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PIM2, PKMYT1 and CCDC112 gene expression levels in multidrug-resistant canine lymphoma

Validation of RNA-sequence data

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ABSTRACT

It is estimated that there are 25-134 lymphoma cases per 100.000 dogs, making lymphoma one of the most frequently diagnosed canine malignant cancers worldwide. Chemotherapy, as first-choice treatment, is often accompanied by drug resistance. If the drug resistance mechanisms can be found, chemotherapy can be adjusted for optimal results. This study aims to validate RNA sequence data in which the genes PIM2, PKMYT1 and CCDC112 show a significant upregulation in chemo-resistant B- and T-cell canine lymphoma. To make a grounded comparison, expression levels of these three genes were measured with quantitative PCR. PIM2, PKMYT1 and CCDC112 did not show an upregulation in chemo resistant B- or T-cell canine lymphoma. Expression levels of PKMYT1 were significantly higher in T-cells compared to B-cells. CCDC112 showed a higher expression level in B-cells compared to T-cells. Also, CCDC112 expression levels differed significantly in age groups. Since the quantitative PCR results did not validate the RNA sequence data, future research should focus on the underlying reason of differences between qPCR and RNA sequence. Also, the idea that multidrug resistance is not caused by one single gene should be further analysed.

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INTRODUCTION

CANINE LYMPHOMA

A common cancer diagnosed in dogs is lymphoma. Lymphoma is a group of disorders affecting the proliferation of either malignant B-cell or T-cell lymphocytes (1). A well-known form of this disease is the multicentric lymphoma in which lymph nodes in the periphery are affected. Other forms of canine lymphoma (cL) occur in the eyes, skin, lungs, central nervous system, mediastinum or abdomen. The latter can be divided into gastrointestinal, renal, hepatic and splenic lymphoma (2). In the Netherlands, it is estimated that there are 33 cases of canine lymphoma per 100.000 dogs (3). The worldwide incidence of canine lymphoma ranges between 25-134 per 100.000 dogs per year, making it one of the most frequently diagnosed malignant cancers in dogs (2,4,5). However, this incidence is only an estimate based on the documented cases. The incidence of canine lymphoma in the whole population could be much higher.

Dogs are not the only species with a high lymphoma incidence rate. Various studies suggest canine lymphoma and human non-Hodgkin's lymphoma (NHL) are similar in many ways (1,6). Microscopical cL and NHL are remarkably akin along with the clinical symptoms and response to chemotherapy. Ito et al. suggests both diseases are possible passed on genetically (1,3). In the Netherlands, 31 new cases of human lymphoma per 100.000 citizens are registered every year (7).

WHO classification

Canine lymphoma can be categorized in several ways. Since the increasing knowledge of different gradations of cL, the need for a reliable classification system emerged. The World Health Organization (WHO) is the most common used classification system for lymphoma in humans and dogs. Besides the difference in anatomic type, lymphoma can be classified in five different stages. The first stage of lymphoma affects a single lymph node (excluding bone marrow). The second stage continues in multiple lymph nodes in a regional area, excluding the tonsils. When lymph nodes all over the body are involved, it is called the third stage. The fourth stage involves any of the previous three stages and involvement of the liver and/or spleen. The fifth stage is defined as any of the previous stages with manifestation in the blood and involvement of bone marrow and/or other organ (extranodal) systems" (8). A publication by Valli validated the WHO classification as a constant reliable method for diagnosing canine lymphoma with an accuracy of 87% similar to human lymphoma classification accuracy (9).

Cause

The exact cause of canine lymphoma remains unknown. Certain risk factors are known, such as chemical exposure, waste incinerators, polluted sites, radioactive waste, magnetic field exposure and residence near industrial areas. Additionally, it could be argued that a defective immune system such as in the case of autoimmune diseases, immunosuppression or immunodeficiency disorders could play a role in lymphomagenesis. Besides environmental risk factors and the immune system, genetics are emerging as a possible causative factor. In the Otterhound, Rottweiler and Scottisch terrier, certain bloodlines show familial presence of

lymphoma (3). A recent European study showed that certain breeds like the Bernese mountain dog, Boxer, Doberman are predispositioned for canine lymphoma due to their breed (10). Nonetheless there is no hard evidence and the exact influence of these risk factors is unclear. More research is necessary to find a predominant cause.

Prognosis

The prognosis of canine lymphoma cannot be judged solely on its location. Other factors like clinical data, pre-treatment chemotherapy, pathology results, histology, immunophenotype, grade, proliferations markers, molecular prognosticators and biomarkers must be considered to establish the most accurate prognosis (2). As the source of the disease remains unknown, the cause cannot be prevented. Therefore, treating and eliminating lymphoma has the highest priority.

CHEMOTHERAPY

Dogs withheld from lymphoma treatment often die within four to six weeks (11). There are different therapies available for canine lymphoma. As lymphoma is seen as a systemic disease, radiotherapy has its limits in eradicating the cancer. Immunotherapy plays a major part in the human treatment of Non-Hodgkin lymphoma, but still has its limitations in canine treatment. Therefore, chemotherapy is seen as a first-choice therapy (2).

Chemotherapy for canine lymphoma can be divided in single-agent or multi-agent therapy. Single-agent therapy, as the name suggests, uses one pharmaceutical substance to induce lymphocyte- and lymphoblast apoptosis (2,12). The single-agent substance is occasionally safer in terms of toxicity of the patient, but the effectiveness, remission and survival times are inferior to the multi-agent therapies (11). Single-agent therapy uses a simpler protocol, making the treatment generally cheaper than the multi-agent therapy. Overall, chemotherapy with one substance gives a partial to complete result but should be regarded as palliative treatment instead of curative treatment (2). Different substances can be used for single-agent therapy. A few examples are L-asparaginase and Doxorubicin. A summary of the remission duration, survival time and percentage of complete response of L-asparaginase and Doxorubicin is presented in **table 1**. It is important to know that the remission time does not mean the patient is cured. Remission time is defined as follows: “The diminution or abatement of the symptoms of a disease; the period during which such diminution occurs” (13).

Single-agent therapy	**Mean first remission duration**	**Survival time**	**% complete remission**
L-asparaginase	126-196 days (63-256)	220-234 days (98-483)	16.6%

Doxorubicin	309 days (105-475) -> for the dogs with complete remission	322 days (5-475)	52%

Table 1 *Single-agent therapy overview (48-50).*

Multi-agent therapy consists of a combination of drugs with different mechanisms of action and different level of toxicity. This way, a higher response rate and longer remission duration are achieved. The combination of cyclophosphamide, vincristine, doxorubicin and prednisolone (CHOP) (or L-asparaginase CHOP) is currently seen as the most effective combination for high grade canine lymphoma (2). This combination is often used as the base for multi-agent chemotherapy with slight adaptations fitting to the patient. A popular multi-agent therapy of lymphoma is a so called COPLA/LVP treatment. It is a mix of L-asparaginase, vincristine, cyclophosphamide, prednisone and doxorubicin called COPLA in combination with LVP, standing for chlorambucil, vincristine and prednisolone. This treatment showed a complete response in 80% of dogs and a median survival time of 36 weeks (14). A summary of the remission duration, survival time and percentage complete remission of CHOP, L-asparaginase CHOP and COPLA/LVP can be found in **table 2**.

Multi-agent therapy	**Mean first remission duration**	**Survival time**	**% complete remission**
CHOP (cyclophosphamide, doxorubicin, vincristine and prednisolone	219 days	323 days	83.1%
(PEG)L-asparaginase + CHOP	217 days	351 days	85.7%
COPLA/LVP (L-asparaginase + CHOP + chlorambucil, vincristine, prednisone)	175 days	252 days	80%

Table 2 *Multi-agent therapy overview (14,46,47).*

Multi-agent chemotherapy shows a complete remission between 80-90%. This sounds positive, but only 20 to 25% of the patient with a complete remission lives longer than 2 years (15). There are several reasons why chemotherapy does not always work, or why a relapse occurs after chemotherapy. This is also known as treatment failure. It could be due to inadequate dosage of chemotherapy or because the frequency of administration does not match with the patient (11,12). Furthermore, cancer cells can become chemo-resistant.

