

DMD #45062

Probabilistic orthology analysis of the ABC transporters: Implications for the development of multiple drug resistance phenotype

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DMD #45062

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DMD #45062

Abbreviations: ABC, ATP Binding Cassette; ABD, ATP Binding Domain; ADME, Absorption, distribution, metabolism and excretion; MDR, Multiple Drug Resistance; MPR, Most Parsimonious Reconciliation; PK, pharmacokinetics; TMD, Transmembrane domain; UPMGA, Unpaired Grouping Method with Arithmetic Mean

DMD #45062

Abstract

Drug transporters are rapidly becoming recognised as central to determining a chemical's fate within the body. This action is a double-edged sword, protecting the body from toxicants, but also potentially leading to reduced clinical efficacy of drugs through multiple drug resistance phenotype. To examine the inter-relationship of this super-family we have constructed phylogenetic trees over an extended evolutionary distance representing each of the seven sub-families. In addition, using protein sequences from species important in the design and evaluation of novel chemicals, namely human, macaque, rat, mouse and dog, we have undertaken probabilistic orthology analysis to examine speciation probabilities within this phylogeny. This data allows us to accurately predict orthologous sequences across these species, an important confirmatory step with implications for cross-species extrapolation of data during drug safety testing. Finally, we present the first complete phylogeny for sub-families within humans constructed utilising the entire coding sequences, at both the DNA and protein levels. We demonstrate for the first time that genes associated with the multiple drug resistance phenotype cluster separately from other genes within the same sub-family, suggestive of a conserved, fundamental, difference in these proteins. Such work may help guide future studies on the mechanisms underlying multiple drug resistance, as well as the development of novel therapeutic approaches to mitigate against its development.

DMD #45062

Introduction

The ATP-binding cassette (ABC) genes comprise a super-family with representatives found in all characterised eukaryotic and prokaryotes; indeed, this superfamily encodes approximately 5% of the *Escherichia coli* genome (Fath and Kolter, 1993; Davidson and Chen, 2004). The majority of ABC genes encode membrane bound transport protein, which act to move polar molecules across the non-polar lipid membrane, utilising the hydrolysis of ATP. As such, these transporters play an important role in the absorption, distribution, metabolism and excretion (ADME) of chemicals (Glavinas et al., 2004). In prokaryotes, ABC transporters may act as both importer and exporter proteins (Fath and Kolter, 1993; Davidson and Chen, 2004). By contrast, in eukaryotes, these proteins act solely as export transporters, and this represents an important functional breakpoint within the super-family. Such efflux is central to the removal of potentially harmful chemicals from cell systems; an action that undoubtedly underlies the biological survival advantage conferred by these proteins and explains their conservation across evolutionary time (Dean and Annilo, 2005).

Although the ability to rapidly eliminate potentially harmful chemicals has obvious survival advantages, it also represents a challenge during long-term chemotherapy. Expression levels of a number of ABC transporters has been shown to contribute towards the development of multidrug resistance (MDR) phenotype, whereby the ADME of administered chemicals is altered, usually resulting in altered pharmacokinetics (PK) and reduced clinical efficacy. MDR has been shown to have a negative impact on the treatment of a number of disease states, including cancer (Deeley et al., 2006; Gillet et al., 2007). Much work has thus been undertaken to understand the molecular mechanisms underlying MDR, and how this can be mitigated during long-term chemotherapy (Coley, 2008). However, translation of these mitigation strategies to the clinic has generally been poor, and MDR still represents a

DMD #45062

significant hurdle to successful chronic chemotherapy regimens (Coley, 2008; Tiwari et al., 2011).

Previous studies on the evolution of the ABC genes have not included all the species utilised in the pre-clinical testing of novel drugs; such a comprehensive analysis would be important for the robust extrapolation of data from preclinical test species to humans. In addition, phylogenetic analysis has often been restricted to only fragments of the total coding sequence, such as the ATP-binding domain, which is likely to be the least variable domain (Dean and Allikmets, 1995; Saier and Paulsen, 2001; Dean and Annilo, 2005), and has relied on distance-based methodologies which are generally accepted to not produce the most robust phylogenetic relationships across super-families (Koski and Golding, 2001).

