Genetic variations in the Canine CYP2B11 gene

Implications for Veterinary Medicine?

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Abstract

In veterinary medicine, the anaesthetic Propofol and the anticancer agent Cyclophosphamide are widely used. These compounds need to be metabolized by a cytochrome and in the canine, CYP2B11 is responsible for this. To further elucidate the cause of variations in metabolism of these compounds, this study aimed to identify and confirm variability in the CYP2B11 gene and predict effects on enzyme phenotype. By PCR reaction, all exonic regions of CYP2B11 in 20 canines of different breeds were amplified and submitted for sequencing. The retrieved sequences were reviewed for variations and these variations were tested to confirm and quantify by Restriction Fragment Length Polymorphism. The sample population consisted of a panel of 100 dogs of 11 breeds and mixed bred dogs, chosen to represent the US dog population. 3 Exonic SNPs were identified; a synonymous G>A SNP in exon 1 (2B11*1) could however not be confirmed and quantified in the larger population. A non-synonymous C>T SNP was found in exon 2 (2B11*2), this was present in the population with an allele frequency of 8% but mainly in Labrador Retrievers. This polymorphism was predicted to have a probable damaging effect on CYP2B11. A potential splice site G>A SNP (2B11*3) was found in exon 7 and showed to be widely distributed, having an allele frequency of 65% in the overall population. Besides these exonic SNPs, two intronic SNPs were identified but not tested to confirm. The reference sequence differed from all tested dogs in exon 6, maybe representing a SNP in the Boxer used for this reference sequence. These polymorphisms could have major effects on the function of CYP2B11 and these results therefore prove a need for further investigation.

Keywords: Cytochrome, CYP2B11, genetic variation, SNP, polymorphism, canine, dog population

Introduction

Cytochromes are a superfamily of enzymes that are found universally, from bacteria and plants to mammals, including humans[17]. In mammals, they are responsible for the metabolism of many compounds, both endogenous such as hormones, and exogenous such as medicines and environmental toxins. Based upon their identity, the cytochrome enzymes are subdivided into families (marked by a number, e.g. CYP2) and subfamilies marked by a letter (e.g. 2B). Although the cutoff values for family and subfamily membership are 40% and 55% amino acid identity [22], members of the same family generally share a sequence homology of ≥59%. Members of the same subfamily are usually ~70% similar.[17]

Cytochrome 2B11 is the orthologue of human 2B6 in the canine. It was first discovered as the major Phenobarbital-inducible liver enzyme in the dog and thus initially named PBD-2.[8] After further investigation, the amino-acid sequence was discovered by Graves et al.[14], it was recognized that this enzyme is actually a cytochrome belonging to the CYP2B subfamily. [14] Functional analyses have shown that the enzyme is involved in the metabolism of many substances. It has been proven to be responsible for the metabolism of endogenous androstenedione and progesterone.[9] Exogenous compounds that are metabolized by CYP2B11 are 2,2',4,4',5, 5'-Hexachlorobiphenyl, a PCB that only dogs can metabolize[8], Propofol[15], one of the most frequently used anesthetics in veterinary medicine, and oxazaphosphorines[4]. As with other hepatic cytochromes, the expression of CYP2B11 is highly responsive to induction and inhibition. Phenobarbital is the major inducer of the enzyme, causing a 9.9-fold increase in activity *in vivo.*[13]An important inhibiting compound is the antibiotic Chloramphenicol[5,15], which thus prolongs Propofol- induced anesthesia.[20]

The oxazaphosphorines Cyclophosphamide (CP) and Ifosfamide (IF) are chemotherapeutica that are widely used in human medicine. They are being used in treating rheumatoid arthritis, malignant lymphomas and various bone and soft tissue sarcomas,[2] among others. These chemotherapeutics are what is called pro-drugs and need to be activated by a cytochrome. In

humans, CYP2B6 is responsible for this, in the canine it is CYP2B11[4]. The activation reaction consists of hydroxylation of CP and IF to 4-Hydroxycyclophosphamide and 4-Hydroxyifosfamide, respectively. These metabolites are not cytotoxic and can diffuse into cells easily. 4-OH-CP is in equilibrium with aldophosphamide, and undergoes spontaneous decomposition, separating into phosphoramide mustard and acrolein. The phosphoramide mustard is the actual cytotoxic component, being a bi-functional DNA-alkylating agent. The byproduct acrolein is a very reactive aldehyde that may enhance the toxicity of phosphoramide mustard by depleting the cell of glutathione[2].

