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Polymorphism in ABC transporter genes of *Dirofilaria immitis*

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ABSTRACT

Dirofilaria immitis, a filarial nematode, causes dirofilariasis in dogs, cats and occasionally in humans. Prevention of the disease has been mainly by monthly use of the macrocyclic lactone (ML) endectocides during the mosquito transmission season. Recently, ML resistance has been confirmed in *D. immitis* and therefore, there is a need to find new classes of anthelmintics. One of the mechanisms associated with ML resistance in nematodes has been the possible role of ATP binding cassette (ABC) transporters in reducing drug concentrations at receptor sites. ABC transporters, mainly from sub-families B, C and G, may contribute to multidrug resistance (MDR) by active efflux of drugs out of the cell. Gene products of ABC transporters may thus serve as the targets for agents that may modulate susceptibility to drugs, by inhibiting drug transport. ABC transporters are believed to be involved in a variety of physiological functions critical to the parasite, such as sterol transport, and therefore may also serve as the target for drugs that can act as anthelmintics on their own. Knowledge of polymorphism in these ABC transporter genes in nematode parasites could provide useful information for the process of drug design. We have identified 15 ABC transporter genes from sub-families A, B, C and G, in *D. immitis*, by comparative genomic approaches and analyzed them for polymorphism. Whole genome sequencing data from four ML susceptible (SUS) and four loss of efficacy (LOE) pooled populations were used for single nucleotide polymorphism (SNP) genotyping. Out of 231 SNPs identified in those 15 ABC transporter genes, 89 and 75 of them were specific to the SUS or LOE populations, respectively. A few of the SNPs identified may affect gene expression, protein function, substrate specificity or resistance development and may be useful for transporter inhibitor/anthelmintic drug design, or in order to anticipate resistance development.

1. Introduction

Dirofilaria immitis, a filaroid nematode that causes dirofilariasis or heartworm disease, is arguably the most important parasite that infects dogs in North America (Wolstenholme et al., 2015). This mosquito-borne nematode affects 30 other mammal species including cats, wild canids, felids and occasionally humans (Lee et al., 2010; Genchi et al., 2011). The disease has worldwide distribution but is more prevalent in temperate, tropical and subtropical regions of the world (Simón et al., 2009). For over 2 decades, prevention of the disease has relied upon a single class of drug, the macrocyclic lactones (MLs). MLs were first used as heartworm preventives in 1987. Concern about possible loss of efficacy (LOE) to this class of drug was reported in 2005 (Hampshire, 2005). Recent studies have unequivocally confirmed ML resistance in *D. immitis* (Bourguinat et al., 2011a, 2015; Pulaski et al., 2014). The ATP binding cassette (ABC) transporters have been implicated in ML resistance (see review Lespine et al., 2012). Specific studies on the role of ABC transporters and ML resistance include studies in *Caenorhabditis elegans* (James and Davey, 2009; Ardelli and Prichard, 2013; Bygarski et al., 2014), *Haemonchus contortus* (Blackhall et al., 1998; Xu et al., 1998; Sangster et al., 1999; Prichard and Roulet, 2007), *Onchocerca volvulus* (Eng and Prichard, 2005; Ardelli and Prichard, 2007; Bourguinat et al., 2008; Osei-Atweneboana et al., 2011; Nana-Djeunga et al., 2014), *Teladorsagia circumcincta* (Dicker et al., 2011), and for benzimidazole resistance in *H. contortus* (Blackhall et al., 2008), triclabendazole resistance in *Fasciola hepatica* (Mottier et al., 2006) and praziquantel resistance in *Schistosoma mansoni* (Messerli et al., 2009). In *D. immitis*, a diplotypic genotype in *Dim-pgp-11* (ABC-B class of the transporter superfamily) gene was found to correlate strongly with a loss of response to ivermectin (Bourguinat et al., 2011a, b).

Members of the ABC transporter superfamily, particularly P-glycoproteins (PGPs) and multidrug resistance proteins (MRPs) have broad substrate specificity (e.g., antibiotics, antimalarials,

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herbicides, antifungals and other chemotherapeutic agents) (Higgins, 2007). ABC transporters, as efflux pumps, influence the bioavailability and disposition of drugs through active efflux of compound out of cells, thereby contributing to a phenomenon called multidrug resistance (MDR) (Lespine et al., 2012; Ardelli, 2013). Overexpression of these transporters has been suggested as a MDR conferring mechanism (Ardelli, 2013). In nematodes, multidrug resistance is suggested to be conferred by members from subfamily B and C, though many reports specifically implicate the role of PGPs in the resistance mechanism (Prichard et al., 2012 Lespine et al., 2012). A study in *B. malayi* has shown that transporters from subfamily A and G may also have a role in resistance, as they were also overexpressed along with PGPs and MRPs following treatment with ivermectin and moxidectin (Stitt et al., 2011; Tompkins et al., 2011).