DRUG RESISTANCE

Drug resistance (DR) can either be present in the animal before treatment (intrinsic) or develop during the time of treatment (acquired). Both forms show a different clinical outcome, but it is thought that the underlying mechanisms work in a similar way (12).

Multiple factors can influence the pattern of drug resistance. It is often more than one mechanism that causes the drug resistance. This is why it is often called multi-drug resistance (MDR).

One role in chemotherapy or drug resistance results from pharmacokinetics reasons. The drug dose and the treatment interval are all patient specific. Not all patients react optimally to the standard dose and interval regime. Without an optimal drug treatment, tumour cells are likely to become chemo-resistant. Patient factors can also influence the optimisation of the drug. Examples are poor drug absorption, an increase or decrease in drug metabolism or an increase in drug clearance (12). The ways in which the tumour surroundings influence the drug's effect on the tumour are presented in **table 3.**

Pharmacodynamics play a bigger role in drug resistance than pharmacokinetics. Tumours are persistent and successful due to their own defence mechanisms. Tumours implement a decrease in cellular drug uptake, increase in drug excretion, drug compartmentalization and/or trigger a change in cellular drug metabolism. All these factors contribute to an insufficient dosage of drugs reaching the tumour. Additionally, tumour cells can influence their response to drugs. Tumours can increase resistance to cell death, increase DNA repair and change their targets, so drugs cannot recognize or bind to their specific targets as can be seen in **table 3** (12). As chemo-resistance greatly influences the success of treatment, knowledge about the underlying mechanisms is important. This article will focus on the failure to achieve cellular response. Two of these mechanisms will be highlighted in the next paragraphs.

Pharmacokinetics			**Pharmacodynamics**	
Iatrogenic	**Host-specific**	**Tumour-specific**	**Failure to reach needed dose of drugs**	**Failure to achieve cellular response**
Wrong drug dose	Poor drug absorption	Insufficient perfusion	Decreased cellular drug uptake	Increased resistance to apoptosis
Wrong treatment interval	Change in systemic drug metabolism	Microenvironment of tumour	Increased drug excretion	Changes in drug target
	Increased drug clearance	Degree of quiescent tumour cells	Drug compartmentalization	Increased repair of drug-induced DNA damage
	Limited drug reach due to organ-barriers		Difference in cellular drug metabolism	

Table 3 *Drug-resistance mechanisms overview (modified from Zandvliet et al. (12)).*

Resistance to apoptosis

Multicellular organisms maintain a balance between cell growth and cell death. A disturbance in this balance can causes uncontrolled cell growth which is a hallmark of neoplasia. When certain pathways prevent cells from going into apoptosis, chemotherapy, often based on inducing the apoptosis pathway, has little effect and drug resistance occurs. It is therefore important to know how apoptosis mechanisms work, how tumour cells can influence apoptosis and how it could be disrupted to maximize chemotherapy.

The apoptosis pathway can be divided in two routes. The extrinsic pathway starts at the cell surface. Tumour-necrosis factor (TNF) receptors, also called death receptors, are a target for TNF ligands and activate the executioner caspases. Executioner caspases go through a complex pathway and eventually initiate cell death. The intrinsic pathway acts through the mitochondria. Members of the BCL2 family, like BID, are transferred to the mitochondria. In the mitochondria, via a complex and not yet fully understood pathway, an apoptotic protease activating factor-1 (APAF1) complex is formed. This complex is cleaved by active executioner caspases and eventually the emerged end-products lead to apoptosis. These pathways are seen as the classic apoptosis pathways. There are also 'apoptotic-like' cell death mechanisms which do not contain caspases, but these pathways are less known (16).

To control such complex pathways a lot of regulators are involved. Certain stress pathways are activated as a response to chemotherapy. This is regulated by members of the protein kinase family. They regulate the AP-1 transcription factors. Genes that are related are AP-1, CD95L and TNF-alfa (16).

Another pathway that is involved is the Janus tyrosine kinase-signal transducers and activators of transcription (JAK-STAT) pathway. STATs are activated by growth-factor receptors similar to the activation of cytokine receptors. STATs have a cytoplasmic signalling role as well as nuclear transcription factors and participate in cell growth and survival (17). Catlett-Falcone et al. provided the first evidence for the role of STAT3 in preventing apoptosis. Inhibition of STAT3 signalling in human myeloma cells induces apoptosis (18). This occurs not solely in myeloma. Holtick et al. adds evidence of the activation of STAT3 leading to apoptosis prevention in classical Hodgkin lymphoma (19).

Drug-induced DNA damage repair.

There are several mechanisms how DNA can be damaged. The daily influence of chemicals, radiation and oxidative stress can cause around 20.000 DNA damages per day (20). A cell limits the amount of damages passing on through checkpoints in the cell division cycle. The cell cycle can be divided in the S, M, G1 and G2 phase. The M phase represents the mitosis (nuclear division) and the cytokinesis (cytoplasmic division). During the S phase DNA is replicated. G1 and G2 function as checkpoints. The regulators of the cell cycle checkpoints are cyclin-dependent protein kinases called Cdks.

During these checkpoints DNA repair mechanisms are active. If DNA is not repaired right, the chance of developing mutations is higher. Mutations can lead to oncogenesis. Excessive chromosome segregation errors are seen as chromosomal instability (CIN). Genome-doubling

is often found in cancer cells and seems to be a precursor of CIN (21). If repair mechanisms are over-active, it has an inhibiting effect on chemotherapy drugs as tumour cells repair the damage easily. There are several repair mechanisms available, including: direct base repair, base excision repair, base pair mismatch repair, interstrand crosslink repair, homologous recombination repair and non-homologous end-joining for double-strand DNA repair (20).

CANDIDATE GENES

Results canine lymphoma research (22) pinpointed three genes as candidate genes for causing chemo-resistance. Namely; PIM2, PKMYT1 and CCDC112. When chemo sensitive cells are compared to chemo resistant cells, all three genes show a significant increase.

PIM2

One member of the protein kinase superfamily is PIM2, also known as PIM-2 Proto-Oncogene, Serine/Threonine Kinase. It is expressed in cells of hematopoietic origin (23). This gene is associated with different types of cancers like lymphoma, leukaemia, hepatocellular carcinoma and colorectal adenocarcinoma (24). PIM2 is part of the JAK-STAT signalling pathway.

The JAK-STAT pathway plays a known variable factor in the success of tumour cells. This pathway is initiated by cytokines oligomerizing their receptor chains, activating a signal. This signal activates the associated JAKs. In turn, JAKs phosphorylate STAT proteins which are then translocated to the nucleus. The STAT proteins stimulate SOCS-1 protein (SOCS-1JABSSI-1). Among other things, SOCS-1 protein works as an inhibitor of JAK (25). In humans, PIM2 phosphorylates and stabilizes the SOCS-1 protein (23). The end goal of the JAK-STAT pathway is to induce apoptosis. Therefore, an upregulation of PIM2 triggers an increase in evading apoptosis.

Cohen et al. found a direct link between the upregulation of PIM2 and non-Hodgkin's lymphoma in humans. With the use of qPCR, PIM2 showed an upregulation of 1.5-2.6 times in non-Hodgkin's lymphoma compared to normal lymphocytes (26). These results are in line with the data of RNA-sequence in canine lymphoma patients. A PIM2 upregulation of 2,38 was seen in B-cell lymphoma ($p=0.02$) (22).

PKMYT1

The gene PKMYT1 encodes for a protein kinase called membrane associated Tyrosine/Threonine 1. PKMYT1 is a member of the WEE-kinase family together with WEE1. It is a membrane-associated kinase located on the endoplasmic reticulum (27). It is part of the cell division cycle and the beta-catenin/TCF signalling pathway.

The cell division cycle has two checkpoints that allows DNA to repair when damaged. To guarantee the right duplication of DNA, the cell cycle is closely regulated by cyclin-dependent kinases (Cdks). Cdks, especially Cdk1, are substrates for the WEE family.