In the quest for new anticancer methods in humans, it has been noticed that the canine CYP2B11 has a particularly high K_m for the hydroxylation of –especially – CP, but also IF. [4,16] This has been the focus point for further investigation and the potential use of CYP2B11 in anticancer gene therapy. In vitro experiments showed that cells expressing CYP2B11 have a 100- to 250- fold lower EC50 for CP and IF than cells expressing CYP2B6 .[16]

The oxazaphosphorines have also become of use in veterinary medicine, with indications similar to those in humans. Cyclophoshamide is used in the treatment of immune mediated diseases such as Immune Mediated Hemolytic Anemia[3] and as an antineoplastic agent. Among other applications, it has been incorporated into a the CHOP protocol. The CHOP protocol is a combination chemotherapy protocol with Vincristine, Doxorubicin, Cyclophosphamide and Prednisone, and is an important basis for many lymphoma treatments in companion animals.[19]

The intrinsic goal of anticancer chemotherapy is to reach toxicity in tumor cells. Unfortunately, it is not (yet) possible to get selective toxicity against the cancer cells only. In order to get the desired anti-tumor effect , it is inevitable that the animal experiences some side effects from the chemotherapy. However, because the primary goal is to prolong the life of the animal and/or increase the quality of life rather than to achieve a complete cure, the limit of acceptable versus unacceptable side effects is reached earlier in companion animals than in humans.

For cyclophosphamide and ifosfamide the most important side effects are myelosuppression (neutropenia, thrombocytopenia) and sterile hemorrhagic cystitis (especially with Ifosfamide)[23,24]. Usually, these toxicities are relatively mild (Grade I or II out of V) but they can result in sepsis and death.[23]

A study in humans has shown that both side effects and tumor response for Cyclophosphamide are negatively associated with the Area Under the Curve (AUC) of this drug's plasma concentration profile.[1] The AUC is measured as the surface area under a plasma concentration-time curve. This is a pharmacokinetic parameter that shows the exposure of the patient to the compound given[25]. This study by Ayash et al.[1] identified two different sets of plasma concentration-time curves for cyclophosphamide in women. The lower curve resulted in a low AUC, and this corresponded with both increased toxicity and increased tumor response. Because a lower curve represents a higher metabolism of cyclophosphamide, it is concluded that its metabolism is not the same for all persons and that these differences result in altered toxicity and efficacy [1]

Because both the side effects and the efficacy of cyclophosphamide have great consequences for the animal being treated, it is of great importance to further elucidate this variance in metabolism. Genetic variation has been documented for many human CYP genes. However, information in the dog is very limited. The current study focuses on a vital step in bridging this gap in knowledge: to identify variations in the coding regions of the CYP2B11 gene in the dog. Changes in these regions can result in changes in the amino acid sequence of the protein and thus have consequences for protein folding and enzyme function.

The hypothesis of the current study is that there is genetic variation in the CYP2B11 gene and that this has implications for the enzymatic function of CYP2B11. The human orthologue CYP2B6 has been shown to have variability in amino acid sequence and these changes have consequences for the metabolism of cyclophosphamide.[21] Also, a study in dogs showed that the metabolism of Propofol, one of the compounds metabolized by CYP2B11 shows a breed-specific variance. This metabolism is

much slower in Greyhounds than in Beagles.[15] Because of the breed-specificity, an underlying genetic mechanism is proposed, and variations in the coding for CYP2B11 may be a likely cause.

