Hepatic microRNA profiles offer predictive and mechanistic insights after exposure to genotoxic and epigenetic hepatocarcinogens

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Keywords: carcinogen; epigenetic; miR-34a; miR-200; liver

List of abbreviations

miRNA microRNA, hepatocellular carcinoma (HCC), MOA mode of action, 2-AAF 2-Acetyl aminofluorene, DEHP Diethylhexylphthalate, DETU Diethylthiourea, MP HCL Methapyrilene HCl, BP benzophenone, PB phenobarbital, MON monuron, Chl. Ac Chlorendic acid, PAM Prediction analysis of microarrays, CAR constitutive androstane receptor, pparα peroxisome proliferation receptor alpha, GO gene ontology

Short Title: miRNAs and chemical hepatocarcinogenesis

Abstract

In recent years accumulating evidence supports the importance of microRNAs in liver physiology and disease; however few studies have examined the involvement of these non-coding genes in chemical hepatocarcinogenesis. Here we examined the liver microRNA profile of male Fischer rats exposed through their diet to genotoxic (2-acetylaminofluorene) and epigenetic (phenobarbital, diethylhexylphthalate, methapyrilene HCL, monuron, chlorendic acid) chemical hepatocarcinogens, as well as to non-hepatocarcinogenic treatments (benzophenone; diethythiourea) for three months. The effects of these treatments on liver pathology, plasma clinical parameters, and liver mRNAs were also determined. All hepatocarcinogens affected the expression of liver mRNAs, while the hepatic microRNA profiles were associated with the mode of action of the chemical treatments and corresponded to chemical carcinogenicity. The three nuclear receptor activating chemicals (phenobarbital, benzophenone, and diethylhexylphthalate) were characterized by the highly correlated induction of the miR-200a/200b/429, which is involved in protecting the epithelial status of cells, and of the miR-96/182 clusters. The four non-nuclear receptor activating hepatocarcinogens were characterized by the early, persistent induction of miR-34, which was associated with DNA-damage and oxidative stress *in vivo* and *in vitro*. Repression of this microRNA in a hepatoma cell line led to increased cell growth, thus miR-34a could act to block abnormal cell proliferation in cells exposed to DNA-damage or oxidative stress. This study supports the proposal that hepatic microRNA profiles could assist in the earlier evaluation and identification of hepatocarcinogens, especially those acting by epigenetic mechanisms.

Introduction

The liver is a frequent target for toxicity induced by exogenous chemicals, due to the metabolizing activities of hepatocytes and the high concentrations of orally ingested chemicals passing to it directly from the gastrointestinal tract (Hardisty & Brix, 2005). Exogenous chemicals can drive carcinogenesis by a variety of mechanisms (Irigaray & Belpomme, 2010; Hernandez *et al.*, 2009). DNA-damaging chemicals drive carcinogenesis by causing detrimental mutations of cancer genes. Conversely, non-DNA reactive chemicals do not damage DNA, but rather drive carcinogenesis by more subtle, epigenetic mechanisms (Boobis *et al.*,2009), including the induction of oxidative stress, of cycles of necrosis/regeneration, or by disrupting physiological mitosis and/or apoptosis (Irigaray & Belpomme, 2010; Hernandez *et al.*, 2009). Oxidative stress can damage DNA and proteins through the generated free radicals, as well as disturbing cellular signalling. Cytotoxic chemicals cause cycles of necrosis/regeneration, which promote the growth of neoplastic lesions, the eventual depletion of telomeres, and the release of pro-inflammatory chemokines and cytokines. Disruption of the normal proliferation and apoptosis balance drives tumor promotion by favoring the expansion of mutated cells.

To protect human health, novel chemicals are evaluated for potential carcinogenicity. DNA-reactive chemicals can be readily and reliably detected by a number of *in vitro* and *in vivo* tests, such as mutation, chromosomal aberration, and micronucleus assays (Boobis *et al.*, 2009). In contrast, no reliable assays exist for the identification of

human epigenetic carcinogens. This situation arises due to the limited understanding of the molecular, biochemical, and cellular events that are associated with epigenetic carcinogenesis. Consequently, risk assessment for carcinogenicity of chemicals (the rodent bioassay) is currently a laborious, complicated, time-consuming, animal demanding, and costly process (Boobis *et al*., 2009). Moreover, this current benchmark for the evaluation of novel chemicals is often irrelevant to humans due to species differences and the high doses which are used (Boobis *et al*., 2009). The development of better assays for epigenetic hepatocarcinogens is dependent on an improved understanding of the molecular events associated with this type of chemical.

MicroRNAs (miRNAs) are a family of evolutionary conserved non-coding RNAs that post-transcriptionally regulate their target genes. In recent years accumulating evidence supports the importance of liver microRNAs in physiology and disease, including cancer (Bala *et al*., 2009). Numerous studies have shown altered expression in liver tumors (e.g. Hou *et al.*, 2011; Pineau *et al.*, 2010); However, so far only a handful of studies have examined the function of miRNAs during chemical hepatocarcinogenesis and these have been mostly restricted to a single chemical treatment (Li *et al.*, 2010; Malik *et al.*, 2011; Moffat *et al*., 2007; Pogribny *et al*. 2009; Shah *et al*., 2007; Yauk *et al.*, 2010). In this study the effects of sub-chronic treatment with a variety of chemical treatments were examined in parallel. Chemical treatments were chosen to represent diverse modes of action (MOA), carcinogenic potential, and effects on the liver. For each chemical its maximum tolerated dose or a

carcinogenic dose, as reported in the literature, for this rat strain were used. PB and BP belong to the PB-type class of compounds that activate the constitutive androstane receptor (CAR) (Chhbra, 2000; Kodama and Negishi, 2006), while DEHP activates the peroxisome proliferator-activated receptor alpha (pparα) (Lapinskas *et al*. 2005). Chronic activation of these receptors drives hepatocarcinogenesis in rodents by disrupting proliferation/apoptosis of initiated cells (Lake, 2010). All three chemicals cause the induction of hepatic tumors in mice, but only PB and DEHP have the same effect in this rat strain (Butler, 1978; NTP TR-217; NTP TR-533). MP HCl is an hepatotoxic hepatocarcinogen that promotes cancer by inducing necrosis/regeneration (Lijinsky, 1984). The potent hepatocarcinogen 2-AAF was the only genotoxic chemical used here, while also having additional promoting faculties (Neuman *et al*. 1997). Also included in this study were two non-DNA damaging hepatocarcinogens with unknown MOA, MON (NTP TR-266) and Chl Ac (NTP TR-304). As a control chemical DETU was included, a kidney carcinogen with few reported liver effects (NTP TR-149). We found that assessing the hepatic miRNAome is a potentially useful avenue for assessing the mechanisms and carcinogenic potential of chemical treatments.

Materials and methods

Animal study

All the chemicals used in this study were purchased from Sigma-Aldrich (Dorset, United Kingdom). Male Fischer (F344) rats were obtained from Harlan Olac (Netherlands) and left to acclimatise for seven days prior to commencement of the

treatments. Animals were then randomly assigned to cages and treatment. The chemical treatments used in this study are summarised in **Table 1**. Nine-week old rats were continually exposed to each chemical in their diet (N=5 animals per group) for a period of up to three months. The animal studies were performed in accordance with the UK "Animals (Scientific procedures) Act" under controlled lighting (12h light cycles), humidity (30-70%), air flow (15 changes per hour), and temperature (22±3 C) conditions. Rats were given access to mains water and diet *ad libitum*. Exposure of the animals to the chemicals was verified by chemical analysis of the diets and daily monitoring of food intake. At the end of the study, animals were killed by an overdose of anaesthetic (halothane Ph.Eur.Vapour) followed by exsanguination. Liver tissue was obtained from the animals immediately upon sacrifice, snap frozen, and stored at -80C until needed.