In the current study, we have used protein alignments of all members of the ABC drug transporter family in humans and a number of important model animals for the testing of novel chemicals to undertake probabilistic orthology analysis. This allows the robust assignment of ortho- or paralogue status to protein pairs, including probability values, thus providing important information for extrapolation of effects between species. In addition, we have expanded on previous phylogenetic studies by using full ABC nucleotide and protein sequences across a range of evolutionarily diverse species. Such an approach not only separates ABC sub-families correctly, but also clusters those ABC transporters associated with MDR, suggesting that this is a specifically evolved design function.

Materials and Methods

Sequence identification and alignment of ABC genes

The Reference Sequences (RefSeq) for all 48 members of the human ABC super-family were identified (Supplemental Table 1) along with their common alternate names and GenBank accession number. Sub-families A-D and G represent those sequences that encode membrane-bound drug transport proteins, whereas sub-families E and F encode ATP binding cassette proteins that do not play a role in drug transport. Using these human sequences as the query, a cross-species megaBLAST was undertaken to identify similar sequences in ten other species (Altschul et al., 1990; Zhang et al., 2000). Where multiple sequences against a single query were identified within a species, all sequences were taken forward, with duplicates trimmed when identified.

Multiple sequence alignments was performed for each sub-family using ClustalX, with an additional alignment being performed solely for the human sequences (Higgins and Sharp, 1988; Thompson et al., 1997): For each sub-family, an *E. coli* ABC gene sequence was included, as the root for the subsequent phylogenetic tree. All alignments were assessed manually to ensure gap insertions were sensible, with subsequent phylogenetic analysis also automatically removing regions containing significant gaps.

Phylogenetic analysis of ABC sub-families

Distance matrices were produced from the ClustalX output using dnadist, including 100 replicates for bootstrap analysis (Felsenstein, 1997). For individual sub-family analysis, these

DMD #45062

matrices were analysed with Neighbor using an unpaired grouping method with arithmetic mean (UPGMA) to produce a consensus tree using the majority rule (Felsenstein, 1997).

For analysis of all sub-families within humans, ClustalX-generated alignments were analysed with Phyml, as this is more computationally efficient for larger analysis (Felsenstein, 1989; Guindon and Gascuel, 2003). One hundred phylogenetic trees were constructed using a maximum-likelihood approach, and Consense was used to generate a consensus tree from the bootstrapped data (Felsenstein, 1989).

Probabilistic orthology analysis of ABC transporter proteins from human, mouse, rat, dog and macaque

Probabilistic orthology analysis is a reconciliation-based orthology methodology that utilises the probabilistic gene evolution model described by Arvestad et al. (Arvestad et al., 2003). The output determines the probability that any given divergence within a phylogenetic tree is the result of speciation as opposed to duplication. This allows the robust assignment of orthologues and paralogues within a tree. Protein sequences for each ABC drug transporter from human, dog, rat, mouse and macaque, were aligned using ClustalX2 (v2.0.12), and ProTest used to determine that LG was the optimum amino acid replacement model for accurate phylogeny resolution (Abascal et al., 2005; Le and Gascuel, 2008). A maximum-likelihood phylogenetic tree was generated with Phyml, which was the input for the probabilistic orthology analysis. In addition to a phylogenetic relationship of genes, a phylogenetic relationship of species is also required for probabilistic orthology analysis, and this was constructed using species divergence times taken from Kumar and Hedges (Kumar and Hedges, 1998) and Jacobs and Downs (Jacobs and Downs, 1994). Probabilistic orthology analysis was undertaken using PrimeGEM, as described in Sennblad and Lagergren

DMD #45062

(Sennblad and Lagergren, 2009), using 10000 MCMC iterations, MCMC-estimated duplication and loss rates, and with an output of posterior orthology probabilities. This output was analysed using the MCMC_analysis perl script available from http://prime.sbc.su.se/primeGEM/downloads/perl/mcmc_analysis.