PKMYT1 phosphorylates Thr14 and Tyr15 of Cdk1 (27) and thereby inhibits the activity of Cdk1/cyclin B. This inhibition causes the cell to enter mitosis. The two checkpoints G1 and G2 ensure the DNA replication goes according to plan, and if not, it can be stopped. Without these checkpoints the cell cycle will continue regardless the existing flaws. Mutations can arise and together with an uncontrollable cell growth cancer arises. Most cancer cells have a defective G1 checkpoint, due to a p53 mutation (28,29) and thus fully rely on the G2 checkpoint. As G2 is regulated by Cdk and cyclin B, PKMYT1 as an inhibitor is a potential gene to investigate for its relation to cancer.

In the beta-catenin/ TFC signalling pathway, PKMYT1 has an inhibiting function. It inhibits GSK3beta and thereby triggers beta-catenin TFC signalling. Activation of this pathway causes growth, metastasis, migration, forming of colonies and epithelial mesenchymal transition of hepatocellular carcinoma cells (30). These findings of Liu add evidence for the oncogenic activity of PKMYT1. PKMYT1 is highly expressed in the immune, nervous, muscle, internal, secretory and reproductive system (31). This could suggest PKMYT1 plays a role in different types of cancer and one of them could be lymphoma. RNAsequence data confirms this suggestion as a significant upregulation of 2,89 is seen in B-cell canine lymphoma patients (p=0.003)(22).

CCDC112

Coiled-Coil Domain Containing 112, CCDC112 for short, has many aliases. It is also known as the Mutated In Bladder Cancer Protein 1 (MBC1) (32). CCDC112 encodes for a satellite protein and is involved in ciliogenesis.

CCDC112 encodes for a protein that balances both positive and negative regulators of centrosome duplication to assist the progress of intraflagellar transport, cilium biogenesis and ciliary vesicle trafficking (33). As stated before, the S phase of the cell division cycle consists of DNA replication. Furthermore, the centrosome is duplicated during this phase. If duplication of the centrosome is not done correctly, the duplication can be either incomplete, overcomplete or matured. This can develop as aneuploidy during the mitosis when multipolar or fragmented spindles appear. Aneuploidy is linked to genome instability and a known hallmark of cancer cells (21,33).

Although no research is yet available on the link between CCDC112 and lymphoma, the facts are promising as it is recently associated with cancer cells. Recent study showed a CCDC112 upregulation of 2,38 in lymphoma patients (p=0,02).

RESEARCH QUESTION

Despite extensive research, the exact role of PIM2, PKMYT1 and CCDC112 in canine cancer remains unknown. This article comprises the expression level of the genes PIM2, PKMYT1 and CCDC112 in chemo-resistant canine B-cell and T-cell lymphoma using qPCR to validate RNAsequence data.

Based on comprehensive and recent literature, it is expected that PIM2, PKMYT1 and CCDC112 show an upregulation in chemo resistant B- and T-cells.

MATERIALS AND METHOD

Patient samples

Eighty dogs diagnosed with canine lymphoma of private owners were presented to the faculty of veterinary medicine, University Utrecht for treatment. After clinical examination, the tumours of the dogs were graded based on cytology performed by the clinical pathologist to define the stage of the tumour. Dogs included in this research did not get any prior treatment for their lymphoma (treatment-naïf) and cooperated for a minimal of one follow-up consult.

Tumour samples were collected using a fine needle aspiration biopsy from a neoplastic lymph node. Samples prior to treatment were named sample 1. When the first tumour relapse showed, it was called sample 2. Tumour relapse was assessed by fine needle aspiration biopsy and cytological examination. When the canine lymphoma did not show any signs of being responsive after more than two chemotherapeutical treatments, it was designated as drug resistant (sample 3). All samples were stored in RNAse free containers, snap frozen using liquid nitrogen and stored at minus 70 degrees Celsius until further use.

cDNA synthesis

RNA was isolated from each sample. Each sample was checked using the Nanodrop (ND-1000; Isogen Life Sciences, Utrecht, the Netherlands) to measure RNA content. To form single strand cDNA, iScript Reverse Transcriptase kit was used according to the manufacturer's manual.

Primers

The primers of PIM2 were already available in the lab and provided by the manufacturer. Primers for PKMYT1 and CCDC112 were designed using Primer Select form DNA STAR Lasergene. Amplicon size was set on 80-150 bases, primer temperature on 58-62 degrees Celsius with a difference of 1 degree. Primer length was 18-24 bases. GC's were excluded, and GC clamp was included in the settings. After selecting a primer, it was blasted using the program primer BLAST from NCBI. The primers were ordered from Eurogentec. The primers that were used for this experiment can be seen in **table 4**.

Primers		**Sequence (5'→ 3')**
PIM 2	**Forward primer (**23 bases)	TGA-TCC-GCC-TGC-TTG-ACT-GGT-TG
	Reverse primer (25 bases)	CCT-AAT-GGG-CCC-TGC-TCT-TGT-GTA-TG
PKMYT1	**Forward primer** (24 bases)	TCC-CCA-TCA-GCC-GTC-TCT-TTC-CTC
	Reverse primer (21 bases)	ACT-CTG-GCC-GGC-TTG-GGT-CAT
CCDC112	**Forward primer** (24 bases)	GCG-GCA-AGG-GGG-CTG-GGA-TGA-CTA

Reverse primer (24 bases)	CAG-GGC-CAG-AAA-TTT-TTG-ATA-CCA

Table 4 Forward and reverse DNA primer sequences for PIM2, PKMYT1 and CCDC112.

Quantitative Real Time PCR

20 µL cDNA was diluted 10 times using 180 µL milliQ (mQ). The optimal temperature of PIM2 is 65 degrees Celsius. First, optimal temperatures for the primers of PKMYT1 and CCDC112 were defined by running a 10 µL well gradient on a 384 well format. Standard line dilutions were prepared in an 8-well strip. S1 contains 240 µL pool, S2 contains 15 µL S1 and 225 µL mQ, S3 contains 15 µL S2 and 225 mQ. The fourth well contains 240 µL mQ. Besides the standard dilutions, a master mix was prepared by adding 3,2 µL forward primer, 3,2 µL reverse primer, 74,6 µL mQ and 400,00 µL iQ SYBR Green Supermix. cDNA was pipetted into the first four columns of a 384 well plate (except row P) using a Viaflow. The Master mix was pipetted in the first four wells of row A up and including O. Next, the 384 well format was sealed and inserted in the BIORAD cfx384 for qPCR analysis. Data was analysed using the program BIOTAD CFX manager program.

Secondly, qPCR was performed in duplo using a mix of 11.13 µL forward primer, 11.13 µL reverse primer, 257 µL mQ and 1380 µL iQ SYBR Green Supermix Bio-Rad. The samples were categorized and pipetted according to **table 5**. BIORAD cfx384 started with a 3-minute cycle of 95 degrees Celsius, then 40 cycles were run with a denaturation of 20 seconds at 95 degrees Celsius at ended with an annealing process at a primer specific temperature for 30 seconds and a 30-second elongation step at 72 degrees Celsius. Data were analysed with the use of BIORAD CFX manager program.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
A	S1	A1-1	A20-2	A26-1	A34-2	A39-1	A47-1	A51-3	A55-2	C3-1	C5a-2	C9-3	C14-1	C17-2	C22-1
B	S1	A1-1	A20-2	A26-1	A34-2	A39-1	A47-1	A51-3	A55-2	C3-1	C5a-2	C9-3	C14-1	C17-2	C22-1
C	S2	A4-1	A20-3	A26-2a	A34-3	A39-2	A47-2	A52-1	A56-1	C3-2	C6-1	C10-1	C14-2	C18-1	C23-1
D	S2	A4-1	A20-3	A26-2a	A34-3	A39-2	A47-2	A52-1	A56-1	C3-2	C6-1	C10-1	C14-2	C18-1	C23-1
E	S3	A6-1	A21-1	A26-2b	A35-1	A40-1	A47-3	A52-2	A57-1	C3-3	C6-2	C10-2	C15-1	C18-3	C23-2
F	S3	A6-1	A21-1	A26-2b	A35-1	A40-1	A47-3	A52-2	A57-1	C3-3	C6-2	C10-2	C15-1	C18-3	C23-2
G	S4	A6-2	A21-2	A26-2c	A36-1	A40-3	A48-1	A53-1	A57-2	C4-1	C6-3	C11-1	C15-2	C19-1	C25-1

