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## Accepted Manuscript

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**Cloning and mRNA expression analysis of an ABCG2 (BCRP)  
efflux transporter in rainbow trout (*Oncorhynchus mykiss*)  
liver and primary hepatocytes**

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**Abstract**

As has been recently demonstrated in mammals, apart from the P-glycoprotein (Pgp, ABCB) and the MRP-like proteins (MRPs, ABCC), another efflux transporter – the BCRP (ABCG2) – expressed in polarized epithelial cells of different tissues, is involved in regulation of intestinal absorption and biliary excretion of potentially toxic xenobiotics. However, no study has been directed towards identification of BCRP (ABCG2) in fish species. In the present study we have cloned the full ABCG2 transcript from rainbow trout (*Oncorhynchus mykiss*) liver, showing a high sequence identity (60%) to human ABCG2 gene. Using Real-Time PCR we measured relative expression of ABCG2 in trout liver and primary hepatocytes and compared these expression levels to

the expression of other ABC transporters expressed in apical membrane of hepatocytes (ABCB1, ABCB11 and ABCC2). ABCG2 expression was 6 fold higher than ABCC2 and almost 42 fold higher than ABCB1, indicating that the ABCG2 probably plays a significant role in the overall disposition and excretion of xenobiotics in fish.

*Keywords:* ABCG2; Trout hepatocytes; Full ABCG2 sequence; Relative expression

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Liver is a major detoxification organ in fish. One important part of detoxification pathways is the extrusion of xenobiotics and their metabolites out of cells by the ABC transport proteins involved in the overall multixenobiotic resistance (MXR) mechanism in trout hepatocytes. Besides the ABCB1 (Pgp) and members of the ABCC (MRP) subfamily, breast cancer resistance protein (BCRP, ABCG2) plays one of the major roles in efflux of xenobiotics in mammals (Doyle and Ross, 2003). Unlike ABCB1 or ABCC2, ABCG2 is a 72-kDa half-transporter protein, consisting of one nucleotide binding domain and one membrane spanning domain (Wakabayashi et al., 2006). Overexpression of this protein in different mammalian cell lines has been linked to the high resistance against a wide variety of anticancer drugs (Ozvegy et al., 2001; Mao and Unadkat, 2005). ABCG2 is prominently expressed in apical membranes of polarized epithelial cells of different organs involved in absorption (small intestine), distribution (placenta and blood brain

barrier) and elimination (liver) (Ito et al., 2005), indicating an important role of ABCG2 in excretion of xenobiotics.

Recently finished analysis of the large zebrafish genome sequencing project revealed that 77% of all human ABC genes have a zebrafish orthologue (Annilo et al., 2006). Although this analysis showed that zebrafish possesses four ABCG2 genes (ABCG2a-d), no study has been directed towards the analysis of gene expression and quantification of ABCG2 in fish so far. In this study we cloned the full gene sequence of rainbow trout (*Oncorhynchus mykiss*) ABCG2, quantified its expression in trout liver and primary hepatocytes and compared this expression to the gene expression levels of ABCB1, ABCB11 and ABCC2.

Total RNA isolation was independently performed from three trout livers. Primary hepatocytes were cultured for 24 h in L-15 medium with FBS (10%) (Ferraris et al., 2002) and RNA was isolated from three independent hepatocyte cultures from three different specimens using the RNeasy Kit (Qiagen). Consensus primers (sense: *CACTCCCTTCCATCGTCTTCAGC*; antisense: *ACTCAACAATACTGGGCAGGTTCA*) were designed based on the EST clone (CR370793) with high sequence similarity to zebrafish ABCG2. The 5'- and 3'-ends of the corresponding cDNA were synthesized using rapid amplification of cDNA ends protocol (RACE, GeneRacer Kit (Invitrogen)) and sequenced. New pair of consensus primers was designed in 5'- and 3'-UTR and full transcript was cloned, sequenced and submitted to the GeneBank (accession number EU163724). The translated sequence was aligned with various ABC proteins using ClustalX and subsequently the phylogenetic tree was generated using the neighbor-joining method (MEGA 3). For the purpose of quantitative real-time PCR analysis total

RNA was subjected to digestion of genomic DNA using Deoxyribonuclease I, Amplification Grade (Invitrogen). First strand cDNA was synthesized using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). The expression of obtained trout ABCG2 was analyzed by quantitative RT-PCR on ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA) using SYBR Green method and  $\beta$ -actin as a housekeeping gene. Gene expression levels of other ABC efflux transporters (ABCB1, ABCC2 and ABCB11) previously identified in trout liver were also quantified and compared to expression of ABCG2 in liver and primary hepatocytes.

