ORIGINAL ARTICLE

An experimental study to identify the potential role of pharmacogenomics in determining the occurrence of oxaliplatin-induced liver injury

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Abstract

Background: Oxaliplatin-based chemotherapy has been linked to the development of sinusoidal obstruction syndrome (SOS), which is detrimental to outcome after liver resection for colorectal liver metastases (CLM). The aim of this study was to determine how the expression of genes involved in the transport and metabolism of FOLFOX chemotherapy impacts on tissue injury in a murine model of CLM.

Methods: Experimental CLM was established in C57/B16 mice and treated with FOLFOX chemotherapy. After 3 weeks, the animals were killed and RNA extracted from liver, spleen and tumour tissue. DNA damage was assessed by immunohistochemistry for γH2AX. Gene expression was determined by reverse transcriptase polymerase chain reaction.

Results: FOLFOX treatment was associated with an increase in the number of γH2AX-positive cells in both the spleen ($P < 0.01$) and tumour tissue ($P < 0.01$), but not the liver. Tissue resistance to injury following FOLFOX was associated with high expression of the copper transporter *ATP7B*. Differences in the expression of genes related to 5-fluorouracil metabolism or DNA repair did not correlate with the severity of tissue injury.

Conclusions: High levels of expression of *ATP7B* are associated with resistance to tissue injury following FOLFOX chemotherapy. Polymorphisms in the *ATP7B* gene may explain varying susceptibility to SOS among patients following oxaliplatin-based chemotherapy.

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Introduction

The use of oxaliplatin-based chemotherapy prior to resection of colorectal liver metastases (CLM) has been associated with the development of sinusoidal obstruction syndrome (SOS) in the non-tumour-bearing liver in up to 45% of patients.[1–4] The presence of SOS is a cause for concern in major liver resection and the use of oxaliplatin-based chemotherapy prior to resection of CLM has been associated with a significant increase in perioperative morbidity.[1,5] More recently, it has been suggested that the presence

of SOS is associated with poorer disease-specific survival and, in particular, an increased risk for intrahepatic recurrence.[2] Thus, there is an urgent need to identify those patients with particular risk for developing SOS in order to ensure that their exposure to oxaliplatin-based chemotherapy is minimized.

Over the last few years, several attempts have been made to develop a murine model of oxaliplatin-induced SOS. However, to date, none of these have been successful in inducing histological changes in the liver despite the administration of high doses of chemotherapy for prolonged periods.[6,7] One potential explanation for this observation is found in early pharmacological studies conducted in healthy mice, which showed the spleen to be the primary organ to take up oxaliplatin and indicated that reduced

concentrations are taken up by the liver.[8] Identification of the factors responsible for the relative resistance of the murine liver to oxaliplatin may represent an important step towards establishing why some patients seem particularly vulnerable to the toxic effects of this drug.

Oxaliplatin is a third-generation platinum compound consisting of a platinum atom conjugated to diaminocyclohexane and oxalate groups.[9] Work by Katano *et al.* and Safaei *et al.* demonstrated, in cancer cell lines, that the cellular influx and efflux kinetics of platinum compounds mirror those of copper.[10,11] Cellular uptake of copper is regulated by the plasma membrane copper transporters CTR1 and CTR2. Cell lines which do not express CTR1 demonstrate high levels of resistance to platinum-based chemotherapeutics, which strongly suggests that this transporter plays a critical role in the cellular uptake of these drugs.[12,13] In support of this observation, it has been demonstrated *in vivo* that dorsal root ganglia with high CTR1 expression are particularly vulnerable to oxaliplatin toxicity.[14] By contrast, high levels of expression of the copper uptake transporter CTR2 have been shown to be associated with relative resistance to platinum-based chemotherapy both *in vitro* and *in vivo* in ovarian cancer.[15–17]

The cellular export of copper is regulated by the transmembrane ATPases ATP7A and ATP7B. Cancer cell lines which demonstrate resistance to platinum-based chemotherapy commonly express high levels of these transporters, thereby preventing the intracellular accumulation of platinum.[10,18,19] The validity of these *in vitro* observations has been confirmed *in vivo*: patients with colorectal cancer, in which high levels of ATP7B are expressed, are more likely to experience disease progression during treatment than those with low levels of ATP7B expression (hazard ratio 3.56; $P < 0.01$).[20]