Evaluation of histopathology and measurement of clinical parameters

For histopathology liver sections were taken from the three main liver lobes, embedded in paraffin wax, sectioned at 5µm, stained with haematoxylin and eosin, and examined by light microscopy. For clinical chemistry plasma was collected and the levels of glutamate dehydrogenase, alanine aminotransferase, and aspartate aminotransferase were measured.

mRNA Microarray analysis

Profiling of liver mRNAs was performed as previously described (Waterman *et al.*, 2010), using the Affymetrix Rat Expression RG-230 v2 GeneChips (Affymetrix, Buckinghamshire, United Kingdom).

miRNA microarray analysis

For miRNA analysis total RNA was extracted from the left lateral liver lobes using Trizol (Invitrogen, Paisley, United Kingdom) following the manufacturer's instructions. The RNA was deemed suitable for microarray analysis if it had a 260:280 ratio above 2.0 (as determined by Nanodrop ND-1000) and a RIN number larger than eight (as determined using an Agilent Bioanalyser). For miRNA profiling three to five liver samples from each group were examined. Profiling of miRNA expression was performed using the Agilent miRNA microarray platform (Agilent, Stockport, United Kingdom). The microarray slides contained the 350 rat miRNAs that were included in the Sanger 10.1 database. The slides were viewed using an Agilent scanner (G2505B) and the data were collected using the Agilent Feature Extraction v.10.1 software. The miRNA hybridisation signals were threshold to 1, $\log_2$ transformed, and normalised to the 75th percentile in Genespring GX 11.0. MiRNAs that were not flagged as present by the Agilent feature extraction software in at least 50% of the samples in any treatment group were filtered out from further analysis. For six liver samples (three control animals and three PB treated animals) the whole process from RNA extraction onwards was performed twice to assess the

reproducibility of the generated microarray data (Control and PB technical replicates).

Quantitative Real Time PCR (qPCR) analysis

For mRNAs reverse transcription was performed using the High capacity cDNA RT kit (Applied Biosystems, Warrington, United Kingdom). The generated cDNA was amplified using the Taqman 2X Universal PCR master mix, No AmpErase UNG (Applied Biosystems), with each PCR reaction performed in triplicate. The qPCR data were analysed in the ABI 7500 Sequence Detection System (Applied Biosystems). The comparative Ct Method ($\Delta\Delta C_T$ Method) was used to quantify miRNA expression. Calibration was based on the expression of the 18s rRNA Taqman. For miRNAs total RNA was reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). Mature miRNA taqman assays were purchased from Applied biosystems. Quantification was performed as per manufacturer's instructions using 18s rRNA or snoRNA as the calibrator gene.

Cell culture

Fao rat hepatoma cells (ECACC 85061112) were maintained in DMEM media supplemented with 10% FBS. Recombinant human tgf-beta1 was purchased from R & D (Abingdon, United Kingdom) and used as described previously (Caja *et al*.,2007). Etoposide and hydrogen peroxide were obtained from Sigma-Aldrich.

Statistical Analysis

Student's t-test and ANOVA were used to test for statistical significance. Deregulated mRNAs were identified as those with $p<0.05$ (t-test from control) and more than 1.5-fold change from control. Over-expressed gene ontology categories were identified as those with $p<0.01$ within Genespring GX 11.0. One-way ANOVA with Benjamini Hochberg multiple testing correction followed by Tukey HSD post hoc test were used to identify differentially expressed miRNAs. To identify predictive miRNA signatures the Prediction Analysis of Microarrays (PAM) software was used (Tibshrani *et al*., 2002).

Results

Effects of chemical treatments on liver pathology, clinical chemistry, and mRNA profiles

To certify that the treatments had the expected hepatic effects we evaluated plasma clinical parameters, liver pathology and biochemistry, and liver mRNA expression of the treated animals (**Tables 2-3**). The nuclear receptor activators (PB, BP and DEHP) resulted in hepatic hypertrophy and significant increases in adjusted liver weight (**Table 2**). PB and BP treatments also induced *cyp2b1* (11.5-fold and 9.5-fold from control respectively), which is a defining characteristic of CAR activation (Lake, 2010). Similarly, DEHP caused changes in the expression of mRNAs that are characteristic of pparα activation, such as the induction of *cyp4a1* (4.7-fold from

control) and an enrichment of genes involved in lipid metabolism (Lake, 2010; Lapinskas *et al*., 2005) (**Table 3**).

In accordance with its known cytotoxic properties MP HCL caused the largest increases in the plasma levels of three markers of hepatotoxicity (glutamate dehydrogenase, aspartate aminotransferase, and alkaline aminotransferase) (**Table 2**); was the only compound for which evidence of necrosis/apoptosis was found upon histopathological examination (**Table 2**); and together with 2-AAF were the only treatments that resulted in an enrichment of the cell cycle Gene Ontology (GO) group affecting a number of cell cycle genes (**Tables 3-4**). 2-AAF was also the only compound to cause an enrichment of the programmed cell death GO group (**Table 3 and Table 5**), although no increase in liver necrosis/apoptosis was detected (**Table 2**).

MON was the only compound besides the nuclear receptor activators to induce a significant, albeit small, increase in adjusted liver weight at three months (113% control), as well as to induce minimal liver vacuolation (**Table 2**). Both MON and Chl. Ac caused an enrichment of mRNAs involved in the oxidation-reduction GO, while Chl. Ac also affected a number of genes involved in lipid metabolism (**Table 3**). The non-hepatocarcinogen DETU had few effects on the examined liver parameters (**Tables 2)**, affected fewer mRNAs (**Table 3**), and did not result in the enrichment of any GO group (**Table 3**). Overall, chemical treatments had the expected hepatic effects after subchronic treatment (Chhbra, 2000; Lake, 2010;

Lapinskas *et al*., 2005; Neuman *et al*., 1997; NTP-TR-149; NTP-TR-266; NTP-TR-304).

Effects of chemical treatments on the liver miRNAome at three months

We next used a microarray platform to examine the effects of the chemical treatments on the liver miRNAome. Box plot analysis demonstrated comparable distributions of the microarray samples (**Fig. 1A**), while two sets of technical replicates revealed a very strong correlation of miRNA expressions (**Fig.1B**), confirming the reliability and reproducibility of the microarray data. As independent validation the expression levels of five miRNAs were determined by qPCR and found to be highly correlated to their expression changes calculated by microarrays (**Fig. 1C**). Therefore, the microarray platform that was utilized here proved to be reliable and reproducible.

With one way ANOVA and Bonferonni-Holm multiple testing correction, 21 miRNAs had significantly altered expression in animals treated with one or more compounds compared to control animals after three months (**Table 6**). Importantly, all the hepatocarcinogens had effects on the liver miRNAome, while no hepatic miRNAs displayed significant deregulation compared to control following treatment with the non-hepatocarcinogen chemical DETU. The deregulated miRNAs included several belonging to the same genomic clusters or families, which as expected showed highly correlated expression in response to the chemical treatments (Pearson's correlations for miR-200b-miR-200a r=0.98; miR-200b-miR-429 r=0.96; miR-96-miR-182 r=0.96; miR-99a-miR-100 r=0.98; miR-29a-miR-29c r=0.95). Importantly, the clustering of the samples based on the differentially expressed

miRNAs follows a logical order, suggesting that hepatic miRNA profiles reflect the mode of action of the chemicals (**Fig.1D**). DETU, the chemical with the least effects on the liver, associated with the control animals. In a second node the two PB-type compounds, PB and BP, clustered together. A third node contained the two compounds which had the largest effect on cell cycle at three months (MP HCL and 2-AAF). Hepatic miRNA profiles also clustered together the three most potent hepatocarcinogens tested in this study (2-AAF, MP HCL, and Chl.Ac).

