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The Post-Transcriptional Regulator EIF2S3 and Gender Differences in the Dog: Implications for Drug Development, Drug Efficacy and Safety Profiles

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Abstract

Canis familiaris, the domestic dog, is a key animal species for preclinical drug development, including toxicity assessment. As data relating to gender dimorphic toxicity in the clinic emerge, the topic of sex differences in relation to drug toxicity will increase in prominence. Much of the emerging clinical data cannot easily be explained by body weight or body fat differences – hence the gender differences may be related to more complex hormonal and/or potential underlying gene expression differences. The dog also demonstrates some gender differences in drug-induced toxicity; hence, in the current study, we investigated differences at the gene expression level between male and female dogs in selected tissues of relevance to toxicity. We attempted to elucidate whether key gene expression differences do exist and if so, whether these gender differences may potentially impact on disease states, drug metabolism and toxicity. We investigated gene expression in the heart (the ventricle and atrium) along with the main tissues of drug absorption, metabolism and excretion, namely, the GI tract (ileum), liver and kidney (medulla and cortex) and performed *in silico* pathway analysis to elucidate key pathways possibly affected by gender dimorphic expression profiles.

Surprisingly, we show that the post-transcriptional regulator, EIF2S3, is consistently highlighted across all six tissues examined: the gene was nearly three times over-expressed in male dogs compared to females, in all the tissues studied. This finding should be contrasted with the observation that the vast majority of genes showed no difference and for those where differences were found it was limited to one or two tissues. Thus, the discovery that EIF2S3 showed such large differences (common to all the tissues studied), was an intriguing finding. Pathway analysis showed tissue-specific gender dimorphic profiles are apparent between male and female canines; interestingly, EIF2S3 appeared to play a key role in these pathways. High homology with the human EIF2S3 raises the prospect of an analogous role for sex-differences in humans.

Introduction

The topic of sex differences in adverse responses to drugs is gaining much attention at the moment, as data in regards to gender dimorphic toxicity in the clinic are rapidly emerging [4,5]. Much of these data rule out some obvious gender differences (such as body weight and body fat) [6,7] as major causal factors. However, it is possible that hormonal signaling underlies these basic differences which are poorly understood at the moment. Hence, there is a compelling need for basic research at the molecular level, in order to comprehend and thus perhaps prevent these sex differences in adverse responses in the future.

Canis familiaris, the domestic dog, is a major preclinical animal species for drug development. The species is used in a wide range of studies involving safety and efficacy, including regulatory toxicology studies and cardiovascular telemetry studies [1] and thereby in translational safety and dose-prediction models to human [2]. Therefore, in the current study, we investigate gene expression in the major drug metabolizing tissues (in addition to heart tissues) to see whether any sex differences occur - and if so - to investigate the potential implications for drug development. Another issue to consider is that apart from regulatory toxicology studies, preclinical animal studies tend to be predominantly male [3] and the concern is that sex differences, which may translate to humans in the clinic, are being overlooked.

In this study we investigated gene expression profiles in dog heart tissues (ventricle and atrium), along with the major drug metabolizing tissues of the kidney (medulla and cortex), liver and small bowel (gut ileum) and used pathway analysis tools to evaluate whether major sex differences were occurring.

Materials and Methods

Tissues

Tissues were harvested from untreated dogs. 3 male and 3 female dogs were analysed for the following organs: heart (ventricle and atrium separately), liver, kidney (cortex and medulla separately) and the GI tract (ileum). Intra and inter-gender variation between the 3 male and 3 female dogs in the 6 tissues was also quantified (see supplementary data). All animal studies were approved by the AstraZeneca animal ethics committee, in accordance with Home Office Regulations.

Gene expression data and statistics

RNA isolation was performed with Qiagen Tissue RNAeasy isolation kit (Qiagen). Chip hybridization and readouts were performed according to the manufacturer's instructions (Affymetrix) on Canine_2 affy U133 chips. QC of raw data and RMA analysis

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performed with R Package (version 2.7.1). RMA data output files expressed in log2-transformed format.

Individual genes were identified using two-tail student t-test to compare log2-transformed expression levels of each gene between males and females. Genes showing at least a 20% difference (over or under) in expression, with a p-value of under 0.05 were considered significant.

Pathway analysis

Pathway analysis was performed with Ingenuity Pathway Analysis (IPA) version 8.6.

Results and Discussion

We found that individual genes exhibited gender dimorphic expression across heart (ventricle and atrium) and drug metabolizing tissues (liver, kidney medulla, kidney cortex and ileum). A variety of genes was under and over-expressed in a gender dimorphic pattern for each tissue. Notably, EIF2S3 was very highly expressed in males versus females in all tissues studied (Figure 1a and supplementary Figure 1).