Oxaliplatin is rarely administered as monotherapy, but, rather, is used alongside a thymidylate synthase inhibitor such as 5-flourouracil (5-FU) or leucovorin (in FOLFOX) or the oral pro-drug capecitabine (in CAPOX/XELOX).[21] Upon cellular entry, 5-FU is converted into its active metabolites by the action of the enzyme thymidine phosphorylase (TP). These metabolites exhibit a cytotoxic effect through the inhibition of the enzyme thymidylate synthase (TS). Many cells also possess the capability to convert 5-FU into biologically inactive metabolites via a pathway that utilizes the enzyme dihydropyrimidine dehydrogenase (DPD).[22]

The aim of the current study was to determine the differential expression of genes involved in the cellular transport of platinum compounds and tissue injury following exposure to FOLFOX chemotherapy.

Materials and methods

The use of animals in this study was approved by the local research ethics committee and the UK Home Office.

Experimental model of CLM

Following isoflurane anaesthesia, C57/Bl6 mice underwent midline laparotomy. Subcapsular injection of the syngeneic MCA38 colorectal cancer cell line, stably transfected with the pGL4.51 luciferase reporting vector (Promega UK, Southampton, UK), was then performed in the left lobe of the liver (10^5 cells per animal suspended in 10 µl of Hank's buffered salt solution). The injection track was sealed with manual compression to achieve haemostasis followed by application of butylcyanoacrylate glue (Indermil®; Henkel Ireland Ltd, Dublin, Ireland). The abdomen was closed in two layers with vicryl.

Five days following surgery, the presence of viable tumour was confirmed using the IVIS® Imaging System (Caliper Life Sciences, Hopkinton, MA, USA). At this point, treatment was commenced with intraperitoneal FOLFOX chemotherapy, which consisted of 6 mg/kg oxaliplatin followed 2 h later by 50 mg/kg 5-FU and 90 mg/kg folinic acid. This drug regimen was based on a review of the literature and the present group's own preliminary toxicity studies. Control animals received vehicle (10% DMSO) alone.

Animals were treated weekly for 3 weeks with FOLFOX and killed 24 h after the final dose of chemotherapy. There were five animals in each experimental group.

Histology and immunohistochemistry

Sections of 5 µm were cut from formalin-fixed, paraffin-embedded blocks of liver, spleen and tumour tissue from each animal. One of these sections was stained with haematoxylin and eosin (H&E) in order to facilitate an assessment of drug-induced tissue injury.

To assess chemotherapy-induced DNA damage in each of these tissues, immunohistochemistry was performed for γH2AX. In brief, sections were deparaffinized and peroxidase activity blocked. Antigen retrieval was performed with 1 mμ EDTA pH 8.0, following which endogenous biotin was blocked by use of a commercial kit (Vector Laboratories, Inc., Burlingame, CA, USA) before incubation in 20% swine serum for 30 min. The primary antibody (#9718; Cell Signalling Technology, Inc., Danvers, MA, USA) was added at a concentration of 1 : 100 and sections incubated overnight at 4 °C. Sections were then incubated for 90 min with a biotin-conjugated secondary antibody (Dako UK Ltd, Ely, UK) and for 45 min with streptavidin biotin-peroxidase conjugate (Vector Laboratories, Inc.). 3,3′-diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories, Inc.) was used as the chromogen. The numbers of positive stained cells in 15 separate high-power fields (HPF; magnification ×20) were counted in a blinded manner.

Reverse transcription polymerase chain reaction

RNA was extracted from snap frozen samples of liver, spleen and tumour from each animal using the RNeasy system according to the manufacturer's instructions (Qiagen Ltd, Crawley, UK). RNA was quantified on a spectrophotometer and 1 µg used to generate cDNA using Moloney murine leukaemia virus reverse transcriptase (RT) and a random hexamer primer (Promega UK) to a final total volume of 50 µl.

Reactions consisted of 12.5 µl polymerase chain reaction (PCR) mastermix, 400 nM each of forward and reverse primers and 1 µl of cDNA to a final volume of 25 µl. Amplification was performed using 35 cycles consisting of denaturation at 95 °C, annealing at 55 °C and elongation at 70 °C, each step of which lasted 30 s. This was followed by a final elongation step at 70 °C for 7 min. The PCR product was then run on 1% agarose gel and detected by staining with ethidium bromide. β-Actin served as the internal control. Primer sequences are listed in Table S1 (online).