The coding regions for CYP2B11 in the DNA of 20 dogs were amplified by PCR reaction. PCR products were submitted for sequencing. To find variance, retrieved sequences were aligned to each other and to the reference sequence for CYP2B11 from the UCSC Genome Browser. Where variations were discovered, the region was amplified for a total of 100 dogs. Restriction endonucleases were used to confirm and quantify the polymorphism by restriction fragment polymorphism. As a final step the changes in amino acid sequence was submitted to the online PolyPhen program[23], that predicted the consequences for the protein function.

Materials and methods

- Reference sequence

The gene coding for CYP2B11 was retrieved from the UCSC Genome Browser (http://genome.ucsc.edu). The most recent Whole Genome Shotgun assembly was used, which was submitted in May of 2005. This sequence is from a female Boxer sequenced by the Broad Institute. The sequence is found under RefSeq NM_001006652.1. It is located on the positive strand of Chromosome 1 from basepair 115726067 to 115740654. The gene for CYP2B11 consists of 15736 basepairs in 9 exons and codes for a protein with 494 amino-acids[18].

The mean quality score of this sequence is 47. Scores higher than 40 correspond with a high confidence in the sequence, having an error rate of <1/10000 (http://genome.ucsc.edu). This means that the reference sequence is overall highly reliable. The image shows a dip in the quality score at the start of exon 6. This is most likely caused by a particularly high GC percentage in that region, also visible in the image.

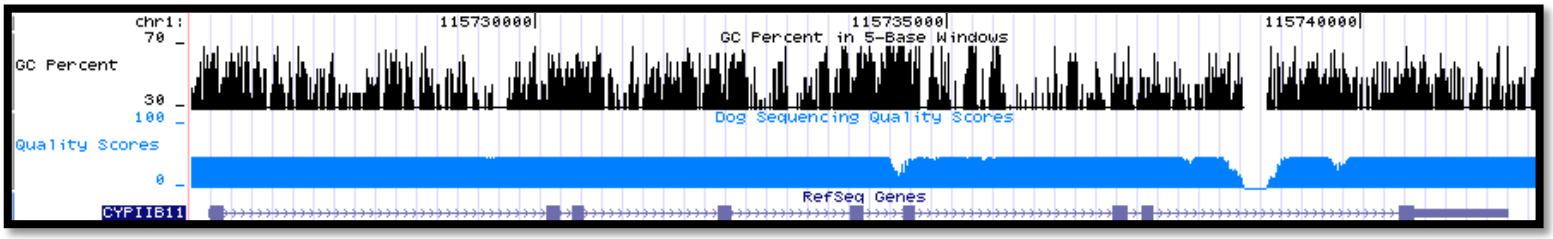

Figure 1: The gene for CYPB11 as retrieved from the UCSC Genome Browser - Above is the position on chromosome 1. Below that the GC percentage ranging from 30 to 70%. The blue bar represents the Quality Score of the sequence and thus the reliability. The lower purple line represents the gene. Boxes represent exons 1-9 from left to right and arrowed lines represent introns. The thick line to the right of exon 9 represents the 3'UTR, the 3'untranslated region

- PCR

For the PCR reactions intronic primers are designed flanking the exons. This was done to allow complete exons and intron/exon junctions to be amplified. For primer design, online Primer3 software v 0.4.0 was used (http://frodo.wi.mit.edu/) entering the reference sequence exons and marking an extra 50bp on each side as the target sequence to be amplified. The resulting primers were entered into the UCSC in silico PCR to verify the product size and location. Furthermore, the

primers were tested for compatibility and self-annealing using OligAnalyzer. After these verifications the oligonucleotides were ordered from the Research Technology Support Facility at Michigan State University.