H	S4	A6-2	A21-2	A26-2c	A36-1	A40-3	A48-1	A53-1	A57-2	C4-1	C6-3	C11-1	C15-2	C19-1	C25-1
I	S5	A10-1	A23-1	A28-1	A36-2	A45-1	A48-2	A53-3	A58-1	C4-2	C8-1	C11-2	C15-3	C19-2	C25-2
J	S5	A10-1	A23-1	A28-1	A36-2	A45-1	A48-2	A53-3	A58-1	C4-2	C8-1	C11-2	C15-3	C19-2	C25-2
K	S6	A11-1	A23-2	A31-1	A37-1	A46-1	A49-1	A54-1	A59-1	C5-1	C8-2	C12-1	C16-1	C20-1	
L	S6	A11-1	A23-2	A31-1	A37-1	A46-1	A49-1	A54-1	A59-1	C5-1	C8-2	C12-1	C16-1	C20-1	
M	S7	A15-1	A25-1	A32-1	A38-1	A46-2	A50-1	A54-2	A59-2	C5-2	C9-1	C13-1	C16-2	C20-2	
N	S7	A15-1	A25-1	A32-1	A38-1	A46-2	A50-1	A54-2	A59-2	C5-2	C9-1	C13-1	C16-2	C20-2	
O	NTC	A20-1	A25-2	A34-1	A38-2	A46-3	A51-1	A55-1	C2-1	C5a-1	C9-2	C13-2	C17-1	C21-1	
P	NTC	A20-1	A25-2	A34-1	A38-2	A46-3	A51-1	A55-1	C2-1	C5a-1	C9-2	C13-2	C17-1	C21-1	

Table 5 *384 well format example.*

Reference genes

Genes B2M, GAPDH, SDHA, TBP and YWHAZ were tested as reference gene using the program CFX Maestro for specificity and efficiency. GAPDH, SDHA and YWHAZ had the best efficiency (range 92.1% - 98.1%) and were most fitted as reference genes.

Statistical analysis

The programs Microsoft Excel and SPSS were used for the analysis of the data. The fold change (fc) was calculated with the formula $2^{(-\Delta\Delta Ct)}$. Results were significant if the p-value was <0.05.

First all data were checked for normality using the program SPSS. The Shapiro-Wilk test, a histogram and a normal Q-Q plot were used to determine the normality of the fold change of PIM2, PKMYT1 and CCDC112. Most data were not distributed normally. Therefore, non-parameter tests were used for further analysis.

The Kruskal-Wallis test was used to determine whether there is a difference in fold change between chemo-sensitive and chemo-resistant cells in the groups age or WHO. The Mann-Whitney test was used to test if there is a difference in fold change between code, sex and MDR for PIM2, PKMYT1 and CCDC112. Unidentified samples with no verification whether it is a B-cell or a T-cell lymphoma, were excluded for further analysis.

Next, samples were paired. The data were divided into two groups; B-cell lymphoma and T-cell lymphoma. Each group consists of sample 1,2 and 3. In order to check the hypothesis it is important to look at the chemo-sensitive cells (MDR0) and the chemo-resistant cells (MDR1). Therefore, the first sample (chemo-sensitive) of a patient was compared to the last sample of the patient, which showed signs of chemo-resistance. This means sample 1 was compared to

either sample 2 or 3, depending which sample showed drug resistance. The Wilcoxon Signed Ranks test was applied for the paired samples within the B-cell group and the T-cell group.

RNA sequence

RNA-seq was used according to the manufacturer's guide. cDNA samples were used for qPCR and RNA-seq. RNA sequence data was first normalized according to gene length, but soon corrected to normalization according to mean reads per sample (set on one million) as advised by the manufacturer.

RESULTS

RESULTS

PIM2

A Kruskal-Wallis test indicated that there were no statistically significant differences between PIM2 fold change of WHO group 1 (*Mean Rank* = 12.00), group 3 (*Mean Rank* = 57.58), group 4 (*Mean Rank* = 55.03) and group 5 (*Mean Rank* = 48.21), *H* (corrected for ties) = 3.633, *df*=3, *N*=104, *p*=0.304. Note: there were no samples for group 2.

A Kruskal-Wallis test indicated that there were no statistically significant differences between PIM2 fold change of age 1 (*Mean Rank* = 12.00), age 2 (*Mean Rank* = 29.20), age 3 (*Mean Rank* = 49.00), age 4 (*Mean Rank* = 41.33), age 5 (*Mean Rank* = 53.06), age 6 (*Mean Rank* = 57.33), age 7 (*Mean Rank* = 50.60), age 8 (*Mean Rank* = 53.11), age 9 (*Mean Rank* = 36.43), age 10 (*Mean Rank* = 70.00), age 11 (*Mean Rank* = 63.83), age 12 (*Mean Rank* = 56.71), age 13 (*Mean Rank* = 87.50) and age 14 (*Mean Rank* = 48.00), *H* (corrected for ties) = 12.967, *df*=13, *N*=104, *p*=0.450.

A Mann-Whitney U test indicated that there were no statistically significant differences between fold change expression in male participants (*Mean Rank* = 53.72, *N*=65) and fold change expression in female participants (*Mean Rank* = 50.46, *N*=39), *U*=1188.000, *z*=-0.534 (corrected for ties), *p*=0.593, two-tailed.

A Mann-Whitney U test indicated that there were no statistically significant differences between fold change expression in B-cells (*Mean Rank* = 48.49, *N*=67) and fold change expression in T-cells (*Mean Rank* = 43.15, *N*=26), *U*=771.000, *z*=-0.856 (corrected for ties), *p*=0.392, two-tailed (**figure 1**).

A Wilcoxon signed rank test indicated that there were no significantly differences in fold change expression of MDR1 paired B-cell samples compared to MDR0 paired B-cell samples, *T*= 56.00, *z*=-1.285 (corrected for ties), *N*-Ties=18, *p*=0.199 (**figure 3**).

A Wilcoxon signed rank test indicated that there were no significantly differences in fold change expression of MDR1 paired T-cell samples compared to MDR0 paired T-cell samples, *T*= 3.00, *z*=0.000 (corrected for ties), *N*-Ties=3, *p*=1.000 (**figure 6**).

PKMYT1

A Kruskal-Wallis test indicated that there were no statistically significant differences between PKMYT1 fold change of WHO group 1 (*Mean Rank* = 65.00), group 3 (*Mean Rank* = 53.65), group 4 (*Mean Rank* = 48.89) and group 5 (*Mean Rank* = 54.50), *H* (corrected for ties) = 0.897 *df*=3, *N*=104, *p*=0.826. Note: there were no samples for group 2.

A Kruskal-Wallis test indicated that there were no statistically significant differences between PKMYT1 fold change of age 1 (*Mean Rank* = 65.00), age 2 (*Mean Rank* = 55.80), age 3 (*Mean Rank* = 36.33), age 4 (*Mean Rank* = 31.00), age 5 (*Mean Rank* = 57.21), age 6 (*Mean*

Rank = 61.58), age 7 (*Mean Rank* = 35.40), age 8 (*Mean Rank* = 47.67), age 9 (*Mean Rank* = 59.43), age 10 (*Mean Rank* = 55.20), age 11 (*Mean Rank* = 40.50), age 12 (*Mean Rank* = 72.43), age 13 (*Mean Rank* = 25.00) and age 14 (*Mean Rank* = 12.00), *H* (corrected for ties) = 15.833, *df*=13, *N*=104, *p*=0.258.

A Mann-Whitney U test indicated that there were no statistically significant differences between male participants (*Mean Rank* = 52.66, *N*=65) and female participants (*Mean Rank* = 52.23, *N*=39), *U*=1257.000, *z*=-0.071 (corrected for ties), *p*=0.944, two-tailed.

A Mann-Whitney U test indicated that the fold change expression in T-cells (*Mean Rank* = 58.38, *N*=26) was higher than the fold change expression in B-cells (*Mean Rank* = 42.58, *N*=67), *U*=575.000, *z*=-2.534 (corrected for ties), *p*=0.011, two-tailed. The formula of Clark-Carter (34) converts *z* into r: $r = \frac{z}{\sqrt{N}} = -0.263$. According to Cohen's conventions, this is considered a medium sized effect (35)(**figure 1**).