It should be noted that the chemical treatments caused both downregulation and upregulation of miRNAs (**Table 6**). In terms of magnitude, most altered miRNAs were changed between 1.5 to 5-fold from control, similar to the changes observed between HCC and benign liver tissue (Hou *et al.*, 2008). Some of the observed miRNA deregulation was specific to a particular compound, for example the induction of miR-107 by DEHP or miR-139-5p by Chl. Ac. We were particularly interested in identifying miRNAs that were affected by more than one chemical, as these could be involved in a more general adaptive or adverse response to the treatment. Compared to control animals, ten miRNAs had significantly altered expression in the livers of animals treated with two or more chemicals: members of the miR-200a/200b/429 cluster (PB, DEHP, BP, 2-AAF, Chl.Ac, and MP HCL) ; members of the miR-96/182 cluster (PB, DEHP, BP, MON); miR-34a (2-AAF, MP HCL, Chl.Ac, MON), miR-99a (2-AAF, MP HCL, MON), miR-203 (2-AAF and Chl.Ac), miR-193 (MON and Chl.Ac), and miR-221 (BP and DEHP). Of these

miRNAs only miR-221 was deregulated in a different direction by treatments, being induced by DEHP and repressed by BP.

It was also observed that there was a significant correlation between the number of mRNA probes and miRNAs that were deregulated compared to control for each treatment ($r=0.74$; $p<0.05$ Spearman's correlation) indicating that there is an association between strength of the perturbation induced by chemical treatments on these two gene classes.

In conclusion, our findings demonstrate that chemical hepatocarcinogens affect liver miRNA profiles after long term treatments, that these profiles are associated with the MOA of chemicals, and that a surprisingly consistent set of miRNAs are affected by chemical treatments.

Effect of nuclear receptor activators on liver miRNAs

The three nuclear receptor activators (PB, BP, and DEHP) had clearly distinct effects on liver pathology, including the induction of hepatomegaly and hypertrophy (**Table 2**). At the miRNA level the characteristic common response elicited by these compounds was a highly positively correlated induction of the miR-200a/200b/429 and of the miR-96/182 clusters ($r=0.99$, $p<0.001$ Pearson's correlation between miR-96 and miR-200b in CON, PB, BP, and DEHP treated samples), something which was not observed for the other compounds (**Fig.2A-2B**).

The two PB-type compounds (PB and BP) are both reported to be mice hepatocarcinogens, but only PB is reported to be hepatocarcinogenic in male Fischer

rats (Butler *et al.*1978; NTP TR-533). The two compounds have overall very similar effects on the liver miRNAome at three months (see significantly deregulated miRNAs for each treatment **Table 6** and clustering **Fig.1D**), probably reflecting their common MOA. Interestingly, it was observed here that PB and BP could be distinguished at the miRNA level by the repression of miR-221 following BP treatment (**Fig.2C**). Several studies have reported that miR-221 is a liver oncogene, driving hepatocyte proliferation and suppressing apoptosis (Park *et al.*, 2011; Pineau *et al.*, 2010; Sharma *et al.*, 2011). The repression of miR-221 after BP treatment could therefore contribute to the non-carcinogenicity of BP in these animals compared to PB.

Regulation and adaptive functions of the miR-200a/200b/429 cluster and of miR-34a

The hepatic miRNAs most commonly affected by the tested chemicals were the members of the miR-200a/200b/429 cluster and miR-34a (**Fig. 3A** and **3B**). MiR-200b was not affected by treatment of male Fischer rats for seven days with 2-AAF or MP HCL (**Fig.3C**), indicating that it is only induced after longer treatments. For miR-34a a significant induction could be observed in animals treated with 2-AAF or MP HCL from this early timepoint (**Fig.3D**), indicating that unlike for the miR-200a/200b/429 cluster, its induction is an earlier and persistent effect.

MiR-34a and miR-200b are known to be p53-regulated miRNAs (He *et al.*, 2010; Kim *et al.*, 2011). This is in agreement with the pronounced and persistent induction of this miRNA in rats treated with genotoxic 2-AAF. Only 2-AAF treatment resulted

in significant induction of a number of p53 regulated genes (e.g. *bax* 2.3 fold from control; *cdkn1a* 3.7 fold from control; *ccng1* 3.3-fold from control). In agreement with this observation, treatment of the rat hepatoma cell line Fao with the DNA-damaging agent etoposide resulted in the induction of both miR-34a and miR-200b (**Fig.4A**).

In contrast, the other three hepatocarcinogens that induce miR-34a are not thought to be DNA-damaging chemicals (Lijinski, 1984; NTP-TR-266; NTP-TR-304), hence their effect on the expression of this miRNA is probably occurring by alternative mechanisms. One such mechanism is the induction of oxidative stress, which has been suggested to be involved with the propagation of MP cytotoxicity (Craig *et al*., 2006). Accordingly, MP HCL treatment resulted in the induction of three genes (*akr7a3* 4.3-fold; *srxn*1 1.9-fold; *trib3* 3.4-fold) which have been associated with glutathione depletion (Gao *et al.,* 2010). Additionally, for both MON and Chl.Ac an enrichment of mRNAs involved in oxidation-reduction were observed, with this GO category being the only enriched one following treatment with the former chemical (**Table 3**). Hence, oxidative stress could be mechanism linking the induction of miR-34a with these chemical treatments. Importantly, hydrogen peroxide treatment resulted in the induction of miR-34a, but not of miR-200b in Fao rat hepatoma cells, indicating that this miRNA can be induced by oxidative stress (**Fig.4B**).

The miR-200 family inhibit epithelial to mesenchymal transition (EMT), a process which facilitates the acquisition of invasive and metastasic properties in numerous tissues, by promoting the expression of *cdh1* (*e-cadherin*) through repression of the

zeb1/zeb2 transcription factors (reviewed in Dykxhoorn.,2010). However, *cdh1* was not among the deregulated mRNAs in the livers of treated animals according to the transcriptomic data (data not shown). To confirm that this miRNA family associates with regulation of *cdh1* in the rat liver we examined a cellular model whereby treatment of Fao rat hepatoma cells with tgf-β1 results in downregulation of *cdh1* and morphological changes that are reminiscent of EMT (Caja *et al*.,2007). As reported (Caja *et al*.,2007), tgf-β1 treatment for 48 hours resulted in the downregulation of the expression of *cdh1* in Fao cells (**Fig.4C**). We found that the downregulation of *cdh1* in tgf-β1 treated Fao cells is also associated with the downregulation of miR-200b (**Fig.4C**), suggesting that induction of these miRNAs are indeed involved in the regulation of the expression of *cdh1* in rat hepatic cells.

MiR-34a is an established negative regulator of the cell cycle (He *et al*., 2010). In agreement with this observation, transfection of anti-miR-34a into Fao cells resulted in an increased number of viable cells after three days of culture (**Fig.4D**). Transfection of miR-99a which was also affected by 2-AAF and MP HCl had no effect on cell growth (**Fig.4D**). The induction of hepatic miR-34a is therefore an early and persistent event that can act to prevent abnormal cell growth due to DNA-damage or oxidative stress.