Statistical analysis

Data are presented as the mean ± standard error. Statistical significance was assessed using Kruskall–Wallis one-way analysis of variance (ANOVA) and the Mann–Whitney U-test as appropriate. A P-value of <0.05 was considered to indicate statistical significance.

Results

FOLFOX-induced tissue injury

Intraperitoneal administration of FOLFOX demonstrated antitumour efficacy in this model, with significant areas of cell death seen on the review of H&E-stained sections (Fig. 1a). In keeping with previous reports, there was no histological evidence of SOS. As the spleen has been reported to be the main organ to take up oxaliplatin-based chemotherapy in pharmacological studies, sections of this organ were also reviewed. These revealed atrophy of the germinal centre of the spleen and relative expansion of the red pulp (Fig. 1b).

The toxic effects of oxaliplatin are mediated through the binding of platinum to DNA, which results in the formation of DNA double-strand breaks and either activation of the cellular DNA repair mechanisms or, if sufficient damage is present, programmed cell death. In order to determine whether the histological changes observed in the tumour and spleen were a consequence of DNA damage, immunohistochemistry was performed for γH2AX. There was a significant increase in the number of γH2AX-positive cells in both the spleen (38 versus nine cells per HPF; $P < 0.01$) (Fig. 2a) and tumour tissue (51 versus 38 cells per HPF; $P < 0.01$) (Fig. 2b) of FOLFOX-treated animals, whereas no such increase was demonstrated in the liver (four versus five cells per HPF) (Fig. 2c). Together, these observations would appear to support the assertion that the murine liver is relatively resistant to FOLFOX-induced injury, whereas the spleen and tumour tissue are more vulnerable.