A Wilcoxon signed rank test indicated that there were no significant differences in fold change expression of MDR1 paired B-cell samples compared to MDR0 paired B-cell samples, *T*= 80.00, *z*=-0.240 (corrected for ties), *N*-Ties=18, *p*=0.811 (**figure 4**).

A Wilcoxon signed rank test indicated that there were no significant differences in fold change expression of MDR1 paired T-cell samples compared to MDR0 paired T-cell samples, *T*= 0.00, *z*=-1.604 (corrected for ties), *N*-Ties=3, *p*=0.109 (**figure 7**).

CCDC112

A Kruskal-Wallis test indicated that there were no statistically significant differences between CCDC112 fold change of WHO group 1 (*Mean Rank* = 3.00), group 3 (*Mean Rank* = 52.58), group 4 (*Mean Rank* = 47.06) and group 5 (*Mean Rank* = 58.17), *H* (corrected for ties) = 5.314, *df*=3, *N*=104, *p*=0.150. Note: there were no samples for group 2.

A Kruskal-Wallis test indicated that there were statistically significant differences between CCDC112 fold change of age 1 (*Mean Rank* = 3.00), age 2 (*Mean Rank* = 80.80), age 3 (*Mean Rank* = 50.33), age 4 (*Mean Rank* = 89.00), age 5 (*Mean Rank* = 61.15), age 6 (*Mean Rank* = 50.08), age 7 (*Mean Rank* = 28.90), age 8 (*Mean Rank* = 40.33), age 9 (*Mean Rank* = 37.43), age 10 (*Mean Rank* = 53.60), age 11 (*Mean Rank* = 56.83), age 12 (*Mean Rank* = 56.86), age 13 (*Mean Rank* = 46.50) and age 14 (*Mean Rank* = 2.00), *H* (corrected for ties) = 26.782, *df*=13, *N*=104, *p*=0.013. The formula $\eta^2 = \frac{H}{\mathrm{N}-1} = 0.260$ was used to interpret the proportion of variance. According to Cohen's (35) conventions, this effect could be considered as "large" (**figure 2**).

A Mann-Whitney U test indicated that there were no statistically significant differences between male participants (*Mean Rank* = 51.52, *N*=65) and female participants (*Mean Rank* = 54.13, *N*=39), *U*=1204.000, *z*=-0.426 (corrected for ties), *p*=0.670, two-tailed.

A Mann-Whitney U test indicated that the fold change expression in B-cells (*Mean Rank* = 54.34, *N*=67) was higher than the fold change expression in T-cells (*Mean Rank* = 28.08, *N*=26), *U*=379.000, *z*=-4.212 (corrected for ties), *p*=0.000, two-tailed. The formula of Clark-Carter (34) converts *z* into r: $r = \frac{z}{\sqrt{N}} = -0.437$. According to Cohen's conventions, this is considered a medium sized effect (35).

A Wilcoxon signed rank test indicated that there were no significant differences in fold change expression of MDR1 paired B-cell samples compared to MDR0 paired B-cell samples, *T*= 65.00, *z*=-0.893 (corrected for ties), *N*-Ties=18, *p*=0.372 (**figure 5**).

A Wilcoxon signed rank test indicated that there were no significant differences in fold change expression of MDR1 paired T-cell samples compared to MDR0 paired T-cell samples, *T*= 3.00, *z*=0.000 (corrected for ties), *N*-Ties=3, *p*=1.000 (**figure 8**).

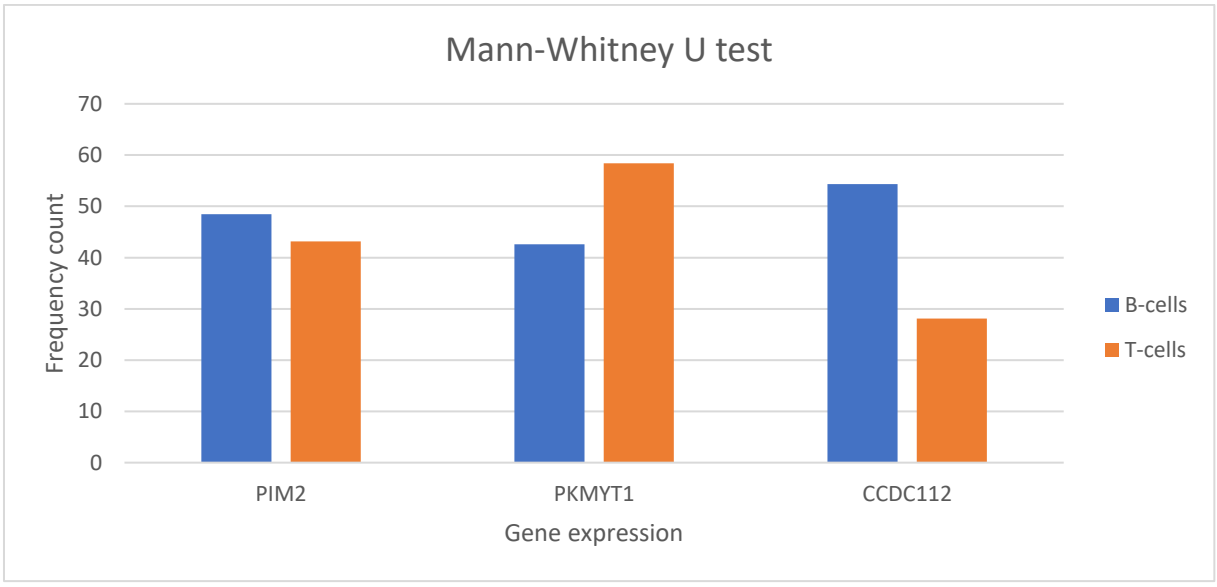

Figure 1 Mann-Whitney U test, frequency count (in mean ranks) of B- and T-cells PIM2, PKMYT1 and CCDC112.

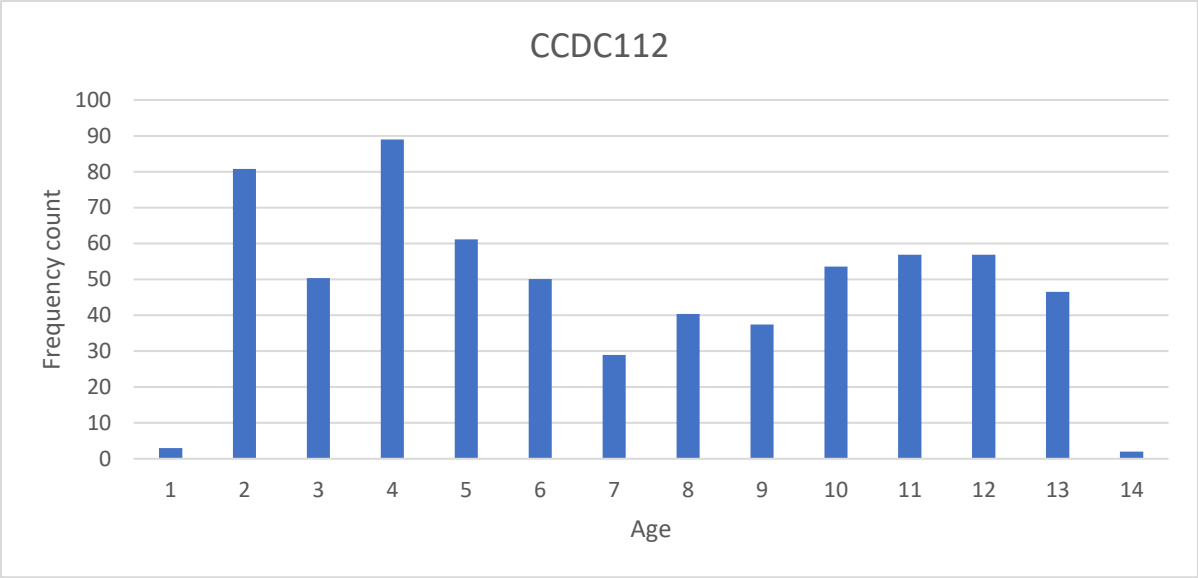

Figure 2 Kruskal-Wallis test, frequency count (in mean ranks) of the ages 1-14 of CCDC112.