Taq polymerase (+ 10X PCR buffer, +$MgCl_2$) and deoxynucleosides were purchased from Invitrogen, Carlsbad, CA 92008. A 25µl reaction volume was used. A typical reaction contained 5 pmoles of both the 5'and 3' primer, 2nmol of each dNTP, 500nmol Tris-HCl, 125nmol KCl, 50nmol $MgCl_2$ and 20ng of DNA. Thermocycling conditions were optimized for each primer set and the conditions determined to be optimal are presented in Table A1

- DNA panel

The DNA from the sample population that was tested is derived from a panel established in the lab in 1990. It was designed to reflect the range of dogs that are encountered in the US- and has representatives from different dog groups, pointer, working dogs, Nordic breeds, etc. Importantly, for each breed, only dogs that were not related for at least 3 generations were included in the panel. The panel consists of 100 dogs; the distribution is shown in table 1. Their DNA was isolated from blood and has a concentration in the working solution of 20ng/µl

Breed	**No. of samples in panel**
Cocker Spaniel	7
Greyhound	8
Sheltie	1
Dobermann Pinscher	5
Collie	9
Labrador Retriever	11
Scottish Terrier	11
Beagle	11
German Shepherd	9
Pointer	8
Siberian Husky	9
Mixed Breed	11

Table 1: Breed distribution of DNA panel

- Sequencing

Once the anticipated PCR fragment was obtained as judged by agarose gel electrophoresis, the products were submitted to the Research Technology Support Facility at Michigan State University for sequencing. In order to get more sensitivity and specificity with sequence analysis, each PCR product was submitted for sequencing in both directions, using either the 5' or 3' primer as sequencing primers.

When the sequences and chromatograms were retrieved, they were inspected for quality of the trace, and were entered into the DNAstar Seqman program for alignment and variation detection. The in-silico PCR product from the UCSC Genome Browser Site was used as the reference sequence. Then, the alignment was reviewed for variations. Variations that were found were critically inspected for quality of the signals and checked to make sure that the variation is seen on both sequences from the same dog. Examples of sequence variations are shown in figure 2. The upper chromatogram in figure 2 shows a chromatogram of a putative homozygote dog that is different from the reference sequence. The lower chromatogram shows an example of a putative heterozygote – there are two simultaneous signals of about equal amplitude which are each about half the amplitudes of other signals.

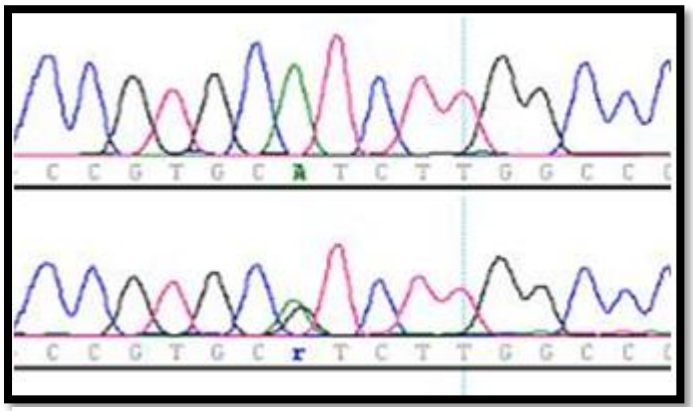

Figure 2: Examples of what is interpreted as homozygote A (upper chromatogram) and heterozygote r (G and A peak, lower chromatogram)

Because sequence analysis is an expensive and possibly error-prone method of genotyping, restriction enzymes have been used to confirm and quantify the polymorphism for all dogs including the sequenced samples. Restriction enzymes were selected for the ability to cut the two different sequences in a different way, which means that the polymorphic site is part of the cut site. This was not readily available for all polymorphisms found. The solution to this problem was to design mutagenic primers that created a cut site in one sequence but not the other. Ideally the restriction enzymes would cut both sequences at least once as an internal control, but given the relative small size of the sequences this might not be possible.

- Gel electrophoresis

To visualize the results from both PCR and restriction digestion, Agarose gel electrophoresis was used. The standard Agarose concentration is 1.5 % but for results of restriction digestions a higher percentage was needed to be able to distinguish smaller fragments from each other better.