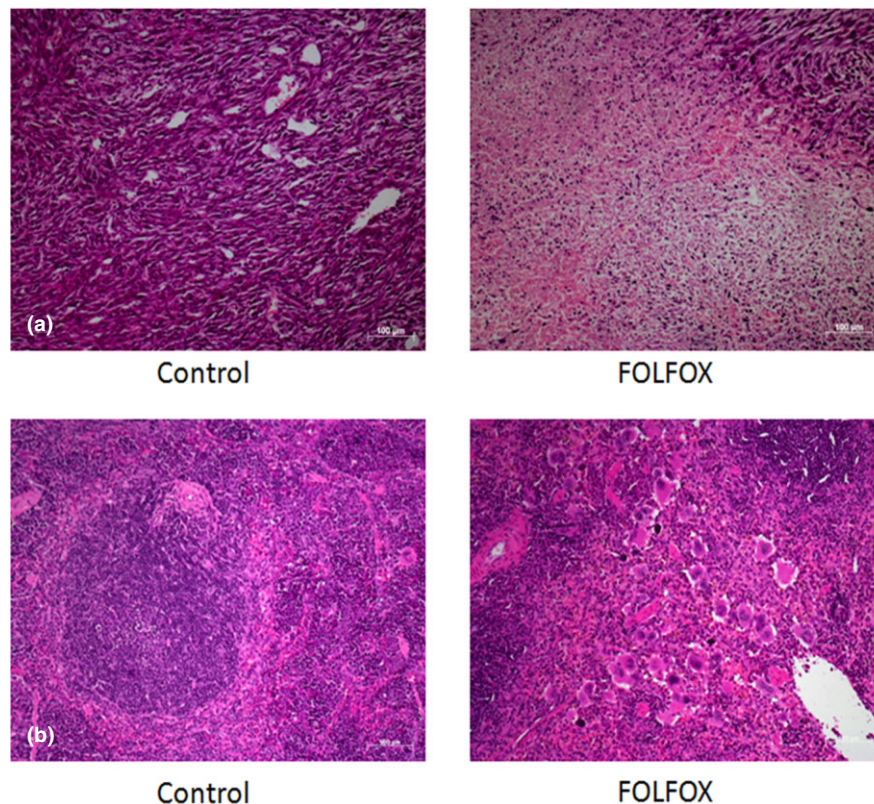
Figure 1 (a) Histopathology demonstrated areas of tumour death in FOLFOX-treated animals compared with control animals, confirming the efficacy of this regimen. (b) Evidence of splenic injury with loss of germinal centres and relative expansion of the red pulp was also apparent. (Haematoxylin and eosin stain; original magnification ×10)
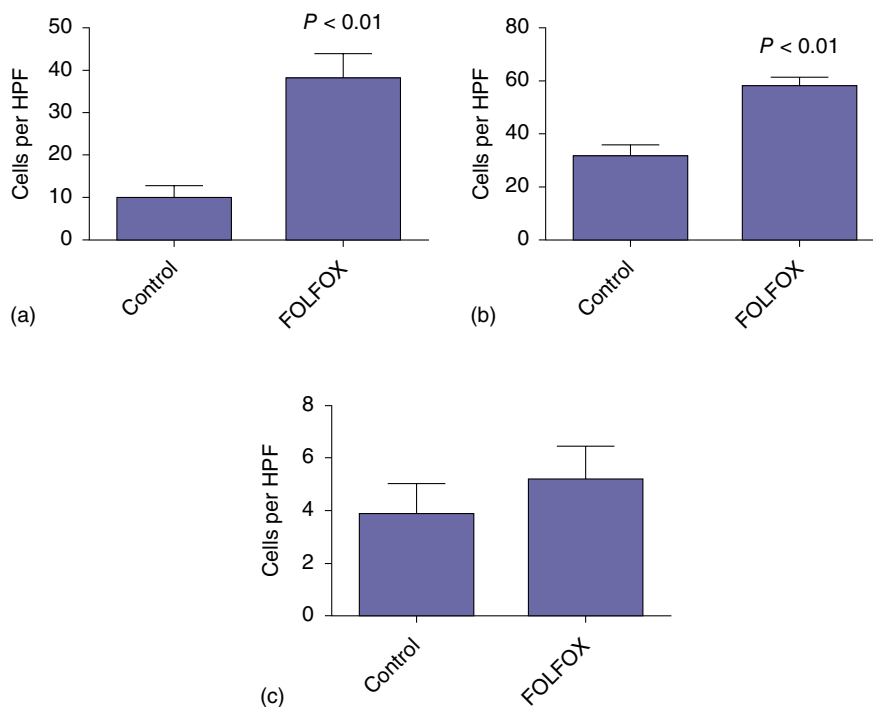
Figure 2 Immunohistochemistry for γH2AX revealed FOLFOX-induced DNA damage in the (a) spleen and (b) tumour tissue. (c) This damage was not seen in the liver. HPF, high-power field
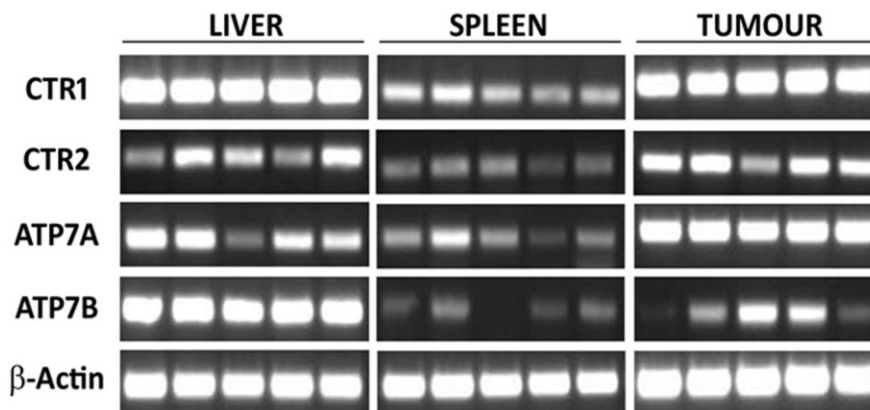
**Figure 3** Expression of copper transporter genes in liver, spleen and tumour tissue

Copper transporter expression

To assess the relationship between the ability of tissues to take up oxaliplatin-based chemotherapy and the development of histological injury, RT-PCR was performed to determine the expression of the copper influx (*CTR1* and *CTR2*) and efflux (*ATP7A* and *ATP7B*) transporters. Despite the absence of injury to the liver, mRNA expression of the influx transporters was such as might be expected in an oxaliplatin-sensitive organ (i.e. high expression of *CTR1* and relatively low expression of *CTR2*) (Fig. 3). Overall, the transcript level of these transporters was similar to that seen in oxaliplatin-sensitive tumour tissue. Together, these findings would suggest that the resistance of the murine liver to FOLFOX-induced injury in this model is not a consequence of a deficiency in oxaliplatin uptake.