DMD #45062

Results

Phylogenetic analysis of ABC sub-families

Using a distance-based method we were able to generate nucleotide-level phylogenetic trees for each sub-family (Supplemental Figures 1-5). Table 1 represents a summary of these data, describing the total number of ABC genes identified in each species, plus the number of sequences for each species that could be clearly demonstrated to lie within a single sub-family through phylogenetic analysis.

Using distance-based methods it is only appropriate to assign individual sequences within a sub-family, and not to predict orthologues. Assignment of orthologues based purely upon phylogenetic trees derived by most parsimonious reconciliation (MPR) has been demonstrated to have poor predictive value (Koski and Golding, 2001). A more appropriate analysis is probabilistic orthology analysis (Sennblad and Lagergren, 2009). In this method, gene evolution is set within the context of a species evolution tree and modelled under variable gene birth-death rate parameters (Rannala and Yang, 2007).

For each human ABC protein, we present the most probable orthologue for macaque, rat, mouse and dog (Supplemental Table 2), along with the probability score for that match. In general, given nomenclature for each protein is consistent with the indicated analysis; for example, the protein named ABCA6 in humans and mice are demonstrated to be the result of a speciation event, and hence be orthologues, with a probability of over 99%. However, some orthologue assignments are not fully supported by the probabilistic orthology analysis: For example, in the case of ABCD2, probabilistic orthology analysis assigns high probabilities to the sequence being an orthologue of either ABCD2 or ABCD3. In such cases, sequences are tentatively assigned as orthologues to the human sequence for which there is the higher

DMD #45062

probability, but alternate high probability matches are noted within the table (Supplemental Table 2) and phylogeny (Supplemental Figure S6). Finally, we are able to expand the current knowledge base, assigning orthologue status to several ‘orphan’ sequences, particularly from the Macaque, which has been relatively poorly investigated until now.

In addition, probabilistic orthology analysis provides an estimate of the most probable gene loss/duplication rates that would result in the given phylogeny. For each combination of duplication and loss rates examined within the analysis, the probabilistic likelihood is determined, producing the 3-dimensional Gaussian distribution seen in Figure 1. The maximum posterior probability of the analysis represents the most likely duplication and loss rate estimates, which were 0.0095Myr^{-1} and 0.0122Myr^{-1} , respectively. It is important to note, that these estimates are relevant for only the species set examined (human, rat, mouse, dog, macaque), with duplications and loss rates often being considerably different over larger evolutionary distances. .

Phylogenetic analysis of the ABC super-family in humans

Following examination of phylogenetic relationships within sub-families across a number of animal species relevant to pre-clinical chemical testing, we next examined the relationship between human sub-families. PhymI was used to confer a logical consensus tree with acceptable bootstrap values at major nodes (Figure 2). This analysis was able to successfully resolve the sub-families, and provide further insight into the evolution of this super-family. Unsurprisingly, sub-families E and F, which lack a transmembrane domain (TMD), cluster separately from other sub-families. Interestingly however, they diverge at different points within the phylogenetic tree, suggesting that these two TMD-lacking sub-families have arisen as independent loss-of-function (TMD) events.

DMD #45062

We have also observed that those sequences that encode proteins associated with the MDR phenotype appear to segregate within the phylogeny. Within the B sub-family, a clear separation of ABCBs 1, 4, 5, and 11 can be seen from the rest of the sub-family (Figure 2), with the former group all being previously demonstrated to play a role in the development of MDR (Childs et al., 1998; Ambudkar et al., 1999; Smith et al., 2000; Huang et al., 2004). In addition, ABCG2 separates from the rest of the G sub-family, and is the only sub-family member associated with the MDR phenotype (Cervenak et al., 2006). Finally, the C sub-family also divides into those sequences associated with the MDR phenotype and those not, although in this case the segregation is not as clear. It is of interest to note that the ABCC9 and ABCC8 sequences appear to segregate with the MDR phenotype group, lending further weight to the suggestion that these genes may indeed contribute to a drug resistance phenotype (Deeley et al., 2006; Zhou et al., 2008).