- Nomenclature

The sequence variations that are found will be described according to the recommendations for human sequence variations .[7] The variations will be described for the genomic sequence (indicated by g.), the mRNA (indicated by r.) and the protein (indicated by p.). A theoretical example would be if a G to A variance was found in position 456 of the genome. This would potentially be noted as g.456G>A, r.456G>A, p.A152B

- PolyPhen prediction

PolyPhen is a Web server that is used to predict the effect of non-synonymous SNPs. It bases its predictions on the function of the amino acid (e.g. active or binding), alignments with homologous proteins and if the substitution is likely to change the 3d-structure of the protein. These three tests result in a prediction of the effect of the substitution.[23]

Results

After optimization of reaction circumstances for each primer set, the PCR amplification of all exons was successful. For each amplicon, sequences were obtained and inspected of 20 dogs .

Inspection of the sequence alignments revealed a number of putative polymorphisms.

In exon 1 a putative SNP was found in basepair 129. One of the Siberian Huskies that was sequenced shows what is interpreted as heterozygosity for GA while the reference sequence shows a G. This polymorphism would change position 123 in the mRNA from G to A also. This does not change the amino acid sequence of CYP2B11 (GGG → GGA = G → G) but it is still of interest to confirm and quantify this polymorphism. This is because the polymorphism can potentially be linked to a variation in another position (for example in the promoter region) which can have effects for (inducibility of) the enzyme. It could thus potentially be a marker for different phenotypes.

The alignment of sequences for amplicon 2 which contains both exons 2 and 3 revealed a putative polymorphism in exon 2, basepair 4121 of the genomic sequence. Where the reference sequence shows a C, both the sequenced Labradors show a T, one being a putative TT homozygote and one probably a CT heterozygote. This polymorphism changes the mRNA in base 220 from a C to a U, which changes the codon for amino acid 74 from Arginine (CGC) to Cysteine (UGC).

The third putative exonic polymorphism was found in the sequences from exon 7&8. The sequences showed that in position 10933 of the genome, which lies in exon 7, 14 out of 20 sequenced dogs are either homozygote AA or heterozygote GA instead of the G of the reference sequence. This polymorphism changes position 966 in the mRNA from a G to an A. Although this does not change the amino acid code (GAG(E) → GAA(E)), a polymorphism in this position could have extensive consequences for the protein. This is because the polymorphism is located on the edge of exon 7, being the second basepair in that exon. It is possible that the change from a G to an A affects splicing and causes an aberrant protein with altered efficacy to be formed.

Although not the major goal of this study, the use of intronic primers made it possible to identify two putative intronic polymorphisms. A G>A polymorphism was located in intron 4, in basepair 6339, and another G>A SNP was found in intron 7 in position 11166 in one dog.

The reference sequence that was used in this study showed to be overall very reliable. However, in Exon 6 at position 8448 there was a difference between it- showing a T- and every sequence that was retrieved from the panel – showing an A. As can be seen in the image of the CYP2B11 gene (figure 1), this is a region with a particularly high GC percentage and this could be a cause of a less reliable sequence. The T at that position could also be a polymorphism that is present in the dog population and possibly not picked up by our sequenced samples. If the A is the major allele in the dog population and the T is a polymorphism present in the Boxer from the reference sequence, it would mean a difference in the mRNA in position 871 from A to T. This changes the amino acid code: p.T291S.

To confirm and quantify allele frequencies for the found SNPs, restriction endonucleases were found as described in Materials & Methods to differentiate between the two possible genotypes. For the SNP in exon 1 (g.129C>T), it was necessary to design mutagenic primers and the enzyme Hpy188III was expected to be successful in cutting the CC phenotype but not the TT. However, results showed only a partial digestion, with 94 of 96 initial tested samples showing a pattern expected for a GA heterozygote and the presumed CT heterozygote showing a TT pattern. Because both this high percentage of heterozygotes and the unexpected pattern of the putative heterozygote did not correspond with the expected results, it was assumed that this enzyme was ineffective for some reason. However, the results did show a difference between the digestions of the putative heterozygote and the supposed CC samples. A different set of mutagenic primers was used to create a restriction site for HinfI – an enzyme that is used very often with high efficacy - but again, the digestion reaction did not reveal the expected results. What the reason is for these unexpected results is not known, this needs further investigation.