The mRNA expression of the efflux transporter *ATP7A* in the liver was somewhere between those of the tumour and spleen, again suggesting that this is not pivotal in mediating resistance to oxaliplatin-induced injury. By contrast, mRNA expression of *ATP7B* was markedly higher in the liver than in the other tissues, which suggests that the liver may be able to rapidly export
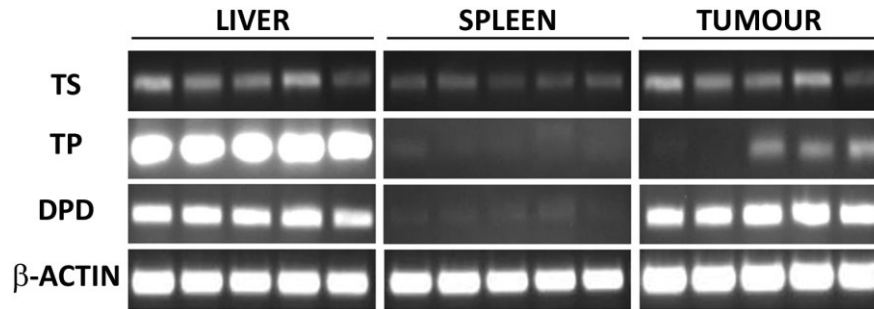
Figure 4 Expression of genes related to 5-fluorouracil metabolism in liver, spleen and tumour tissue. TS, thymidylate synthase; TP, thymidine phosphorylase; DPD, dihydropyrimidine dehydrogenase
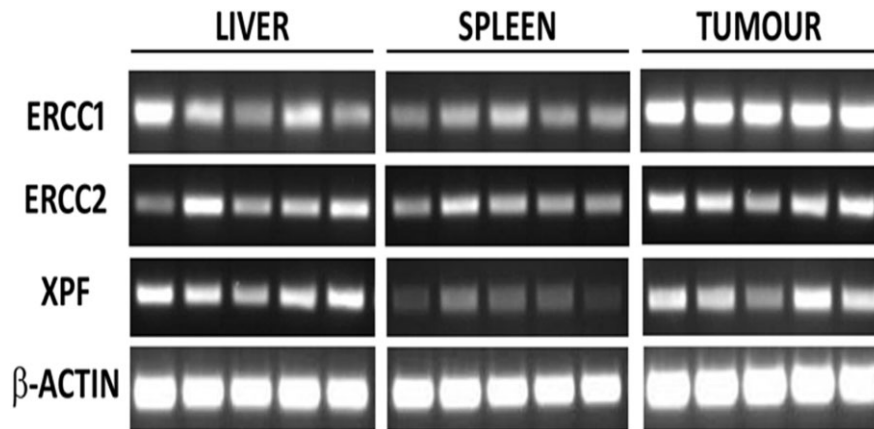
Figure 5 Expression of genes from the nucleotide excision repair pathway

oxaliplatin, thereby providing a mechanism through which intracellular exposure to the drug is limited and thus minimizing toxicity (Fig. 3).

5-FU metabolism

In order to assess the relative sensitivity of the liver to the 5-FU component of the chemotherapy regimen, expression of TS, TP and DPD were compared across all three tissues. The liver was found to express the highest amount of TP transcript, suggesting there should be no deficiency in converting 5-FU into its active metabolites. The expression of DPD was similar in liver and tumour tissue, and the expression of TS was similar across all three tissues (Fig. 4). It therefore seems unlikely that 5-FU resistance prevents the development of histological liver injury in this model.

Nucleotide excision repair pathway

The cellular response to DNA–platinum adducts is to attempt repair by making use of the nucleotide excision repair (NER) pathway in which, following recognition of DNA damage, the DNA helix is unwound to allow excision of the damaged DNA by proteins including ERCC1/2 and XPF. Following excision, the damaged DNA is resynthesized by DNA polymerase and the helix rewinds. Impairment of the NER pathway is associated with increased sensitivity to oxaliplatin-mediated DNA damage.[23,24] It has recently been suggested that decreased expression of ERCC2 transcript may be associated with the development of SOS in patients with CLM treated with preoperative chemotherapy.[25]

In the current model, mRNA expression of the NER components ERCC1/2 and XPF in the liver was generally lower than that seen in tumour tissue, but was broadly similar to that seen in the spleen (Fig. 5). This would seem to suggest that differences in this regard are not responsible for the relative resistance of the murine liver to FOLFOX.