Sequencing of the cloned 1965 bp transcript and its subsequent analysis using BLASTX revealed that the obtained sequence corresponds to ABCG2 found in mammalian species. Translation of the sequence resulted in a 655 amino acid length polypeptide. Multiple alignment of the obtained trout sequence with mammalian ABCG2 proteins and 4 predicted ABCG2 proteins found in zebrafish genome, revealed a high degree of similarity to mammalian ABCG2 and zebrafish ABCG2a (Table 1). The results of phylogenetic analysis also indicated grouping of the identified sequences within ABCG subfamily, showing this sequence as evolutionary closest to zebrafish ABCG2a (Fig. 1).

The expression level of identified ABCG2 gene was measured in trout liver and compared to three other ABC proteins recently cloned in our laboratory (Fig 2A). The ultimately highest expression was determined for ABCB11 – 18 fold higher than for ABCG2, more than 100 fold higher than for ABCC2, and almost 800 fold higher than ABCB1. High ABCB11 expression was expected due to its physiological role in

transport of bile salts. However, ABCG2 expression in trout liver was more than 6 fold higher than ABCC2 and almost 42 fold higher than ABCB1.

In order to further evaluate primary trout hepatocytes as a suitable model for ABC transporters studies, the expression of the same four transporters was determined in hepatocytes and the results compared with the liver expression profile. The same relative difference in the transporters' expression was detected in primary hepatocytes cultured for 24 h. Nevertheless, all four transporters exhibited lower expression in cultured hepatocytes (Fig. 2B).

In conclusion, our results showed that ABCG2 is highly expressed in trout liver and primary hepatocytes. Furthermore, since ABCC2 (MRP2), and especially ABCB1 (Pgp), were previously considered the major transporters implicated in the extrusion of unmodified xenobiotics and their metabolites, this study points to the ABCG2 as possible key mediator of MXR defense in apical membrane of fish hepatocytes. Its substrate specificity and true role in metabolism of xenobiotics in fish, however, remain to be addressed in further research.

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**Figure captions**

Fig. 1. Phylogenetic tree based on the multiple alignment (ClustalX) of ABCG subfamily (dr, *Danio rerio*; gg, *Gallus gallus*; hs, *Homo sapiens*; om, *Oncorhynchus mykiss*; xt, *Xenopus tropicalis*; rn, *Rattus norvegicus*). The tree was generated using neighbor-joining method and the percentage concordance based on 1000 bootstrap iterations is shown at the nodes.

Fig. 2. Results of quantitative gene expression analysis of four trout ABC transporters expressed in liver and primary hepatocytes. (A) Relative expression of four trout ABC transporter and  $\beta$ -actin mRNAs in liver. The values represents means  $\pm$  standard deviations of three independent RNA isolations. (B) Expression of four trout ABC transporter mRNAs in primary hepatocytes relative to their expression in liver. Quantities are normalized on  $\beta$ actin mRNA expression. Values above the bars represent fold down regulation in comparison to the liver.

Table 1.

Percent similarity matrix for several ABCG proteins from different organisms (dr, *Danio rerio*; gg, *Gallus gallus*; hs, *Homo sapiens*; om, *Oncorhynchus mykiss*; xt, *Xenopus tropicalis*).

	drABCG1	drABCG2a	drABCG2b	drABCG2c	drABCG2d	ggABCG1	ggABCG2	hsABCG1	hsABCG2	omABCG2	xtABCG2
drABCG1		26.3	27.3	26.3	26.3	88.3	24.8	87.9	26.3	27	23.8
drABCG2a			47.9	46.6	62.4	28	63.9	26.6	61.5	72.3	63.9
drABCG2b				63.4	46.2	27.7	47.2	27.3	48	47.1	47.8
drABCG2c					44.9	26.8	44	26	47.4	44.9	45.9
drABCG2d						25.9	55	25.9	55.5	63	55.9
ggABCG1							26.1	85	26.9	27	24.1
ggABCG2								25.2	62.8	61.5	65.5
hsABCG1									26.5	26.7	23.6
hsABCG2										60.3	69.7
omABCG2											62.4
xtABCG2											

Figure 1.