Using hepatic miRNA profiles to identify hepatocarcinogenic chemicals

We finally examined whether hepatic miRNA biomarkers or signatures can facilitate the earlier identification of chemical hepatocarcinogens. An important limitation for such an analysis was the relatively small training set of compounds, which are

insufficient for the evaluation of sensitivity and specificity of any identified miRNA signatures. Nevertheless, such an analysis can be a preliminary indicator of whether miRNA data can be useful for deriving predictive models using data from a larger training set. For that purpose we tested the predictive abilities of the set of 10 miRNAs which were significantly affected by two or more of the chemical treatments (**Table 6**). We utilized the prediction analysis of microarrays (PAM) software within the BRB-array tools developed by Dr. Richard Simon and the BRB-ArrayTools Development Team. PAM is a statistical technique that uses a gene signature (classifier) to assign class to samples using the nearest shrunken centroid (Tibshirani *et al*., 2002). Using this 10 miRNA signature the PAM algorithm could correctly classify all six hepatocarcinogenic treatments, as well as the non-hepatocarcinogen DETU (**Fig.5A-5B**). However this signature is probably too stringent, as it also classified BP as a hepatocarcinogen (**Fig.5A-5B**). In conclusion, this 10 miRNA signature was successful in correctly classifying the hepatocarcinogens, but gave ambiguous predictions for BP.

Discussion

An important finding of this study was that all the tested carcinogens affected the expression of hepatic miRNAs at three months. In this context, it is important to note that the only two studies in which hepatocarcinogenic chemicals were reported not to affect hepatic miRNAs liver had examined livers following acute treatments (Moffat *et al.*, 2007; Yauk *et al.*, 2010), in contrast to acute treatment with the hepatotoxins acetaminophen and carbon tetrachloride, which deregulated miRNAs within twenty

four hours (Fukushima *et al.*, 2007). Additionally, it has recently been reported that treatment with the genotoxic hepatocarcinogen ENU has minimal effects on liver -miRNAs after one or three days, but has much more prominent effects after 14 and 28 days (Li *et al.*, 2010). Together, these observations suggest that liver miRNAs appear to be initially refractory to acute hepatocarcinogenic treatments, although the reason behind this discrepancy is currently unknown. However, the data collected here make it clear that sub-chronic and chronic chemical treatments eventually induce prominent effects on the liver miRNAome that are discernable well before the appearance of hepatic tumors.

While some effects of the treatments on the liver miRNAome were chemical specific, a small set of miRNAs were commonly affected by the diverse carcinogenic treatments which were examined here. It is noteworthy that this set did not include miRNAs which have been previously strongly associated with HCC- such as miR-122, miR-21, and miR-199a-3p (Hou *et al.*, 2011; Pineau *et al.*, 2010). These miRNAs may not be important in chemically-induced hepatocarcinogenesis or alternatively they may not be deregulated until the later stages of the disease.

The most commonly chemical-affected hepatic miRNAs at three months were members of the miR-200a/200b/429 cluster and miR-34a (**Fig.3A-3B**). In recent years numerous studies have reported that the miR-200 family act as a positive regulator of the expression of *cdh1*, an important cell-to-cell adhesion molecule (reviewed in Dykxhoorn ,2010). Downregulation of *cdh1* is a key event implicated in epithelial-mesenchymal transition (EMT), a developmental process involved in the

acquisition of invasive faculties in liver cancer (Ding *et al*.,2010; Kim *et al*., 2011). We showed here that the miR-200a/200b/429 cluster is downregulated during tgf-β1 induced repression of *cdh1* in Fao rat hepatoma cells. The induction of the miR-200a/200b/429 cluster could be an adaptive response protecting the epithelial state of hepatocytes by sustaining high levels of *cdh1* expression.

MiR-34a has been reported to be upregulated in HCC (Pineau *et al*., 2010), as well as in response to treatment with hepatocarcinogens (Li *et al*., 2011; Malik *et al*., 2011; Pogribny *et al*., 2007; Shah *et al*., 2007). Here it was unaffected by the two non-hepatocarcinogenic treatments at three months, while it was significantly induced by all four non-nuclear receptor activating hepatocarcinogens (**Fig.3B**). This miRNA has widespread effects on the proteome of hepatic cells, resulting in a cell cycle block (Cheng *et al*.,2010), and has also been reported to be involved in terminating liver regeneration after hepatectomy (Chen *et al*.,2011). The *in vivo* and *in vitro* findings in this study suggest that this miRNA is involved in the hepatic response to DNA-damage and oxidative stress. Paradoxically, we found here that this miRNA was persistently induced by the two chemical treatments that resulted in increased cell cycle at three months, MP HCl and 2-AAF (**Table 2-4**). One possible explanation of this phenomenon is that the proliferating liver cells are a hepatic sub-population in which this miRNA is not induced. Alternatively, the proliferative and anti-apoptotic effects of these carcinogens may overcome the cell cycle block that is associated with miR-34a activity. Interestingly, the expression of miR-34a is progressively increased

in HCC (Pineau *et al.*, 2010), indicating that hepatic cancer cells can bypass the tumor-suppressive properties of this miRNA.

This study therefore suggests that liver miRNAs have fundamental roles in directing adaptive responses during the early stages of chemical hepatocarcinogenesis (**Fig. 6**). This does not exclude the possibility that other deregulated miRNAs that were identified here may have adverse functions, for example miR-99a which is commonly repressed in HCC (Hou *et al*., 2010).

Omics technologies are being pursued as potential methodology that can complement or replace the 2-year rodent bioassay (Blomme *et al*., 2009; Ellinger-Ziegelbauer *et al*., 2008). It has been reported that the mRNA profiles of livers reflect the MOA of the chemicals with which they are treated (Ellinger-Ziegelbauer *et al.*, 2008). An important question for this study was whether examination of hepatic miRNA profiles could offer mechanistic information about the tested chemicals. The observations collected here suggest that a similar link exists between chemical mechanisms and hepatic miRNA profiles. Firstly, the tested nuclear receptor activators and non-nuclear receptor activators had clearly distinct miRNA profiles at three months of treatment (Correlated induction of miR-200a/200b/429 and miR-96/182 cluster vs. induction of miR-34a). Second, hierarchical clustering analysis demonstrated the similar miRNA profiles of two structurally diverse PB-type compounds (PB and BP) and of the chemicals which had the strongest effect on the cell cycle and apoptosis (2-AAF and MP HCL). Third, expression of miRNAs was linked to carcinogenic mechanisms e.g. miR-34a with genotoxicity and oxidative stress. Fourth,

hepatocarcinogenic PB treatment could be discriminated from non-hepatocarcinogenic BP treatment from differential effects on miR-221, a miRNA which is strongly linked with liver cancer development.

Given the observations discussed above, a reasonable hypothesis would be that miRNA biomarkers or signatures can be identified that will facilitate the safety evaluation of chemicals, with due respect to the caveat that only a small number of chemicals were tested here. However, it is important to note that this study supports, as a proof-of-principle, that miRNA expression contains predictive information. Of the hepatic miRNAs found to be deregulated in this study miR-34a showed the greatest potential to be a useful biomarker for those chemical hepatocarcinogens which act through the induction of DNA-damage or oxidative stress. We also used the PAM software to evaluate the predictive powers of hepatic miRNA signatures (**Fig.5**). This analysis indicates that hepatic miRNA profiles could classify the tested hepatocarcinogens and correctly classify DETU as a non-hepatocarcinogen. This could be especially useful for non-DNA reactive hepatocarcinogens such as MON and Chl.Ac which are difficult to identify based on their effects on liver pathology and clinical chemistry. In this context, the recent reports that circulating miRNAs could offer biomarkers of tissue injury and hepatocarcinogenesis (Laterza *et al.,* 2009 and Sukata *et al*., 2011) are important.