To complement the nucleotide-level analysis, we also undertook a protein-level analysis for the human ABC transporters. In this analysis we also included the protein sequences from the pre-clinical species rat, mouse, dog and macaque. The derived phylogenetic tree is consistent with the orthologue assignment, with tight clustering of orthologues at the end of branches (Figure S6). In addition, simplification of the phylogenetic tree to illustrate the overall structure (Figure 3) is consistent with the conclusions drawn from the nucleotide-level analysis of human sequences. Whilst the multi-species amino acid-level analysis produces a different tree topology, clustering of MDR-associated sequences is still observed within the phylogeny (Figure 3).

DMD #45062

Discussion

Proteins encoded by the A-D and G sub-families of the ABC transporter super-family play a central role in chemical ADME, affecting a compounds PK profile and, potentially its clinical efficacy (Hembruff et al., 2008; Kalliokoski and Niemi, 2009). It is important to understand the phylogenetic relationships of this super-family for two reasons: Firstly, the testing of novel chemical entities for both efficacy and toxicity is routinely undertaken in non-human mammalian species, with the data extrapolated to humans (Barille, 2008). In order for such extrapolations to be undertaken, complex physiologically-based pharmacokinetic models have been developed (Dressman et al., 2011). However, at present the role of drug transport proteins is poorly represented in many of these models, often being either encompassed in a generic ‘active transport’ term, or limited to very few specific transporters (Pang et al., 2009; Fan et al., 2010). One reason for this limitation is that the relationship between transporters in pre-clinical test species and humans is still relatively poorly understood, and as such this work aid in the focussing of experimental work to identify kinetic differences between orthologues. Once coupled with data on species differences in transporter expression (Takahashi et al., 2008; Cedernaes et al., 2011), this will allow far more robust cross-species extrapolation of drug ADME (Glavinas et al., 2004). in model species and humans. Secondly, understanding the mechanisms underlying MDR is an important step in identifying potential means to mitigate this important limitation to chemotherapeutic intervention (Coley, 2008).

Phylogenetic analysis over an extended evolutionary distance allows the clear assignment of sequences to sub-families for mammalian species. However, for species with a larger divergence time from humans, such as *Strongylocentrotus purpuratus*, *Caenorhabditis elegans* and *Drosophila melanogaster*, robust sub-classification is not possible in the majority of

DMD #45062

cases. Work by Sheps and colleagues also attempted to identify human orthologues for ABC drug transporters in *Caenorhabditis elegans*, using the amino acid sequence of ABC proteins (Sheps et al., 2004). They were able to assign orthologues to 8/43 human ABC drug transporters, and in the current study we confirm five of these using a different analysis methodology. The use of the entire coding sequence for phylogenetic analysis has several benefits over the use of only a selected region (e.g. TMD or ABD): Firstly, use of only a portion of the coding sequence excludes any variability seen within the rest of the sequence, which may result in bias in the generated phylogenies; secondly, whilst the TMD is most likely to be the most variable region, and hence main driver for the phylogenetic trees, use of this alone would exclude the non-TMD containing subfamilies (ABCE and ABCF), reducing the completeness of the analysis; thirdly, for robust assignment of orthologues through probabilistic orthology analysis it is crucial that all variability is accounted for within the analysis.

Probabilistic orthology analysis was able to robustly identify paralogues and orthologues between humans and several pre-clinical species, including the macaque which is currently poorly annotated. In addition, this analysis provides information on the rate of sequence change within the super-family, with gene duplication and loss rates of 0.0095Myr^{-1} and 0.0122Myr^{-1} , respectively, being estimated for the ABC superfamily in humans, mouse, rat, dog and macaque. Cotton and Page have previously estimated that average duplication and loss rates in the vertebrate lineage over the last 200Myr to be 0.00115 Myr^{-1} and 0.00749Myr^{-1} (Cotton and Page, 2005), meaning that for the ABC super-family both duplication and loss rates appear to be considerably higher than the average for all genes. It should be noted that other papers have estimated higher average duplication and loss rates (Lynch and Conery, 2000; Lynch and Conery, 2003), but there are potential confounders in these studies and, in

DMD #45062

general, the averages are still lower than the estimate for the ABC super-family derived herein. These high duplication and loss rates supports the assignment of orthologue status via probabilistic analysis, as opposed to a simple MPR-based approach; Sennblad and Lagergren demonstrated that the rate of false orthology predictions from an MPR-based approach increased with the duplication and loss rates (Sennblad and Lagergren, 2009). The presence of significantly higher duplications and loss rates for the ABC super-family could be reflective of a fluid phylogeny that can alter relatively rapidly, which would be logical for a protein family providing protection against chemicals in an ever-changing environmental milieu.