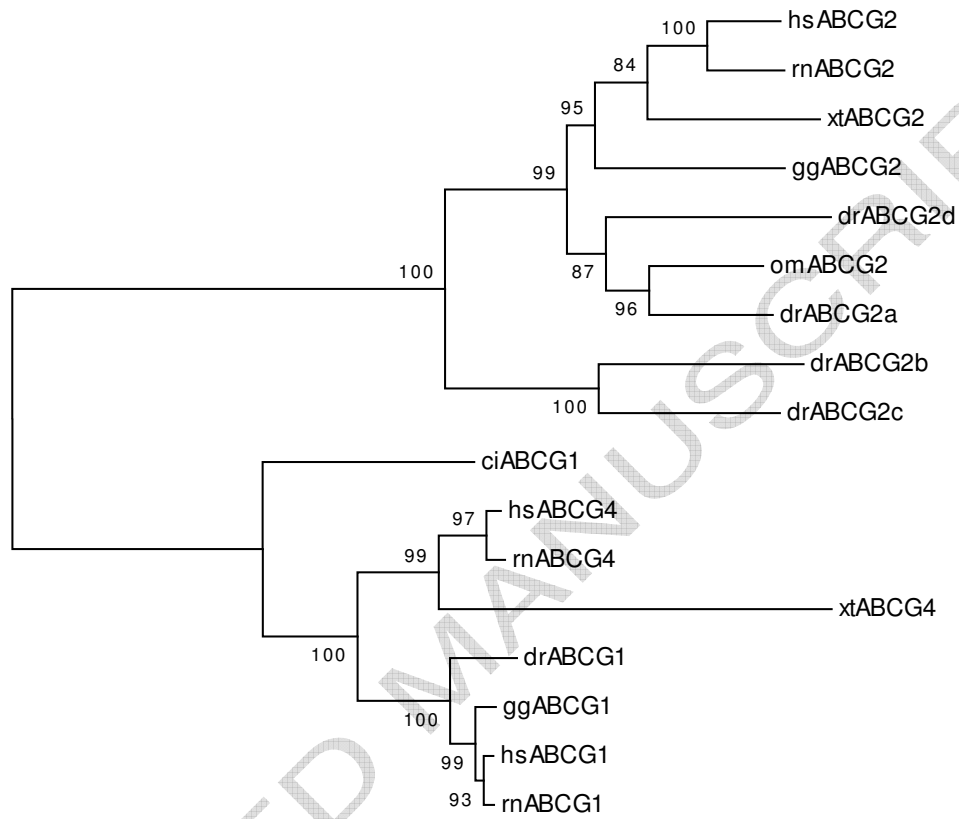
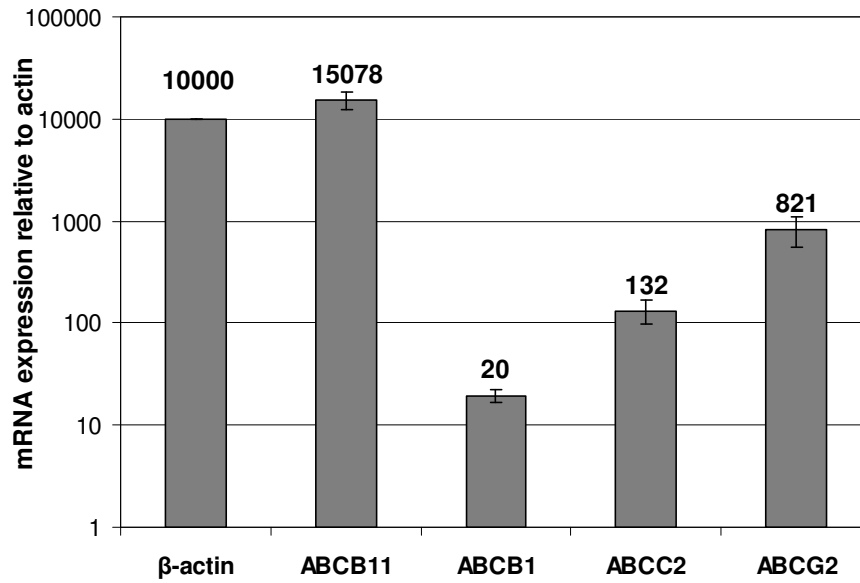


Figure 2.

(A)



(B)