The DNA-damaging and mutagenic consequences of exposure to genotoxic hepatocarcinogens are well characterized, but the molecular events associated with non-genotoxic chemicals are not so well understood. To the best of our knowledge

this was the first study that has investigated in depth the effects of treatments with diverse genotoxic and non-genotoxic chemical hepatocarcinogens on the liver miRNAome. The data generated here support the hypothesis that hepatic miRNA profiles can facilitate an improved mechanistic understanding and the earlier prediction of chemical hepatocarcinogens, including non-genotoxic chemicals. Currently such predictions are very difficult.

Funding:

This work was supported by funding from the Biotechnology and Biological Sciences Research Council (BBSRC, UK) in association with Syngenta.

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Tables

Table 1: Summary of chemical treatments

Compound	Dose (ppm)	Number of neoplasias/carcinomas in 2-year bioassay in male Fischer rat	Reference
Diethythiourea (DETU)	250	0/50 (0%)	(NTP TR-149, 1979)
Benzophenone (BP)	1250	0/50 (0%)	(NTP TR-533, 2006)
Monuron (MON)	1500	9/50 (18%)	(NTP TR-266, 1988)
Diethylhexylphthalate (DEHP)	12000	12/49 (24%)	(NTP TR-217, 1982)
Phenobarbital (PB)	1000	11/33 (33%)	(Butler, 1978)
Chlorendic Acid (Chl.Ac)	1250	23/50 (46%)	(NTP TR-304, 1987)
Methapyrilene hydrochloride (MP HCL)	250	18/20 (90%)	(Lijinsky, 1984)
2-Acetylaminofluorene (2-AAF)	40	23/23 (100%)	(Ogiso *et al.*, 1985)

Table 2: Effect of chemical treatments on liver pathology and plasma clinical parameters

	CON	DETU	BP	MON	DEHP	PB	Chl. Ac	MP HCL	2-AAF
Terminal Bodyweight (g)	349.8±21.3	350±20.8	353.8±33.2	308.8±26.2	313.4±22.1	356.8 ±9	319±18.7	346.6 ±5	334.6± 4.3
Liver Weight (g)	12.4±1.3	12.5± 1.6	15.5±2.1**	11.7±1.2	18.7±2.1**	15.8± 0.8	10.6± 0.5	12.5± 0.5	12.1±0.8
Adjusted liver weight	11.6	11.7	14.7*	13.1*	19.9*	14.7*	11.5	11.9	12.2
Hypertrophy	0/5	0/5	5/5	0/4	5/5	4/4	0/4	0/5	0/5
Vacuolation	0/5	0/5	4/5	4/4	0/5	4/4	0/4	0/5	0/5
Necrosis/apoptosis	0/5	0/5	0/5	0/4	0/5	0/4	0/4	4/5	0/5
Biliary hyperplasia +/- inflammation	0/5	0/5	0/5	0/4	0/5	0/4	0/4	5/5	5/5
Alanine amino-transferase (IU/L)	71.4	82.6**	83.0**	85.2**	61.4*	85.3**	81.5*	96.8**	80.8**
Aspartate Amino-transferase (IU/L)	68 ± 9.2	70.2 ± 8.1	75.4 ± 14.2	66.6 ± 2.1	53.8 ± 3.6**	69.5 ± 4.5	70.3 ± 4.2	88.8 ± 10.3**	67.8 ± 3.1
Glutamate dehydrogenase (IU/L)	4.64 ± 1.09	7.85 ± 2.32	4.48 ± 0.45	4.78 ± 1.21	4.32 ± 0.59	5.93 ± 0.42	7.70 ± 1.57*	16.72 ± 5.62*	3.52 ± 0.33

Liver weights were analysed by analysis of covariance on final bodyweight. For the other measurements ANOVA was used. CON is control group, for the other treatments see abbreviations in Table 1. Mean values and standard deviations are shown, n=3-5 animals per group. * $p<0.05$ ** $p<0.01$

Table 3: Effects of chemical treatments on mRNA profiles

Treatment	Number of significantly deregulated probes *	Enriched GO groups #
DETU	100	None
BP	186	Response to stimulus
MON	127	Oxidation reduction
DEHP	771	Numerous including oxidation reduction, fatty-acid metabolic process, carbohydrate metabolic process
PB	280	Glutathione transferase activity, monoxygenase activity, oxidoreductase activity, catalytic activity
Chl.Ac	320	Carboxylic acid, lipid metabolic process, oxidation reduction
MP HCL	188	Cell cycle; Mitosis
2-AAF	271	Cell cycle and programmed cell death

* Deregulated probes were identified as those with a p value <0.05 and more than 1.5 fold-change by a T-test between treated and control groups. For treatment abbreviations, see Table 1. #Enriched GO groups were those with p<0.01.

Table 4: Cell cycle mRNAs affected by 2-AAF and MP HCL

Gene name	Fold change in 2-AAF treated	Fold change in MP HCL treated	Significant change by other treatments
ccnd1	2.9	1.9	Chl.Ac (2.1)
cdca3	30.3	41.6	None
kif11	2.6	2.9	Chl.Ac (2.3)
ccnb2	5.2	5.7	None
tubb5	1.5	1.9	None
cdkn3	16.7	18.7	Chl.Ac (8.1)
cdc2	6.8	7.0	DETU (3.9); BP (2.9); Chl.Ac (3.9)
ccnb1	4.7	5.9	DEHP (1.9); DETU (3.4); BP (2.1); PB (2.4); Chl.Ac (3.5)
cdkn2c	2.1	1.9	DEHP (1.6)
nusap1	2.8	3.7	BP (2.0); DETU (1.7)
stmn1	2.2	3.2	DETU (1.5); PB (2.3); BP (1.5); Chl.Ac (2.3)
ube2c	2.3	3.7	DETU (1.7)
mdm2	2.3	-1.9	None
pttg1	n.s.	5.1	None
smc2	n.s	2.4	None
uhmk1	n.s.	-2.0	None
txnl4b	n.s.	1.5	BP (1.6); MON (1.5)
ncapd2	n.s.	2.7	None
nampt	-2.4	n.s.	PB (-1.8); Chl.Ac (-2.4)
steap3	-1.7	n.s.	BP (-1.7)
pkd1	-1.6	n.s.	None
anapc11	-1.6	n.s.	DEHP (-1.7); BP (-1.5);
nek6	1.6	n.s.	None
rprm	1.9	n.s.	PB (-2.0)
wee1	1.9	n.s.	Chl.Ac (2.2)
rhob	2.0	n.s.	None
rgs2	2.1	n.s.	None
tp53inp1	2.1	n.s.	Chl.Ac (2.5)
ccng1	3.3	n.s.	None
cdkn1a	3.7	n.s.	None
phlda3	4.1	n.s.	DEHP (-3.1)

Significantly deregulated probes were identified as those with a p value <0.05 and more than 1.5 fold-change by a T-test between treated and control groups at 90 days. Values shown are the mean fold change between control and chemical-treated animals as determined by microarray analysis of mRNAs which were affected by MP HCL and 2-AAF treatment and are involved in the "cell cycle" GO category.