Eukaryotic translation initiation factor 2 (EIF-2) is known to function in the early steps of protein synthesis [11,12], where it forms a ternary complex with GTP and initiator tRNA and binding to a 40S ribosomal subunit [13] EIF-2 is composed of three subunits: alpha, beta and gamma (EIF2 gamma is also known as EIFS23). We found that EIF2S3 was over-expressed significantly and consistently in all the male dog tissues studied relative to female tissues (see Figure 1a). Growth factor stimulation of EIF2 in response to hormonal signaling, including GH, has been observed in previous studies [14,15].

At the molecular level, growth hormone (GH) is secreted (as plasma 'pulses') from the pituitary differently in males and female rodents (see Figure 1c) [8], whereas the rodent male 'pulse' tends to be episodic, the female can be characterized as more 'continuous'. These GH profile differences between males and females are known to influence drug metabolising gene expression profiles between the sexes and result in very distinct drug metabolising differences in rodent species [9,10]. There is some evidence that these GH profiles have an influence in other animal species, though it is thought that

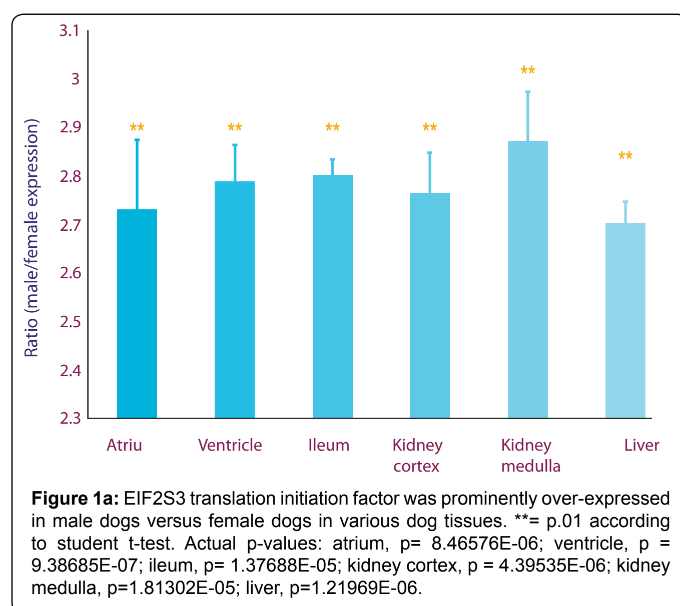

Figure 1a: EIF2S3 translation initiation factor was prominently over-expressed in male dogs versus female dogs in various dog tissues. **= p.01 according to student t-test. Actual p-values: atrium, p= 8.46576E-06; ventricle, p = 9.38685E-07; ileum, p= 1.37688E-05; kidney cortex, p = 4.39535E-06; kidney medulla, p=1.81302E-05; liver, p=1.21969E-06.

these effects are likely to be subtle in humans [10]. The potential impact of GH profiles between male and female humans will not be known until more research on human subjects can be done in this area. Given the strong animal data along with the very compelling clinical data in the clinic, it does remain a distinct possibility that GH profiles play a role in transcriptional and post-transcriptional differences between males and females, in humans as well as other animals, including the dog. Thus, given these results, it is a likely candidate for sexually dimorphic gene expression on the GH-hormonal axis, in a similar manner to that described for rodent models [8,9,16], as outlined in (Figure 1c).

Other factors that may influence EIF2S3 include phosphorylation: PKR has been reported to regulate the EIF2 complex[17,18], It is thought that phosphorylation of EIF2S1 triggers inhibition of protein synthesis by blocking the ability of EIF2B (EIF2S2) to initiate GDP/GTP exchange on EIF2S3 [19]. It is also possible that the EIF2S3 subunit is phosphorylated directly to intiate translation and potential phosphorylation of unknown proteins may result in the downstream activation (or inactivation) of the EIF2S3 subunit. Interestingly in our studies, PDK4 was up-regulated in the male heart tissues and the small intestine relative to female tissues, but not in the kidney and liver tissues – it is possible that PCK1 (which was significantly up-regulated) may fulfill a phosphorylation role in the EIF2 pathway in these tissues.

Pathway analysis on genes in individual tissues demonstrating gender dimorphism was performed with IPA version 8.6 (see supplementary data, materials and methods**)**. All the most significant pathways shown to be influenced by gender dimorphic expression also involved EIF2S3. In the atrium and ventricle, pathways associated with lipid metabolism and small molecule signaling were prominent (supplementary Figures 2a and 2b). The ileum showed a substantial influence on the cell death and development pathways, whilst pathways affected by gender dimorphic gene expression in the kidney appear to be associated with glucose and small molecule signaling, as well as cell death pathways (supplementary Figures 2d and 2e). The liver, which is the major site of xenobiotic and drug metabolism, demonstrated that lipid metabolism and small molecule signaling pathways were particularly affected by gender dimorphically expressed genes. These early results may help to give insight into the genetic basis of male and female susceptibility to various metabolic disease states.