Discussion

This study has confirmed the observation that the murine liver appears to be relatively resistant to the development of injury following exposure to FOLFOX chemotherapy. By contrast, the spleen, along with implanted tumour tissue, appears to be particularly sensitive. By comparing the expression of various genes in these sensitive tissues with that of the liver, this study has been able to demonstrate that this resistance appears to be related to high expression of the copper efflux transporter ATP7B, which

enables the rapid clearance of oxaliplatin from the liver. There were no differences in the expression of genes related to 5-FU metabolism or the NER pathway which would otherwise explain this resistance.

The liver plays a central role in copper homeostasis; in particular, its export is tightly regulated by the liver through the actions of ATP7B, which transfers copper to the secretory pathway whereby it is conjugated to caeruloplasmin or, when copper is in excess, is regulated through biliary excretion.[26,27] For example, Wilson's disease is characterized by excessive hepatic accumulation of copper and occurs as a result of a functional deficit in the *ATP7B* gene.[27] As already discussed, ATP7B also plays a key role in the cellular export of platinum compounds, the overexpression of which is associated with chemo-resistance in cancer cell lines.[19] As ATP7B is normally highly expressed in the liver, it would be reasonable to expect that it would also be relatively protected from the platinum toxicity.

Polymorphisms in the gene *ATP7B* are relatively common in the general population. One study that examined genomic DNA isolated from the peripheral leukocytes of 203 cancer patients found that 61 patients had a polymorphism in *ATP7B*.[28] This study made no attempt to correlate these polymorphisms with chemotherapy side-effects, however, and thus the functional consequence of such polymorphisms is unknown.

Although it is entirely logical to believe there is a direct correlation between the number of cycles of chemotherapy administered to patients and the development of SOS, the evidence for this is not particularly clear. In a multivariate analysis performed in a series of 90 patients undergoing resection of CLM following preoperative chemotherapy, Nakano *et al*. identified the receipt of more than six cycles of oxaliplatin-based chemotherapy as an independent risk factor for the development of SOS.[29] By contrast, a separate study of 196 patients, conducted by Tamandl *et al*., failed to identify any association between the number of chemotherapy cycles and SOS development.[2] One possible explanation for these divergent observations is that pharmacogenomics and, in particular, polymorphisms affecting *ATP7B* function may play a key role in determining individual susceptibility to the toxic effects of oxaliplatin-based chemotherapy.

The concept of modifying a chemotherapy strategy based on the presence of genetic polymorphisms is not new. Irinotecan is an alternative chemotherapeutic widely applied in the management of patients with CLM, the use of which can be associated with several significant complications, including febrile neutropenia and severe diarrhoea. The active metabolite of irinotecan, SN38, undergoes metabolism itself utilizing a group of enzymes known as UDP-glucuronosyltransferase (UGT) 1A. Single-nucleotide polymorphisms (SNPs) in the genes encoding members of this family are common and, when present, particularly in the gene *UGT1A1*, have been associated with an increased incidence of irinotecan toxicity. When these SNPs are detected, it is usual practice to reduce the administered dose of irinotecan.[30,31]

The identification of a cohort of individuals at high risk for developing SOS following oxaliplatin-based chemotherapy would be extremely useful in establishing which patients will be best served by the provision of alternative and equally effective regimens in place of prolonged and detrimental chemotherapy. Screening for SNPs is an attractive way of doing this as genomic DNA can be easily isolated from peripheral blood leukocytes for analysis.

It should be noted that the current study is intended to generate hypotheses rather than to provide a direct causative link between ATP7B expression and the occurrence of oxaliplatin-induced SOS. Nonetheless, despite these limitations, it does appear to offer justification for human studies to explore whether SNPs in *ATP7B* could be used to identify a cohort of patients at high risk for oxaliplatin-induced SOS.

Conflicts of interest
None declared.

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Supporting information

Additional supporting information may be found in the online version of this article.

Table S1. Primer sequences for quantitative reverse transcription polymerase chain reaction (qRT-PCR).