Table 5: Genes involved in programmed cell death which are affected by 2-AAF treatment

Gene ID	2-AAF (Fold change compared to control)	Other treatments (Fold change compared to control)
bax	2.3	None
fas	4.2	None
cdkn1a	3.7	None
tp53inp1	2.1	Chl.Ac (2.5)
cd44	2.0	None
zmat3	2.1	None
sox4	2.4	None
tubb2c	2.5	None
cck	-1.6	None
hmox1	-1.6	none
aldh1a3	2.1	None
phlda3	4.1	DEHP (-3.1)
c6	-3.5	DEHP (-4.4); MON (-2.9); Chl.Ac (-2.9)
nupr1	-2.3	DEHP (-2.8); Chl.Ac (3.9)
aen	1.8	DEHP (-1.7)
steap3	-1.7	BP (-1.7)
ripk1	-1.6	MP HCL (-2.4)
cdkn2c	2.1	MP HCL (1.9); DEHP (1.6)
aldh1a1	3.4	PB (5.8); DEHP (5.5); BP (4.4); MON (3.3)
top2a	4.7	MP HCL (5.5); DETU (2.2); Chl.Ac (2.9)

Significantly deregulated probes were identified as those with a p value <0.05 and more than 1.5 fold-change by a T-test between treated and control groups at 90 days. Values shown are the mean fold change between control and chemical-treated animals as determined by microarray analysis of mRNAs which were affected by 2-AAF treatment and are involved in the "programmed cell death" GO category.

Table 6: Differentially expressed microRNAs after 90 days of chemical treatment

microRNA	Corrected p.value	DETU	BP	PB	DEHP	MON	Chl. Ac	MP HCL	2-AAF
miR-34a	>0.01	-1.1	-1.3	1	-1.2	3.9	2.3	1.7	27.9
miR-96	>0.01	1.3	2.0	3.6	1.6	1.6	-1.5	1.0	-1.3
miR-221	>0.01	-1.5	-1.8	-1.1	3	-1.2	1.5	1.0	-1.1
miR-200a	>0.01	-1.2	2.0	3.7	1.7	1.1	2.0	1.7	2.0
miR-107	>0.01	-1.1	1.1	-1.1	2.0	1.0	1.1	-1.2	-1.3
miR-200b	>0.01	-1.0	2.5	4.5	2.0	1.2	2.3	1.6	2.0
miR-429	>0.01	-1.1	2.4	3.8	1.9	1.2	2.3	1.8	2.7
miR-182	>0.01	2.4	4.3	6.3	3.1	3.3	1.1	1.3	1.4
miR-375	>0.01	-2.0	-2.3	-2.0	1.8	2.2	1.7	-2.1	-1.5
miR-193	>0.01	-1.3	-1.1	1.2	-1.7	-2.4	-4.9	-1.9	-1.3
miR-17	>0.01	-1.1	1.1	-1.1	1.1	1.0	-1.6	-1.2	-1.2
miR-497	>0.01	1.0	-1.1	-1.1	-1.2	-1.3	-1.7	-1.3	-1
miR-203	0.01	-1.2	-1.1	-1.4	-1.4	-1.2	-2	-1.2	-1.6
miR-31	0.01	1.1	1.1	-1	1.3	-1.0	1.3	-1.4	-1.5
miR-29a	0.01	-1.1	-1	1.1	1.3	1.0	-1.2	-1.2	-1.2
miR-29c	0.01	-1.1	1.0	1.2	1.3	-1.0	-1.3	-1.2	-1.2
miR-99a	0.02	-1.0	-1.3	-1.3	-1.3	-1.5	-1.3	-1.5	-1.6
miR-20a	0.03	-1.0	1.0	-1.1	1.1	-1.0	-1.6	-1.2	-1.3
miR-139-5p	0.03	1.3	-1.1	1.1	1.3	1.3	3.2	1.3	-1.3
miR-28	0.04	1.1	1	1.1	-1.1	1.0	1.4	1.0	-1.3
miR-505	0.05	-1.1	1.1	1.5	1.5	1.1	2.8	1.2	-1.6

Differentially regulated miRNAs were identified using one-way ANOVA with Bonferonni-Holm multiple testing correction and $p<0.05$. For treatment abbreviations, see Table 1. Values shown are average absolute fold change from control in that treatment. Underlined are miRNAs with significantly altered expression in livers of treated animals compared to control animals (Tukey HSD post test $p<0.05$).

Figure legends

Figure 1 Verification of miRNA microarray data and clustering analysis (**A**) Each box-plot on the x-axis represents a microarray sample. The lower end of the box is the 25th percentile, while the highest end is the 75th percentile. On the Y-axis is plotted the normalised log2 intensity from the microarrays. The black line in the middle of each box-plot is the median value for that sample. The open circle signifies the log2 value of the miRNA with the highest intensity in each sample. End of the whiskers contain 1.5 interquartile range; (**B**) Comparison of the expression of detected miRNAs for two sets of control and PB technical replicates for which RNA was extracted twice and analysed independently, n=3 per group; (**C**) Correlation between fold changes for selected miRNAs calculated by microarrays and qPCR, Pearson's correlation is shown; (**D**) Hierarchical analysis of control and chemical-treated animals based on the expression of 21 differentially expressed miRNAs. The tree was constructed using correlation uncentred and average linkage. The expression of miRNAs was median centred and normalised in cluster 3.0. Red colour indicates upregulated miRNAs, the green colour downregulated, and the black colour no change.

Figure 2 Hepatic miRNA profiles associated with tested nuclear receptor activators. (**A**) Comparison of the expression of miR-200b and miR-96 in livers of animals treated with nuclear receptor activating compounds (**B**) Comparison of the expression of miR-200b and miR-96 in livers of animals treated with non-nuclear receptor activating compounds (right); (**C**) Mean fold change in expression of miR-221 compared to control after three months of PB and BP treatment, error bars indicate s.d., N=3-5 animals

Figure 3 Effect of chemical treatments on the expression of miR-200a/200b/429 cluster and miR-34a (**A**) Mean fold change in expression of members of the miR-200a/200b/429 clusters compared to control after three months for each treatment group; (**B**) Mean fold change in expression of miR-34a compared to control after three months for each treatment group; (**C**) Effect of 2-AAF and MP HCL treatment on the expression of miR-200b after seven days; N=3 animals per group ; (**D**) Effect of 2-AAF and MP HCL treatment on the expression of miR-34a; N=3 animals per group. Mean values ± s.d. for (**A**) and (**B**) and s.e.m. for (**C**) and (**D**). * $p<0.05$ ** $p<0.01$ from control by one way ANOVA with Benjamini Hochberg multiple testing correction and Tukey HSD post test.

Figure 4 Regulation and functional effects associated with induction of miR-200a/200b/429 and of miR-34a (**A**) Effect of etoposide (50μM for 48 hours) on the expression of miR-34a and miR-200b in the Fao rat hepatoma cell lines; (**B**) Effect of hydrogen peroxide treatment (500μM for 24 hours) on the expression of miR-34a and miR-200b in the Fao rat hepatoma cell lines; (**C**) Expression of *cdh1* and miR-200b in Fao cells treated with 2ng/ml TGF-beta1 for 48 hours. Representative result of two independent experiments is shown; (**D**) Effect of transfection with 50nm of anti-miR-34a, miR-96, miR-99a, and negative control oligo on the number of viable cells after three days. Mean values ± s.e.m are shown, N=3, * $p<0.05$ ** $p<0.01$.