Genes exhibiting gender dimorphic expression were analysed for toxicological risk profiling. Many of the genes demonstrating gender dimorphic expression in the dog are associated with both biological disease states and adverse drug outcomes. These are shown in (Figure 1b).

Adverse event associated genes

An adverse event can be defined as an unfavourable and unanticipated problem, leading to higher risk and likelihood of harm in subjects. An analysis of adverse event-related genes with those genes demonstrating gender dimorphism was undertaken. We found that UCP3, KCNH7 and BDNF showed significantly altered expression in male heart tissues relative to female tissues (Figure 1b). All of these genes have previously been associated with heart failure, Long QT or arrhythmia (see supplementary figure 3 for references). Interestingly, KCNH7 under-expression in may be a factor in the female preponderance for both acquired and heritable Long QT syndrome [6].

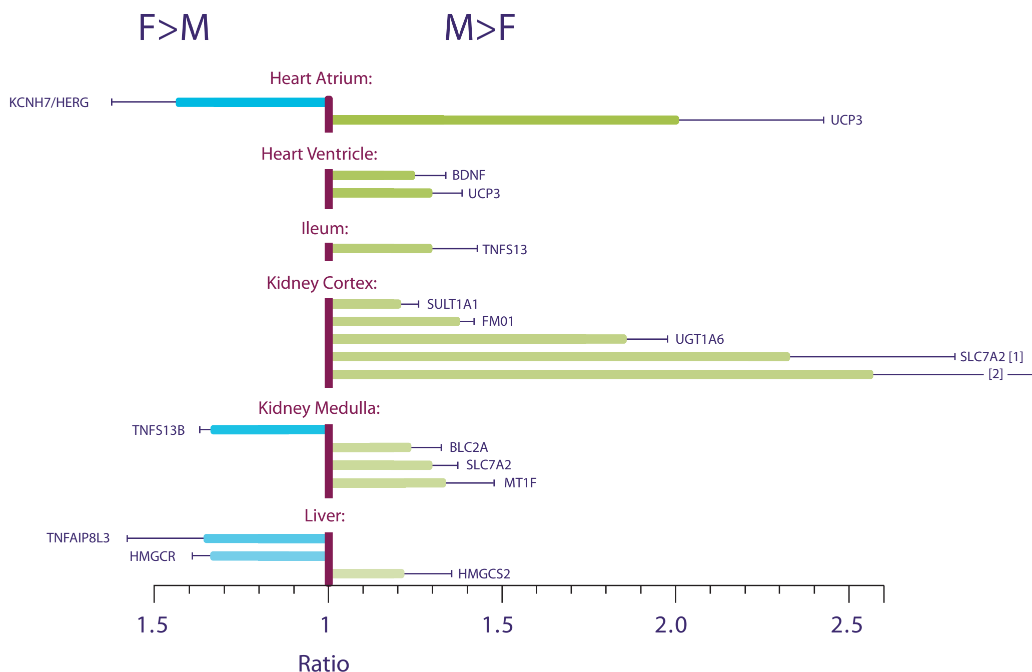

Figure 1b: Gender dimorphic genes in the dog associated with adverse event risk and thus the potential for gender risk-associated drug toxicity (left) and those associated with DMPK activity (right). Genes above the line (at ratio 1.0) represent significantly over-expressed genes in male dogs versus female dogs; genes below represent genes significantly under-expressed in male versus female dogs. DMPK – Drug Metabolism and Pharmacokinetics; AE – Adverse Event.