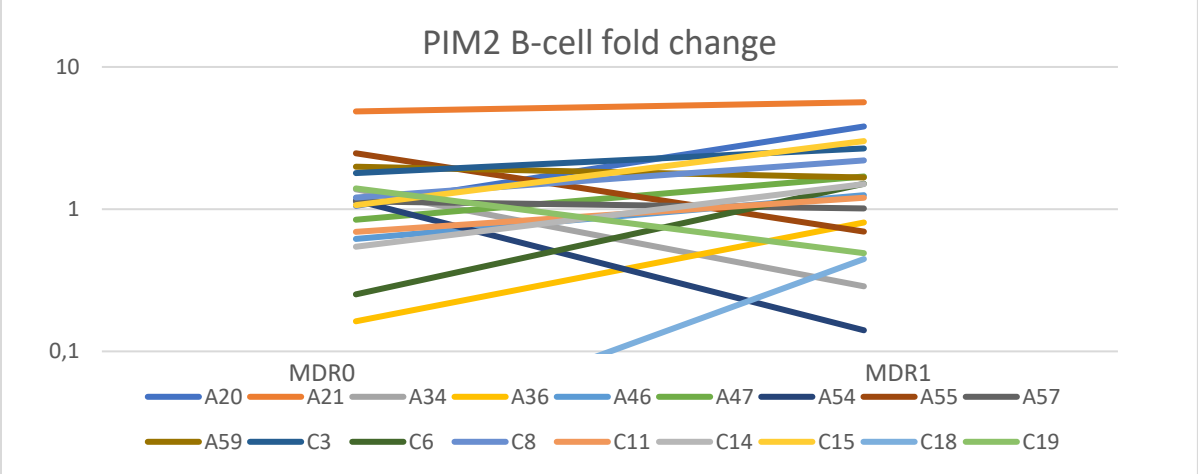

Figure 3 Difference in *fold change between chemo-sensitive cells (MDR0) and chemo-resistant cells (MDR1) of B-cell PIM2 in paired samples*. One line represents two samples of a patient.

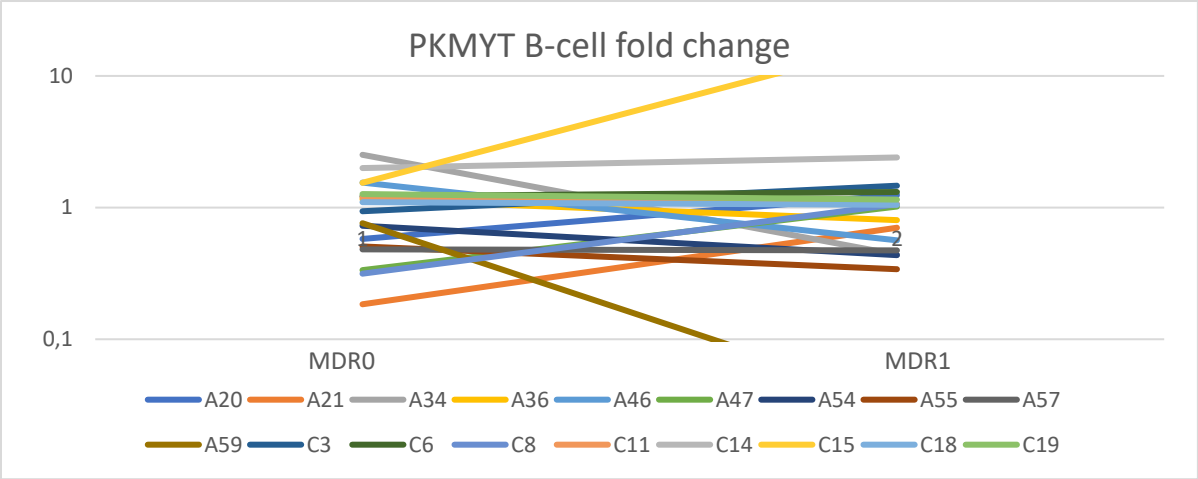

Figure 4 Difference in *fold change between chemo-sensitive cells (MDR0) and chemo-resistant cells (MDR1) of B-cell PKMYT1 in paired samples*. One line represents two samples.

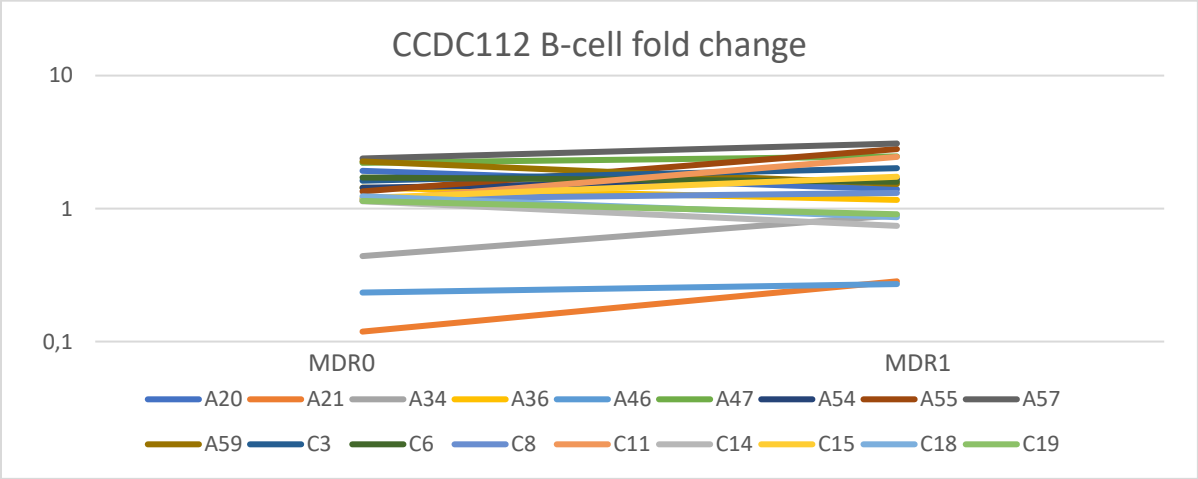

Figure 5 Difference in *fold change between chemo-sensitive cells (MDR0) and chemo-resistant cells (MDR1) of B-cell CCDC112 in paired samples*. One line represents two samples of a patient.

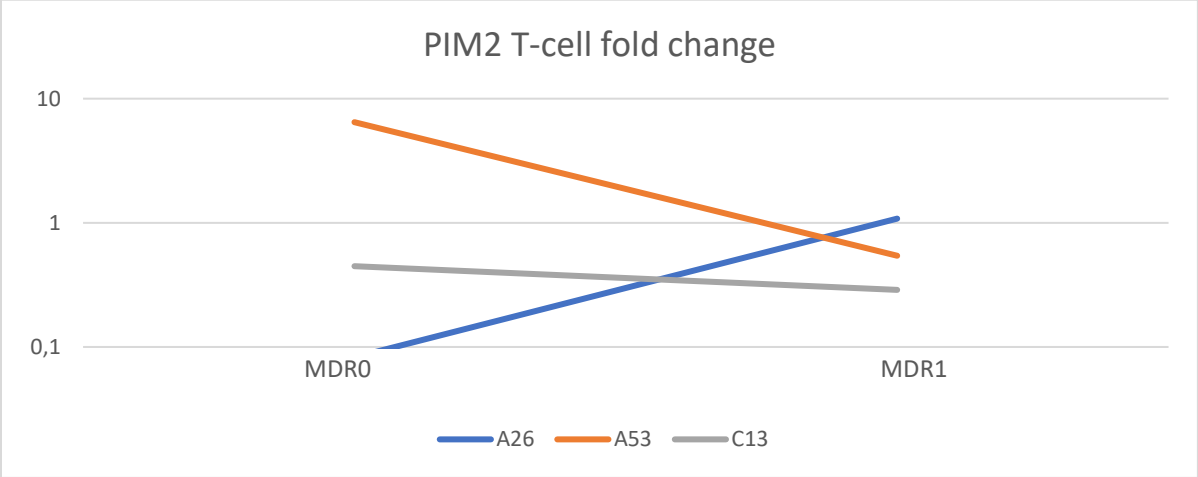

Figure 6 Difference in *fold change between chemo-sensitive cells (MDR0) and chemo-resistant cells (MDR1) of T-cell PIM2 in paired samples*. One line represents two samples of a patient.