In comparison to previous publications, we have aligned the entire mRNA/protein sequence for phylogenetic analysis, rather than selected fragments. We demonstrate that robust phylogenies can be inferred from the alignment of full gene sequences (Supplemental Figures 1-5), distinguishing between full and half transporters within sub-families. In addition, for a single species, human, we have reconstructed the entire super-family. It is possible to successfully resolve the individual sub-families, and some interesting implications arise from this analysis. As noted within the introduction, two sub-families within the ABC super-family do not encode drug transporters, and indeed it has been argued that these genes should be excluded from the super-family (Rees et al., 2009). We demonstrate that these two sub-families have arisen by independent events, most probably through loss of the TMD. This loss of TMD has, obviously, led to an altered localisation of protein products from these sub-families, whilst their retention of an ABD allows them to undertake ATP-dependent processes. In the case of the ABCE sub-family, the sole gene encodes and ribonuclease L inhibitor, an important regulator of interferon action (Bisbal et al., 1995). In the case of the

DMD #45062

three ABCF gene products, these proteins are all members of the GCN20 family and appear to play roles in TNF α -mediated signalling (Richard et al., 1998).

In addition, we provide data to support a clustering of those genes that encode transporters associated with MDR phenotype. This clustering, supported by both transcript and protein level analysis, could indicate that the MDR associated genes/proteins have features that set them apart from other genes within their sub-families. As this relationship translates to the protein level it also suggests that these features may be important in determining the molecular function(s) required to contribute to an MDR phenotype, although these features are as yet unelucidated. Whereas this separation from the main sub-family can be seen clearly in the B and G groups, it is undoubtedly less well defined within the C sub-family; this may be of interest considering that the B and G sub-family members encode proteins that generally have parent chemicals as their substrates, while those transporters encoded by the C sub-family generally transport conjugated products of metabolism (Choi, 2005; Deeley et al., 2006). Further examination is required to fully understand the impact of these different roles in chemical ADME, and on the development of a MDR phenotype.

In summary, the phylogenetic analyses contained herein extend current data on ABC gene orthologues in pre-clinical species, both identifying novel orthologues and correcting previous errors in annotation. Such information is important for the extrapolation of chemical effects in model organisms to humans and hence accurate risk assessment. In addition, we present the first complete human phylogeny across the entire super-family, demonstrating segregation between sequences that encode ABC transporters evoking the MDR phenotype and those which do not, at both the gene and protein level. This leads to the exciting possibility of focussing further on those transporters most likely to result in MDR and the development of strategies to mitigate this.

DMD #45062

Author Contributions

Participated in Research Design: Plant, Fisher, Coleman

Conducted Experiments: Plant, Fisher

Performed Data Analysis: Plant, Fisher

Contributed New Reagents: Coleman

Wrote, or contributed to the writing of, the manuscript: Plant, Fisher, Coleman

DMD #45062

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DMD #45062

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DMD #45062

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DMD #45062

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DMD #45062

Footnotes

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DMD #45062

Figure Legends

Figure 1: Gene duplication and loss likelihood scores within the ABC transporter encoding sub-families. Probabilistic Orthology analysis was undertaken for ABC-transporter proteins, using the gene evolution method of Arvestad (Arvestad et al., 2003). MCMC-estimated duplication and loss rates were calculated for every 10 iteration of a 10000 iteration analysis.

Figure 2: A rooted consensus tree of the gene sequences for all human ATP-binding cassette super-family members. Phylogenetic tree was generated using the maximum-likelihood based program Phyml, with bootstrap values (100 replicates) shown at the major nodes. ABC genes whose proteins products have been positively associated with multiple drug resistance phenotype are highlighted in solid boxes.