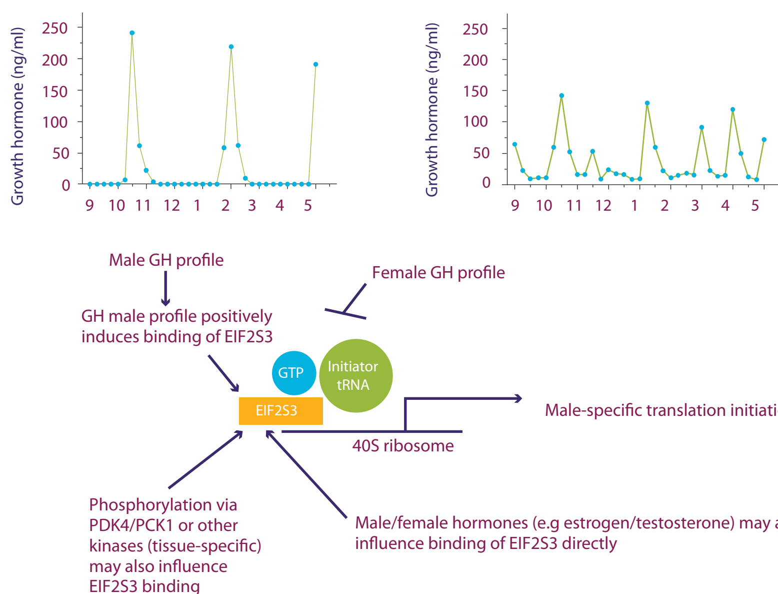

Figure 1c: Hypothethical interaction of EIF2S3 with GH hormone axis in the dog. Male ('erratic') and female ('continuous')-specific GH profiles influence the binding of transcriptional factors (potentially STAT5 as established for rodents8), leading to transcription of the post-transcriptional initiation factor EIF2S3 – which, with GTP and initiator tRNA forms a tertiary binding complex to a 40S ribosomal subunit. The high levels of EIF2S3 in the male influence a variety of gender dimorphic effects at the post-transcriptional level. The complicated nature of the interaction may include a direct influence of hormones and phosphorylation.

Other genes associated with inflammation and tissue injury (that demonstrate gender dimorphic expression) in the Ileum, kidney and liver tissues include MT1F, TNFS13B, SLC7A2, BCL2 and TNFalpha (see supplementary Figure 3). Hence, all of these may potentially influence inflammatory disease and drug response in disease and clinical therapies.

Drug metabolism-associated genes

An analysis of genes associated with drug metabolism (Figure 1b) showed only four genes that displayed a statistically significant difference between the sexes. In the kidney, UGT1A6 and FMO1 metabolic enzymes, found to over-expressed in the male; these are unlikely to have a significant impact on drug metabolism, since the

major site of drug metabolism occurs in the liver, not the kidney.

In the liver, two closely associated genes, HMGCR/HMG CoA (down-regulated in males with a ratio of 0.75) and HMGCS2 (slightly up-regulated in the male dog with a ratio of 1.21) were found to be significantly altered. HMGCR/HMG CoA is the site of statin binding and activation. HMGCS2 on the other hand, may affect statin binding and activation via modulation of statin target HMGCR/HMG CoA). The up-regulation of this gene may also serve to amplify the effect of HMGCR under-expression in the male to affect drugs involved or associated with this pathway - most obviously the statins. This finding could possibly translate to males requiring less and females more of statin drugs to achieve appropriate efficacy and toxicity windows. In fact, statin drugs have shown large gender differences in the clinic in regards to toxicity [20] and – should the data translate to humans -these findings may help to partially account for this. It is also interesting to note that HMGCS2 is located in the PPAR gamma pathway (see supplementary Figure 2f), which is of great interest, since gender dimorphic effects have been observed with PPAR gamma-targeted drugs (rosiglitazone and pioglitazone) in the clinic [21]. This class of drug displays significant gender differences in clinical and pharmacokinetic trials [21].

Our results underline the prominence of the EIF2S3 gene across all tissues studied, where it showed an extremely consistent 2.7-2.8 times greater expression in male dogs when compared to females (Figure 1a). EIF2S3, a known translation initiator in humans, is an X-chromosome expressed gene, located on Xp21. It has been shown that EIF2S3 can escape X-inactivation [22] though our results indicate that - at least in the dog - EIF2S3 works in the opposite direction driving higher transcription in males than females. It is possible that over-efficient X-inactivation or psuedoautosomal (Y-chromosomal) EIF2S3 genes exist in the male and account for the over-expression; however, such chromosomally-linked phenomena generally occur at chromatin boundaries: as such they would be expected to cluster with many genes [22] and our studies indicate that EIF2S3 clearly stands out alone across all the tissues. This strongly suggests that other, more specific mechanisms – such as hormonal interaction with transcription factors, as we suggested earlier – are at play. Based on previous research performed by others [8,10], we postulate that EIF2S3 may be involved in the GH signaling axis (see Figure 1c) in the manner described for rodents.

The gender differences in drug metabolism and adverse event-related genes described in this study also raise the question as to whether current preclinical protocols are appropriately reflective and predictive of clinical outcome. The male dominance in preclinical efficacy and safety pharmacology studies and to some extent clinical trials is driven by the need for a homogenous test population, avoiding female hormonal fluctuations which may pose a challenge for analysis and interpretation.

The results described here, in combination with increasing clinical data describing gender as a risk factor in clinical therapy, as described herein, suggest that it is time to consider increasing our assessment of both sexes in preclinical trials – despite (or even because of) the extra level of complexity. For personalized drug regimens to ever be a reality, this level of complexity should be incorporated into future clinical trial design.

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