For the SNP in exon 2 (g.4121C>T) DNA of 100 dogs was amplified with the same primers and digested with AhdI. A sample of the results is shown in figure 2. The results confirmed that this polymorphism is present in the population. Heterozygous CT or homozygous TT individuals were found mainly in Labrador Retrievers, but one Pointer and one mixed breed dog had a CT genotype. The overall allele frequencies in the sample population and per breed were as shown in Table A3.

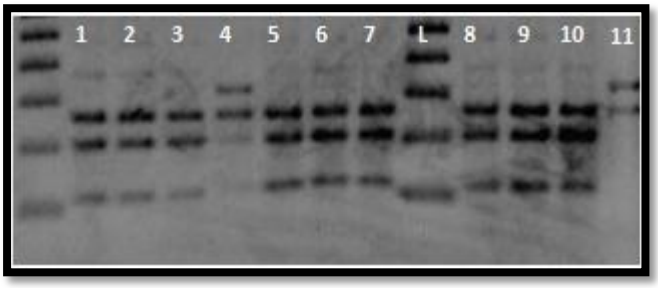

Figure 3: Sample of agarose gel showing results of digestion of exon 2-3 with AhdI. Lane 4 shows results as expected for a heterozygous CT genotype. Lane 11 shows results for a TT genotype, while all other lanes show a CC genotype.

Because the discovered difference in exon 6 (g.8448A>T) could be a polymorphism present in the population but not in the samples used for sequencing, this was tested for in the entire sample population. The same primer set was used as for the initial amplicon, and the products were digested with HinfI. A sample of the results is given in figure 4. Results showed that none of the dogs in the tested population had the T allele.

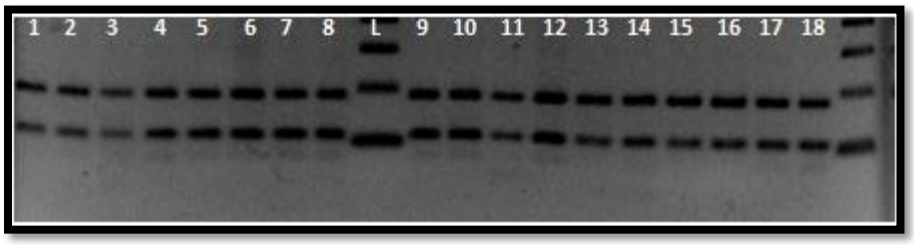

Figure 4: Results of amplification of exon 6 and digestion with HinfI. All lanes (1-18) show a TT genotype.

For the confirmation of the SNP in exon 7 (g.10933G>A) a mutagenic forward primer was needed. After PCR was successful, the products for 98 dogs were restricted with DdeI. This polymorphism was present in a large part of the sample population with highly variable allele frequencies. The allele frequency for the A allele in the overall population was 65%. Collies all showed a GG genotype

whereas German Shepherds all showed the AA genotype. Figure 4 shows a sample of the results as showed by agarose gel electrophoresis.

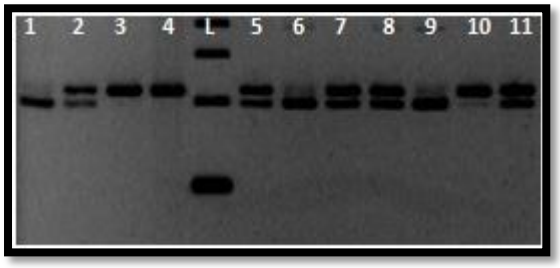

Figure 5: Agarose gel showing results of restriction of exon 7 with DdeI. Lanes 1, 6 and 9 showing a GG phenotype, Lanes 3,4 and 10 showing an AA genotype and lanes 2,5,7,8,11 showing heterozygosity.