Figure 3: A rooted consensus tree of the protein sequences sub-families A, B, C, D and G of the ATP-binding cassette super-family in humans, macaques, rat, mouse and dog. A multiple alignment was generated of ABC proteins from human, macaque, rat, mouse and dog using ClustalX2. Optimum amino acid replacement model was determined by ProTest, and then an LG algorithm and Phyml used to generate a phylogenetic tree using a maximum-likelihood approach. The full tree is presented as supplementary information (Figure S6), with a simplified cartoon showing only the overall general structure shown here. ABC genes whose proteins products have been positively associated with multiple drug resistance phenotype are highlighted in solid boxes.

Table 1: Total number of individual genes identified for each species.

Species name	Sub-family								Total
	A	B	C	D	E	F	G	?	
<i>Homo sapiens</i>	12	11	12	4	1	3	5	-	48
<i>Pan troglodytes</i>	11	11	11	4	1	3	5	2	48
<i>Macaca mulatta</i>	13	11	12	4	1	3	3	-	47
<i>Canis lupus familiaris</i>	13	9	12	4	1	2	5	2	46
<i>Mus musculus</i>	14	11	11	4	2	2	5	2	51
<i>Rattus norvegicus</i>	14	10	9	4	1	2	5	3	48
<i>Bos Taurus</i>	11	9	10	3	1	3	5	3	45
<i>Stronglocentrous purpuratus</i>	1	4	6	1	1	2	2	12	29
<i>Danio rerio</i>	5	5	9	3	1	2	5	9	40
<i>Caenorhabditis elegans</i>	-	-	1	1	1	1	-	29	32
<i>Drosophila melanogaster</i>	-	-	-	2	1	1	1	38	43

Using human ABC transcript RefSeq sequences as the query term a cross-species mega-BLAST was undertaken to identify homologues in each of the listed species. Significant hits, not inclusive of transcript variants or pseudogenes, were included in an initial phylogenetic analysis, allowing designation of sequences into most probable subfamilies. Numbers in the ‘?’ column indicate sequences identified in the megaBLAST search that cannot be conclusively identified as belonging to a specific sub-family.

Figure 1

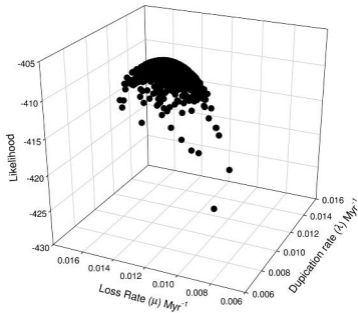


Figure 2

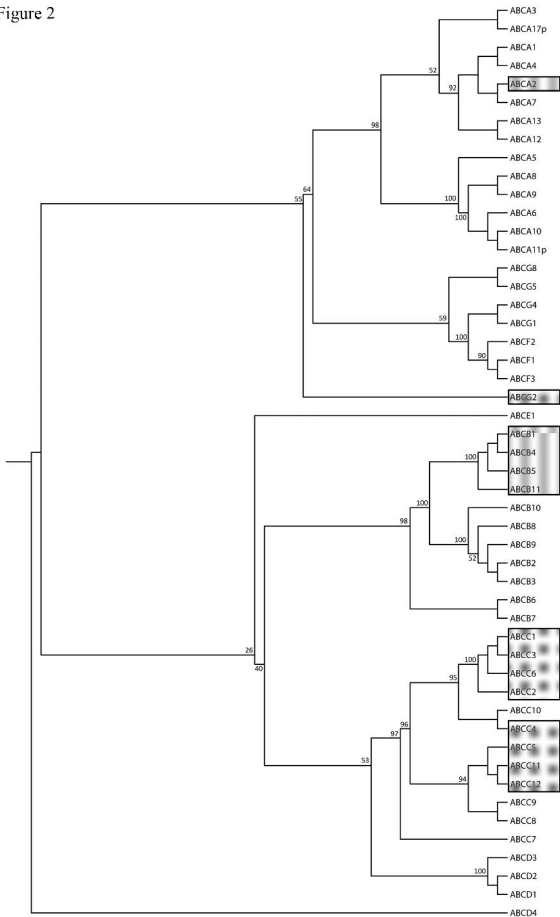


Figure 3