Although ABC transporters may play a role in developing and maintaining anthelmintic resistance, it is believed that their main physiological function is to protect neurons and other tissues in which they are expressed, from a broad spectrum of toxins (Prichard and Roulet, 2007; Ardelli, 2013). Unlike vertebrates, which have a vascular system contained within epithelial cells which line the blood capillaries and other blood vessels where PGPs and MRPs are commonly expressed, nematodes do not have a similar barrier cell layer expressing ABC transporters. The number of efflux transporters is greater in nematodes, compared to mammals, as they may have evolved this diversity as part of their protective mechanism for a variety of different tissues and specific cells. ABC transporters can regulate anthelmintic efficacy by modulating the intracellular or intra cell membrane concentration of lipophilic drugs. Therefore, a deletion or inhibition of such transporters may partially overcome resistance (Lespine et al., 2012; Ardelli, 2013; Greenberg, 2013a, 2014a). For example, deletion or disruption of mammalian and nematode Pgp genes leads to increased sensitivity to ivermectin (Schinkel et al., 1994; Janssen et al., 2013). Also, substrates of PGP that are competitive inhibitors (e.g., verapamil), or that block the transport function directly, have restored anthelmintic sensitivity (Ardelli and Prichard, 2013). In *B. malayi*, ABC transporter inhibitors have potentiated sensitivity to ivermectin (Tompkins et al., 2011). Similar reversal mechanisms have been shown for *H. contortus* resistant to ivermectin (Molento and Prichard, 1999; Lifschitz et al., 2010; Bartley et al., 2012). Inhibitors of MRP, such as MK571 and buthionine sulfoxamine, have also reversed resistance (Prichard et al., 2012). In nematodes, ABC transporters are believed to be essential for the survival of nematodes as they are known to be involved in a wide range of processes. For example, in *C. elegans*, they play critical roles in apoptotic cell corpse removal (Wu and Horvitz, 1998), dauer formation (Yabe et al., 2005), RNAi (Sundaram et al., 2006), directed sperm motility (Kubagawa et al., 2006), apart from resistance to toxins (Broeks et al., 1995) and heavy metals (Broeks et al., 1996; Vatamaniuk et al., 2005), and resistance to pathogens (Mahajan-Miklos et al., 1999). Taking all of this into consideration, ABC transporter genes may prove to be attractive therapeutic targets for new or repurposed MDR reversing agents, and/or for new anthelmintics (Lespine et al., 2012; Ardelli, 2013; Greenberg, 2014b).

Knowledge of allelic variants in ABC transporter genes may be useful information to have during drug design. Knowledge about polymorphism in these genes may help ensure that a new drug will be active against all the allelic forms of these targets. Some polymorphism in MDR transporter genes may influence their level of expression and/or the functional characteristics (Gerloff, 2004), and such knowledge may be useful to understand the mechanism of resistance. Also, heterogeneity of a drug target may determine the drug selection process (Prichard, 2001) and knowledge of polymorphism in drug transporters may help to anticipate resistance development. In order to address these possibilities, the objective of this study was to identify putative ABC transporter genes/subunits from subfamilies A, B, C and G in *D. immitis*, by comparative genomic approaches, and to analyze the genes for single nucleotide polymorphisms (SNPs).

2. Materials and methods

2.1. Identification of all of the ABC-A, -B, -C and -G transporter genes of D. immitis

The genome of *D. immitis* is not fully annotated (Bourguinat et al., 2015) and therefore a complete inventory of ABC transporter genes is not yet available. In a recent study (Bourguinat et al., 2016), eight ABC transporter genes including a pseudogene from *D. immitis* were described. To identify the remaining, un-annotated homologs of ABC transporter genes in *D. immitis*, we followed a similar approach as previously described (Mani et al., 2016). Briefly, all the nucleotide sequences that encode genes or subunits of ABC transporter subfamilies (A, B, C and G) of all nematodes were extracted from available databases such as NCBI (http://www.ncbi.nlm.nih.gov/), Wormbase (http://www.wormbase.org/#01-23-6), Broad Institute (https://www.broadinstitute.org/) and NEMBASE4 (http://www.nematodes.org/nembase4/). These sequences were then used in the nucleotide BLASTt server v2.2 (http://nematodes.org/genomes/dirofilaria_immitis/) to indicate putative protein type. These BLAST searches were helpful to locate each of the putative ABC transporter genes in the scaffolds of the *D. immitis* nuclear genome (version nDi.2.2.2) (http://salmo.bio.ed.ac.uk/cgi-bin/gbrowse/gbrowse/nDi.2.2.2/).

2.2. Synchronized file generation from whole genome sequencing data

Whole genome data from diverse pooled samples of 122 adult *D. immitis*, from 17 dogs from the USA, Grand Canary, Grenada and Italy, and approximately 32,000 *D. immitis* microfilariae from 4 dogs from USA, described previously (Bourguinat et al., 2015; Mani et al., 2016) were used. More information on the *D. immitis* samples and their classification into ML susceptible (SUS) and loss of efficacy (LOE) populations is given in the Supplementary table ST1. The adult worms were mixed sex populations characterized as susceptible to macrocyclic lactone heartworm preventives, while the microfilariae were termed loss of efficacy samples as it was suspected that the dogs may have become infected despite being on macrocyclic lactone heartworm preventives, as previously described (Bourguinat et al., 2015). Some of the adult female worms were gravid. This would only enhance the possibility for detecting polymorphism in the samples. The method followed for synchronized file generation has been described elsewhere (Mani et al., 2016). Paired-end reads were assembled, filtered and trimmed. *D. immitis* nuclear genome v2.2 (http://nematodes.org/genomes/dirofilaria_immitis/) was used as the reference genome. BAM files that consisted of aligned sequences from each population against the reference genome were generated. The program Popoolation2 was used to generate a synchronized file that contained nucleotide frequencies for each population at every base in the reference genome. This information was obtained for each gene in a concise format after filtering for base quality.

2.3. Nomenclature and classification of ABC transporter genes identified

Previously 8 ABC-B transporter genes in *D. immitis* had been