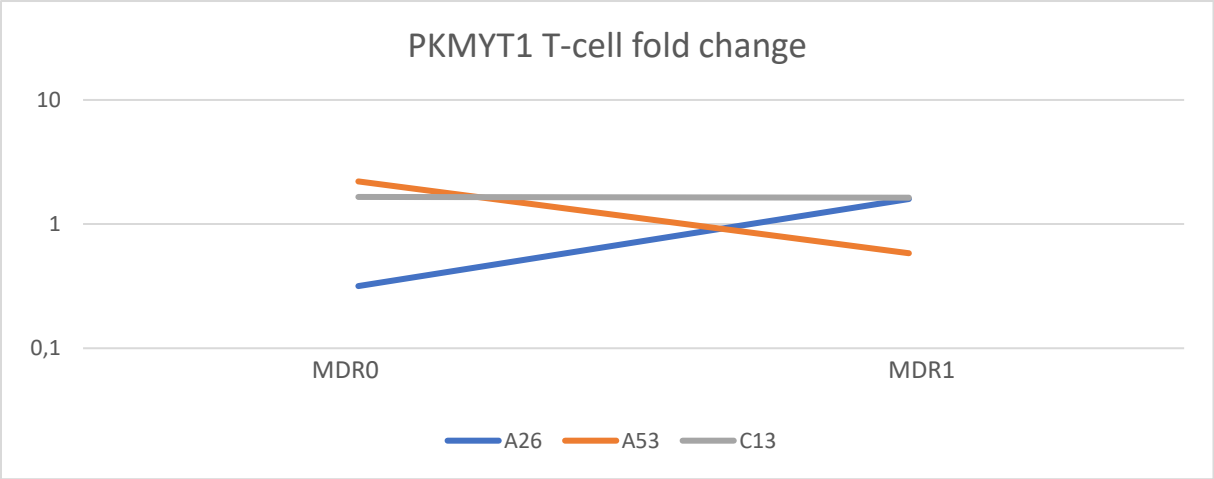

Figure 7 Difference in *fold change between chemo-sensitive cells (MDR0) and chemo-resistant cells (MDR1) of T-cell PKMYT1 in paired samples*. One line represents two samples of a patient.

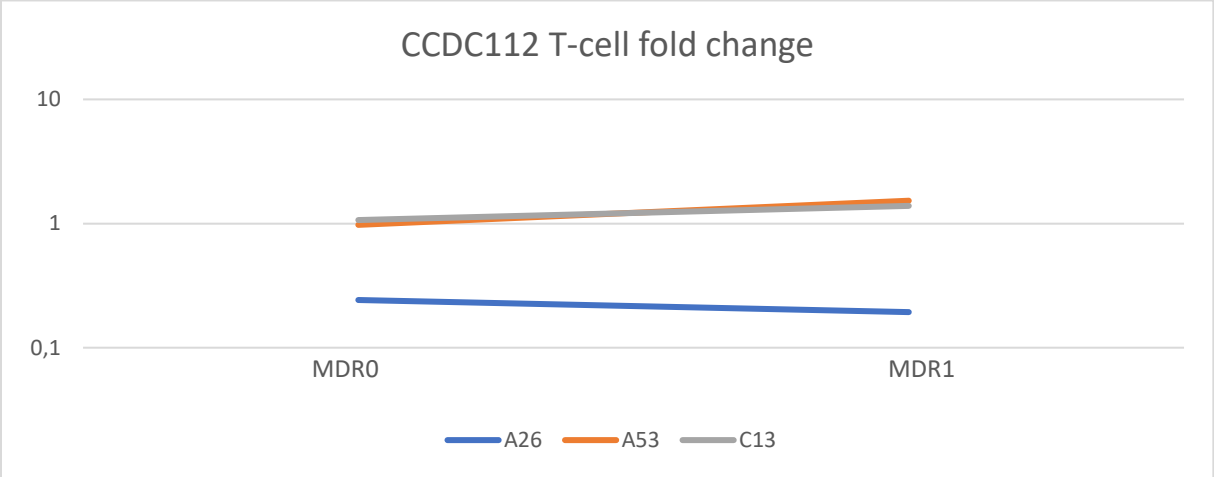

Figure 8 Difference in *fold change between chemo-sensitive cells (MDR0) and chemo-resistant cells (MDR1) of T-cell CCDC112 in paired samples*. One line represents two samples of a patient.

RNA SEQUENCE VERSUS QPCR

To validate the RNA sequence data (22), expression levels of RNAseq and qPCR were compared. RNAseq is expressed in Reads Per Kilobase Million (RPKM) and qPCR in delta CT. Both are shown in percentages of expression levels in the MDR1 sample compared to the MDR0 sample being 100% (**table 6**).

	PIM2		**CCDC112**		**PKMYT1**	
	RNAseq	qPCR	RNAseq	qPCR	RNAseq	qPCR
A20	X	-1,6	X	-16,7	X	-160
A34	-43,2	+48,2	+104,3	-15,4	+91.6	+38,1
A47	+92,4	-18,6	+626,7	-3,7	X	-16,8
A55	-100	+47,3	-82,2	-21,2	X	+6,5
A57	0	+3,4	X	-8,9	X	+2,3
C3	+435,3	-13,2	+9	-6,6	X	-8,1
C6	+392,6	-36,1	+148	+2,4	+357,1	-1,4
C11	+180,6	-14	+178,2	-20,9	-52,3	+2,2
C14	+431,3	-24,1	X	+11,6	+98,3	-3,9
C18	+102,5	+15,5	+2,2	+10,4	+302,8	+0,8
C19	-26	+32,2	-87,5	+6,3	+350	+1,8
C23	+124,8	+34,8	+106,6	-0,7	+610	+27,1

Table 6 *Comparison between RNA sequence and qPCR expression levels of PIM2, CCDC112 and PKMYT1 in percentages* (%). MDR1 samples are compared to MDR0 samples being 100%. X= no data was measured, so it cannot be set as 100% (22).

DISCUSSION AND CONCLUSIONS

Therapeutic results after lymphoma treatment are still not perfect. Reoccurrence rate of tumour relapse is high, even after initial complete treatment. There is little knowledge about which genes contribute to (multi)drug resistant. If certain genes could be highlighted, therapy can be adjusted to prevent drug resistant cells. In this study, the expression of the genes PIM2, PKMYT1 and CCDC112 were measured with qPCR in chemo-sensitive B- and T-cells before chemotherapy and in chemo-resistant B- and T-cells during or after chemotherapy in canine lymphoma patients.

PIM2

Results from qPCR showed no significant difference in expression level in WHO group, age, sex, MDR and code (**figure 1**) for PIM2. **Figures 3** and **6** represent the shift in foldchange for PIM2 between chemo-sensitive B- and T-cells and chemo-resistant B- and T-cells. Both figures imply that there is no significant shift in foldchange for the gene PIM2. A Wilcoxon Signed Ranks tests confirms there is no significant increase or decrease in PIM2 expression between MDR0 and MDR1 in B- or T-cells. In contrast with the expectations, PIM2 expression was not increased in chemo-resistant tumour cells.

The qPCR results do not match with the RNAsequence data. **Table 6** shows the percentage of expression levels of MDR1 when MDR0 is set on 100%. Both RNA sequence data and qPCR data are present and show differences in expression level. Eight samples are contradicting each other in up- or downregulation. When looking at the whole lymphoma patient group, RNA sequence shows a significant increase in PIM2 expression of 2.38 times in MDR1 samples compared to MDR0.

The qPCR results are also not in line with the upregulation of PIM2 in chemo resistant lymphocytes found in a study about non-Hodgkin lymphoma in humans (26). PIM2 upregulation is not only seen in lymphoma (26,36), other cancer types have promising results of PIM2 upregulation as well. Examples are an upregulation of 3.7 times PIM2 expression level when compared to MDR0 in Kapsosi's sarcoma-associated virus (KSHV) (37) and a PIM2 upregulation in multiple myeloma (38).