The PolyPhen program was used to predict the potential effect of changes in the amino acid sequence on protein function. The only confirmed polymorphism that changed the amino acid sequence was found in exon 2 (g.4121C>T), that changes amino acid 74 from an Arginine to a Cysteine. The prediction of the PolyPhen program was that this change in amino acids has a "probably damaging effect" based on alignments with other Cytochrome enzymes. The potential polymorphism shown in the reference sequence was entered also, this substitution was predicted to be benign.

Discussion

This study was designed to identify and confirm polymorphisms in the coding regions of the canine CYP2B11 gene. A total of three SNPs were identified in the exons, and two were confirmed and quantified in a population of 100 dogs of 11 breeds. One SNP could not be confirmed with certainty but is very likely to be present due to differences in restriction fragments. It is clear from the results that there is variance in coding regions and amino acid sequences of the cytochrome 2B11, and that this variance has some degree of breed specific distribution. An overview of the polymorphisms found is shown in Table A2.

The polymorphism in exon 2 results in a change in amino acid sequence. Although the change is not in one of the 6 Substrate Recognition Sites for CYP2B enzymes[12], according to PolyPhen predictions, the change probably has damaging effects on the protein function. Because this can have major consequences for the animals that have this polymorphism, further investigation is needed to confirm changes in protein function *in vivo*.

Exon 7 shows a polymorphism at the second basepair of that exon, which is present in a high percentage of the tested population. The nucleotides around the splice site play a big role in determining the actual splice site, so changes here can have consequences for splicing. This may be that the location of the splice site is changed, or the situation of exon skipping. Both scenarios cause an abnormal protein to be formed with altered efficacy.

This study also identified two putative intronic SNPs (g.6339G>A and g.11166G>A). These polymorphisms are not present in the mRNA and thus have no immediate effects on amino acid coding. Therefore it was out of the range of this study to confirm and quantify them. However, they could still be associated with an altered efficacy of the CYP2B11 enzyme. Associations between intronic SNPs and enzyme function have been described for the human orthologue 2B6 (Nakajima).

This study is limited, mainly because of the small sample population. Currently, there are 158 different breeds registered by the American Kennel Club (akc.org). In this study, 100 dogs of 11 different breeds were tested. This sample represents a very small part of the population, and there may be variations that were not discovered but are present in the total dog population in the US. This was most apparent with the potential polymorphism that was discovered in exon 6. Although this potential SNP was not confirmed in our population, it may very well be present in Boxers and more breeds in the US dog population. However small, the sample population was founded with the purpose of representing the US population, and thus has representatives from a very wide range of breeds, with Nordic breeds, working hounds, pointers etcetera. Although it will be useful to test in a larger part of the population, it will probably never be feasible to test all breeds for presence and frequency of polymorphisms.

Another limitation is that this study only focused on the genomic differences. A number of variances was found and these possibly have an effect in vivo. However, further studies are needed to confirm this effect before implications for veterinary medicine are obvious.

To confirm an effect on splicing for the SNP 2B11*3, liver tissue is needed. It will be possible to use reverse-transcription PCR to amplify the mature mRNA that has been transcribed and spliced, and a size difference may be seen upon gel electrophoresis. Furthermore, it will be possible to sequence both cDNA products and see if both the G and A allele are present.

A next step in finding a genomic basis for differences in enzyme function is to see if in a clinical setting, differences in reactions to cyclophosphamide and ifosfamide are associated with the presence of variations in the CYP2B11 gene. A follow-up study is currently in progress where DNA from patients that have reacted differently to Cyclophosphamide or Ifosfamide was isolated from blood. The DNA will be RFLP tested for the presence of the polymorphisms found in this study and sequenced to find others.

If further investigations show that these polymorphisms have an effect on metabolism of Veterinary compounds in vivo, this implicates a need to test for these polymorphisms and adjust the dose or choose another compound. This study has shown that for 2B11*2 and 2B11*3, PCR followed by restriction digestion is a simple, relatively fast and effective way to distinguish two genotypes. This way of genotyping may thus have a use in a clinical diagnostic setting.

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