Figure 5 PAM prediction of hepatocarcinogenic and non-hepatocarcinogenic chemical treatments based on signature of 10 miRNAs (**A**) Cross-validated probabilities of a sample classified as undergoing a carcinogenic (indicated by a red circle) or carcinogenic (indicated by a green circle) by the PAM software using as the PAM classifier the miRNA signature of 10 miRNAs which were significantly differentially regulated in animals exposed to two or more treatments; (**B**) List of number of examined liver samples that were correctly or incorrectly classified.

Figure 6 proposed model of the adaptive functions of hepatic miR-34a and miR-200a/200b/429 during the early stages of chemical hepatocarcinogenesis

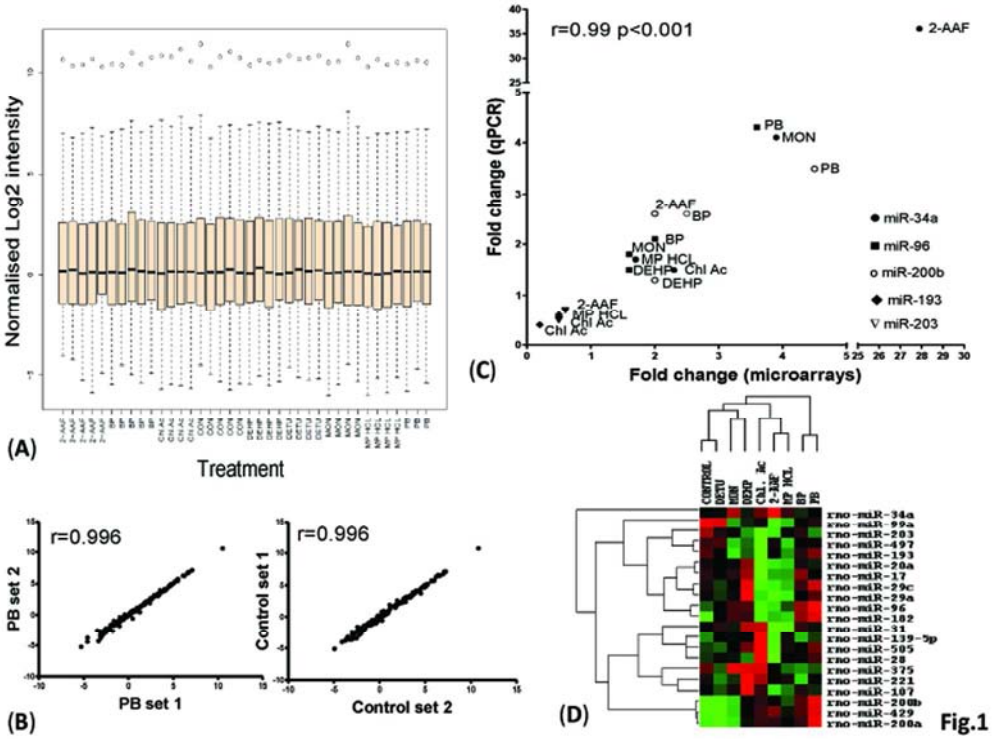

Figure 1. Verification of miRNA microarray data and clustering analysis (A) Each box-plot on the x-axis represents a microarray sample. The lower end of the box is the 25th percentile, while the highest end is the 75th percentile. On the Y-axis is plotted the normalised log2 intensity from the microarrays. The black line in the middle of each box-plot is the median value for that sample. The open circle signifies the log2 value of the miRNA with the highest intensity in each sample. End of the whiskers contain 1.5 interquartile range; (B) Comparison of the expression of detected miRNAs for two sets of control and PB technical replicates for which RNA was extracted twice and analysed independently, n=3 per group; (C) Correlation between fold changes for selected miRNAs calculated by microarrays and qPCR, Pearson's correlation is shown; (D) Hierarchical analysis of control and chemical-treated animals based on the expression of 21 differentially expressed miRNAs. The tree was constructed using correlation uncentred and average linkage. The expression of miRNAs was median centred and normalised in cluster 3.0. Red colour indicates upregulated miRNAs, the green colour downregulated, and the black colour no change.
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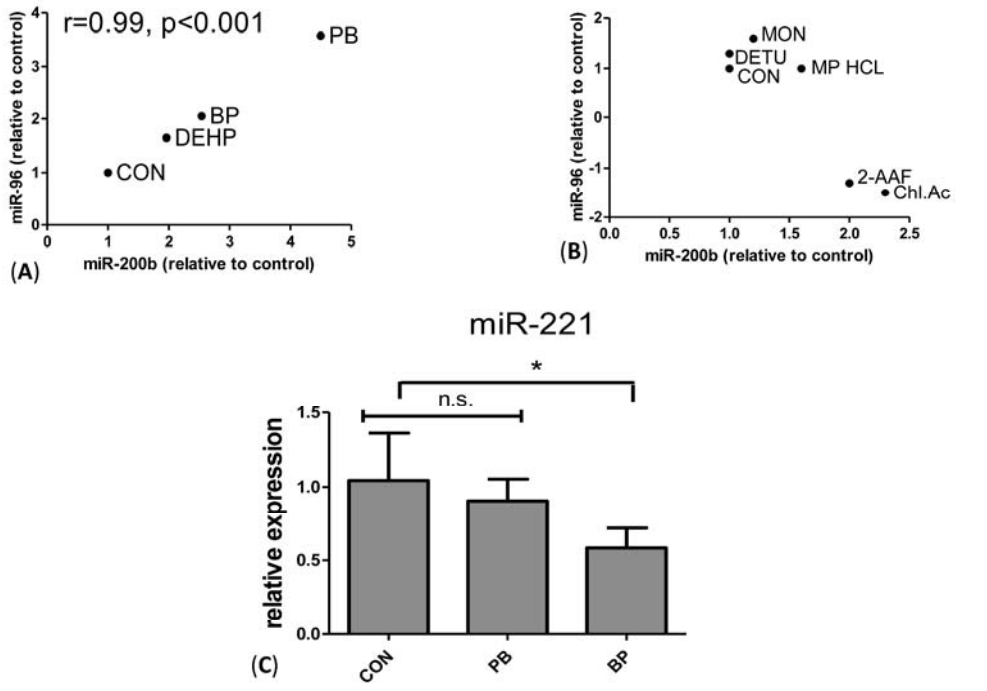


Fig.2

Figure 2. Hepatic miRNA profiles associated with tested nuclear receptor activators. (A) Comparison of the expression of miR-200b and miR-96 in livers of animals treated with nuclear receptor activating compounds (B) Comparison of the expression of miR-200b and miR-96 in livers of animals treated with non-nuclear receptor activating compounds (right); (C) Mean fold change in expression of miR-221 compared to control after three months of PB and BP treatment, error bars indicate s.d., N=3-5 animals
254x190mm (300 x 300 DPI)