In conclusion PIM2 appears not to be significantly upregulated in chemo-resistant lymphoma B- or T-cells. Although changes are seen in individual dogs.

PKMYT1

No significant differences were found in expression levels for WHO group, age, sex and MDR. However, the fold change expression in T-cells is significantly higher than the fold change expression in B-cells **(figure 1**). **Figures 4** and **7** display no significant difference in fold change expression for PKMYT1 in neither paired MDR1 B- nor paired MDR1 T-cells. The cut-off value on the logarithmic scale was set on 0,1 and 10. This result can be confirmed by the outcome of the Wilcoxon signed rank test. No significant positive or negative ranks could be identified between MDR0 and MDR1.

This result is contradicting the results found in the same set of canine lymphoma patients using RNAsequence. As can be seen in **table 6**, the percentage of expression levels of chemo-resistant cells in RNA sequence show a different percentage when compared to the qPCR results. Two samples contradict each other, one states an upregulation and the other states a downregulation. When the whole RNA sequence lymphoma data is checked, a significant upregulation of 2,89 times PKMYT1 expression compared to MDR0 was seen.

There is no similar research available. However, there are promising results showing the carcinogenic effect of overexpressed PKMYT1. Research of Liu showed that the upregulation of PKMYT1 enhanced the migration and growth of cells, promoting the malignant phenotypes of hepatocellular carcinoma (30).

To conclude, PKMYT1 appears not show a significant upregulation in chemo-resistant B- or T-cells. Nevertheless, expression level of PKMYT1 is higher in T-cells compared to B-cells. Changes in expression level are seen in individual dogs.

CCDC112

No significant differences in expression level is observed in WHO, sex and MDR. However, a significant difference is observed in age and code. The expression level is high in the ages of two and four, but minimal in ages one, seven to nine and fourteen (**figure 2**). According to **figure 1**, CCDC112 has almost doubled in expression level in B-cells compared to T-cells. **Figures 5** and **8** represents the fold change expression of CCDC112 in MDR0 and MDR1 samples. These figures imply that there is no significant up- or down-regulation of CCDC112. This suggestion is endorsed by the Wilcoxon signed rank test. The qPCR results of CCDC112 did not show a significant upregulation in chemo-resistant B- or T-cells, thus in contrast with the expectations, CCDC112 expression was not increased in chemo-resistant tumour cells.

The qPCR results contradict the data collected with RNAsequence of the same group of patients as can be seen in **table 6**. Six samples have contradicting results as either RNA-seq stating an up- or downregulation and qPCR the opposite. RNAsequence data of the complete set of lymphoma patients showed a significant CCDC112 upregulation of 2,38 times CCDC112 expression in chemo-resistant cells compared to MDR0.

Although research has not linked CCDC112 expression level to lymphoma, recent results link CCDC112 to cancer hallmarks (33). This can be seen as a step in the right direction.

In conclusion, CCDC112 has a higher expression in B-cells compared to T-cells, but appears not to show a significant upregulation in chemo-resistant B- or T-cell lymphoma. Although changes are seen in individual dogs.

RNA sequence data versus qPCR results

PIM2, PKMYT1 and CCDC112 all show inconsistencies when qPCR and RNA sequence data are compared, as can be seen in **table 6**. Surprisingly, eight out of ten PIM2 samples seem in contrast with each other. Whereas one test shows a down-regulation, the other shows an upregulation of the gene. This is also the case for PKMYT1 where three out of seven sample are in contrast with each other. **Table 6** shows a six out of nine inconsistency for

CCDC112. For example, CCDC112 expression has doubled six times at the time of chemo-resistance according to RNA-seq. The qPCR results however imply CCDC112 expression increased with 3.7 percent.

Nagalakshmi et al. compared gene expression levels with RNAsequence and qPCR as well. A strong correlation of 98% was found between both results (39). Likewise, Wu et al. demonstrated strong similarities of more than 84% with a small standard error between foldchange data of qPCR and the RNA sequence data (40).

Literature implies that it is possible to use qPCR to validate RNA sequence but does not always correlate strongly. In this article qPCR and RNA-seq results do not match significantly One theory is that the increase in gene expression levels in RNA sequence is more or less a factor two, which could be insufficient for RNA sequence to notice. The total amount of reads was 1.2 million on average. The low amount of reads per cell of RNA sequence could give different results between RNA-seq and qPCR as suggested by Wu (40). Low total amount of reads could be due to low quality or low quantity of RNA. This is a known challenge of RNA sequencing. An example of low quantity RNA samples is from tumour samples (41). Adiconis et al. compared five methods suitable for low-input RNA samples. For gene expression RNA-sequence, the SMART method showed a r=0.860 correlation with the control library for a low-quantity library. The low-quality RNA correlated to the control library by RNase H method (r=0.962) and Ribo-Zero method (42). Every method has an own protocol as can be seen in previous research by Adiconis (42). Future research should acknowledge these methods to optimize RNA-seq results for gene expression.

Another theory is that small differences in expression cannot be tested reliably with RNA sequence. Specific gene markers for B-cell or T-cell lymphoma are clearly distinguishable in RNA-seq and qPCR. CD19 expression is significantly higher in B-cell lymphoma compared to T-cell lymphoma with a factor 19.22. TCF1 expression is five times higher in T-cell lymphoma compared to B-cell lymphoma (22). PIM2, PKMYT1 and CCDC112 show a gene expression of factor two. Research of Nagalakshmi et al. showed a strong correlation between RNA-seq and qPCR, but found differences in the low expressed genes (39). The precise boundary between small and big differences is not clear. Factor two could be too subtle, but factor five just adequate.

Surprisingly, some qPCR reference markers showed a zero-expression level in RNA sequence. For example, few patients showed a zero expression of SDHA and YWHAZ. These markers are tested for efficacy (E) and the coefficient of determination (R^2) through qPCR and are thought to be stable reference markers.

Challenges of research

There were certain challenges along the road of research. Samples which could not be defined as T- or B-cell lymphoma were excluded from the data. The data which has been included, were divided in B-cell lymphoma and T-cell lymphoma. RNA-sequence has shown distinct gene expression levels between these two groups. Dividing the group in B-cell and T-cell

lymphoma, narrowed down the T-cell group remarkably. The presence of only three patients, giving 6 samples, made it difficult to find significantly important results.

As the research question specified chemo-sensitive and chemo-resistant cells, a remarkable amount of lymphoma patient data was excluded for the second part of the research. Only patients with a chemo-sensitive first sample and a chemo-resistant second or third sample were included. It is debatable whether the excluded data is in fact not fitting to the research. It could be questioned whether the norm or cut-off value for chemo resistance is in fact fitting. Some might argue that every cell is chemo-resistant to a certain extent. Chemo resistance is a complex process. This research, previous research and unpublished data (22, 43) acknowledged that the probable cause of drug resistance is not due to one gene. One theory implies that there are always drug-resistant cells present in tumour cells. This explains why patients with the diagnosis 'complete remission', show a 75-80% relapse within two years after treatment (15).

Another theory elaborates on the first theory and implies that drug resistance evolves during time. If a patient gets a certain chemotherapy X, it is likely resistant cells are present for chemotherapy X. When cDNA samples are taken at that moment, it is likely that genes activating or interfering with pathway X will show high gene expression levels. When the same patient gets a new chemotherapy Y, it is probable that other cells in the tumour have defence mechanisms against chemotherapy Y. Samples taken differently in time could show different genes being activated (22, 44)

Perhaps, when taking these theories into account multidrug resistance is not the perfect category. Based on the assumption every tumour has drug-resistant cells, it is best to look at length of complete response instead of the response itself. The disease-free period ((DFP) is an indicator of the success of chemo-therapy (45) and should be prolonged to achieve smaller tumour size after treatment and a longer success ratio.

Ultimately, different tumours cells can use different tumour mechanisms at the same time or after each other. This makes it difficult to use analysis methods like RNAseq and statistics for a small onset of patient samples. Future research should focus on finding overarching pathways in a large set of patients. A cluster analysis will help with the formation of chemo-resistant subgroups. Finding overarching pathways can eventually help to personalize chemotherapy and thereby prolonging the disease-free period for each patient.

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