause of death was not always lymphoma, therefore the OST data were influenced by other diseases and euthanasia based on owners' decision. In the goup of T-cell lymphoma patients, 5 dogs received chemotherapy treatment after relapse. The cause of death in this group was lymphoma in all cases. All 31 mastocytoma patients had their tumors surgically removed. After surgery, 16 patients received chemotherapy; 10 patients were treated with masitinib, 11 patients treated with vinblastine, and 2 dogs recieved lomustine. In case of severe side effects, dose reduction was applied or the drug was discontinued. The cause of death was not always related to mastocytoma.

2.2. Methods

The P-gp expression of lymph node and mastocytoma samples was detected by immunohistochemistry method using the C494 clone monoclonal antibody. The binding of primary antibody was visualized with a brown chromogen-linked secondary antibody. Staining was assessed manually. In addition to the expression level (%), the staining site (membrane or cytoplasm dominant) and intensity were recorded.

P-gp activity was determined by calcein assay. The calcein-acetoxymethyl ester can diffusely enter into living cells, and is converted by esterases. The fluorescence of active calceine molecule can be measured by flow cytometry. This calcein is exported by cell-surface P-gp molecules, resulting the decrease of fluorescence. However, with the addition of the P-gp inhibitor verapamil, these transporters are inhibited and the strong signal of calcein becomes detectable again. The factor obtained from the difference in intensity of the two fluorescence is the multidrug resistance activity factor (MAF).

$$(IF_{verapamil} - IF_{calcein}) / IF_{verapamil}$$

3. New scientific results of the study

Based on the international literature, P-gp expression and activity have never been studied simultaneously in such a large canine tumor patient population.

The intensity of P-gp staining was significantly increased in lymphoma and mastocytoma samples expressing a higher percentage of the protein. Increased expression may indicate a higher number of receptors, which also cause a proportional increase in signal intensity. In the lymphoma population, higher levels of expression were found in tissues from T-cell patients, and dominant cytoplasmic P-gp staining was more frequently observed in this group. The intracellular P-gp expression suggests that cells with higher malignancy may be able to sequester cytotoxic compounds in their cytoplasmic organelles by proteins expressed in their membranes.

There was a significant positive correlation between P-gp expression and MAF in both tumour types. Thus, the increase in activity detected by the calcein assay reliably indicates the function of the expressed receptors. The two assay methods compare well with each other. The degree of malignancy (grade) in mast cell tumor patients also showed a strong positive correlation with both P-gp

expression and MAF values. It can be expected, that higher malignancy leads higher therapeutic resistance to chemotherapeutic regimens.

Based on our hypothesis, higher P-gp expression and enhanced function is also associated with more pronounced therapeutic resistance, because the higher number of transmembrane proteins is capable of transporting more cytotoxic drugs into the extracellular space. To verify this hypothesis, comparisons with survival times were performed. The tests were performed using cut off values. We expected that patients with higher expression and higher MAF values would have shorter median OST and RFP times. T-cell lymphoma patients meet this hypothesis.

The main results of the statistical analyis to prove the hypothesis are as follows:

Above the cut off of 8% P-gp expression, the OST was 158 days, while patients under this expression lived for a 177 days. For P-gp activity, patients above the cut off of 0.19 MAF lived 158 days, while the OST for the group below this was 181 days. As before, the RFP was shorter in the group above 8% (median 56 days) compared to patients below the cut off (median 93 days).

The MAF population above 0.19 had RFP of 47 days, while patients below 0.19 did not relapse for median 140 days. However, when examining the whole lymphoma patient population and B-cell population, we found that P-gp expression and MAF gave different results when comparing OST and RFP data. When considering the data from all lymphomas, a negative correlation was observed, above a cut off of 6.4%, the OST was 351 days, while below cut off it was 398 days. In contrast, there was a positive correlation in the RFP comparison. Patients above the cut off had a median of 301 days without recurrence and below 6.4% had 263 days of recurrence-free survival. In the functional analysis, both survival times showed a positive correlation. A MAF above 0.19 produced OST of 417 days compared to 349 days below 0.19 MAF. Above 0.19, RFP was 323 days and below 0.19, patients lived for 198 days without recurrence. Similar correlations were obtained during analysis of the B-cell lymphoma group. The group below 6% OST was 398 days, while patients below the cut off lived for 445 days. Thus, a negative correlation was confirmed here. In contrast, RFP and P-gp expression were positively correlated, above cut off patients did not recur for 315 days, below cut off patients had 283 days

RFP. The MAF of 0.19 and survival times used for the functionality test were positively correlated. The MAF group above cut off had OST of 485 days, while patients below cut off had a survival of 381 days. In the RFP comparison, the group above the cut off did not recur for 326 days, while the recurrence-free survival of the population below 0.19 MAF was 263 days.

In mastocytoma patients, both P-gp expression and MAF were positively correlated with OST data. Patients above the 37.5% cut off P-gp had survival of 1274 days compared to survival of 330 days for the group below the cut off. The overall survival of the group above the MAF 0.17 cut off was 398 days, while patients below the cut off lived 245 days. An interesting finding was that RFP showed a negative correlation with MAF values. The median recurrence-free survival of patients above 0.17 was 131 days, while RFP of the group below 0.17 was 212 days. This was of great significance in mastocytoma patients, because in dogs who were treated with chemotherapy, the occurrence of recurrence was primarily related to drug resistance. It can be taken as a confirmation of our hypothesis that RFP decreases with higher activity.

The statistical comparisons do not suggest that knowledge of P-gp expression or activity is prognostic for patients with lymphoma or mastocytoma. In the conclusion of our results, we put a prominent emphasis on discussing the influencing factors that affect patient survival. Among these, we discussed the cause of mortality, which was not an influencing factor in the T-cell lymphoma population, where the expected associations were confirmed. In the B-cell lymphoma patients, we found that patients with lower MAF were much more susceptible to adverse effects of chemotherapeutic drugs, and treatment of these dogs had to be delayed or dose reduced much more frequently. In this context, the patients in this group relapsed much earlier, and had the lowest OST. The dose reductions and treatment delays may be accompanied by an increase in resistance and lower P-gp activity may implies stronger sensitivity to the adverse effects of chemotherapeutic agents.

Other influencing factors for both lymphoma and mastocytoma may be the sampling, tissue processing and evaluation methods. Although technically the calcein test is a well-standardized test, transcutaneus sampling of lymph nodes may include epithelial, mesenchymal

and adipose cells in addition to lymphoid cells. Besides that, tumour cells with low initial P-gp expression and activity may increase efflux function due to chemotherapy (substrate vincristine, doxorubicin, vinblastine). In the future, it would be very interesting to characterise the changes in P-gp function during chemotherapeutic treatments, similar to our previous *in vitro* studies, and to investigate other proteins and factors that also enhance therapy resistance in veterinary clinical oncology.

4. List of publications related to the subject of the dissertation

Dékay V., Karai E., Füredi A., Szebényi K., Szakács G., Vajdovich P.: **P-Glycoprotein activity at diagnosis does not predict therapy outcome and survival in canine B-cell lymphoma**. Cancers, 13, 14 (16):3919., 2022. IF (2022): 6.575

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Karai E., Szebényi K., Windt T., Fehér S., Szendi E., Dékay V., Vajdovich P., Szakács G. és Füredi A.: **Celecoxib Prevents Doxorubicin-Induced Multidrug Resistance in Canine and Mouse Lymphoma Cell Lines**, Cancers, 12(5). 1117, 2020.; IF (2020): 6.162

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