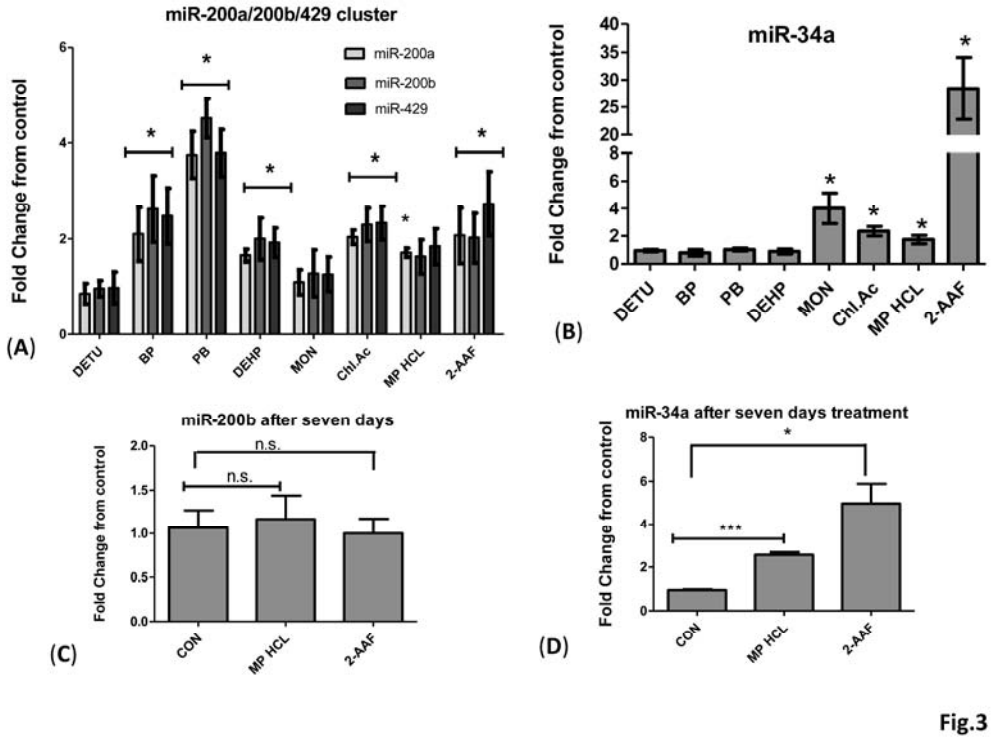


Fig.3

Figure 3. Effect of chemical treatments on the expression of miR-200a/200b/429 cluster and miR-34a (A) Mean fold change in expression of members of the miR-200a/200b/429 clusters compared to control after three months for each treatment group; (B) Mean fold change in expression of miR-34a compared to control after three months for each treatment group; (C) Effect of 2-AAF and MP HCL treatment on the expression of miR-200b after seven days; N=3 animals per group ; (D) Effect of 2-AAF and MP HCL treatment on the expression of miR-34a; N=3 animals per group. Mean values ± s.d. for (A) and (B) and s.e.m. for (C) and (D). * $p<0.05$ ** $p<0.01$ from control by one way ANOVA with Benjamini Hochberg multiple testing correction and Tukey HSD post test.
254x190mm (300 x 300 DPI)

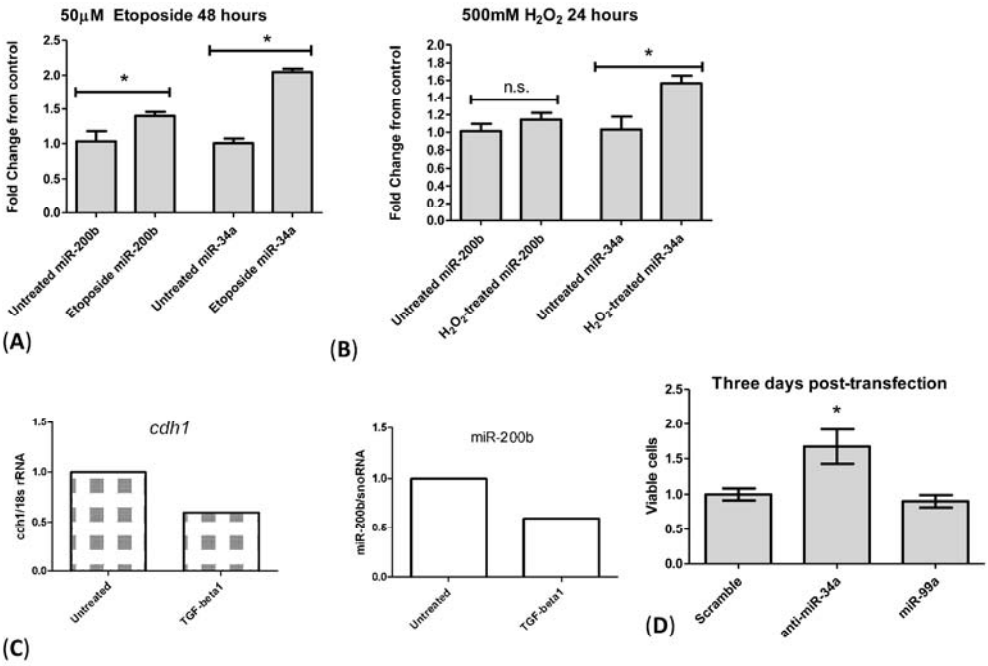
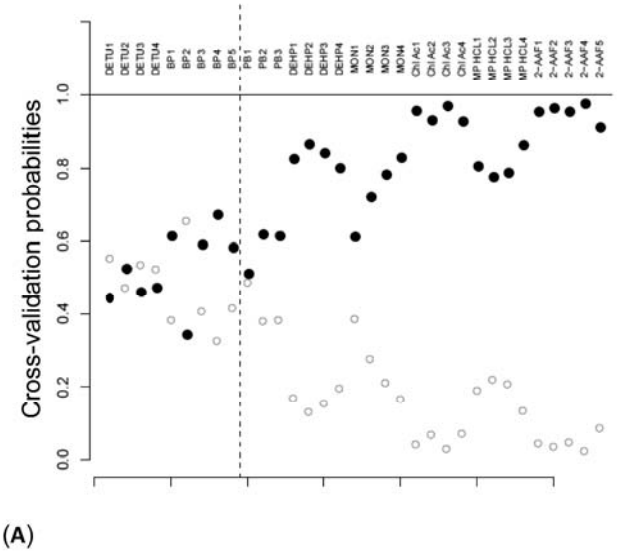


Fig.4

Figure 4. Regulation and functional effects associated with induction of miR-200a/200b/429 and of miR-34a (A) Effect of etoposide (50µM for 48 hours) on the expression of miR-34a and miR-200b in the Fao rat hepatoma cell lines; (B) Effect of hydrogen peroxide treatment (500µM for 24 hours) on the expression of miR-34a and miR-200b in the Fao rat hepatoma cell lines; (C) Expression of cdh1 and miR-200b in Fao cells treated with 2ng/ml TGF-beta1 for 48 hours. Representative result of two independent experiments is shown; (D) Effect of transfection with 50nm of anti-miR-34a, miR-96, miR-99a, and negative control oligo on the number of viable cells after three days. Mean values ± s.e.m are shown, N=3, * $p<0.05$ **$p<0.01$.
254x190mm (300 x 300 DPI)



(A)

(B)

Sample Classification

	Correctly	Incorrectly
DETU	3/4	1/4
BP	1/5	4/5
PB	3/3	0/3
DEHP	4/4	0/4
MON	4/4	0/4
Chl.Ac	4/4	0/4
MP HCL	5/5	0/5
2-AAF	5/5	0/5

Fig.5

Figure 5. PAM prediction of hepatocarcinogenic and non-hepatocarcinogenic chemical treatments based on signature of 10 miRNAs (A) Cross-validated probabilities of a sample classified as undergoing a carcinogenic (indicated by a red circle) or carcinogenic (indicated by a green circle) by the PAM software using as the PAM classifier the miRNA signature of 10 miRNAs which were significantly differentially regulated in animals exposed to two or more treatments; (B) List of number of examined liver samples that were correctly or incorrectly classified.
254x190mm (300 x 300 DPI)

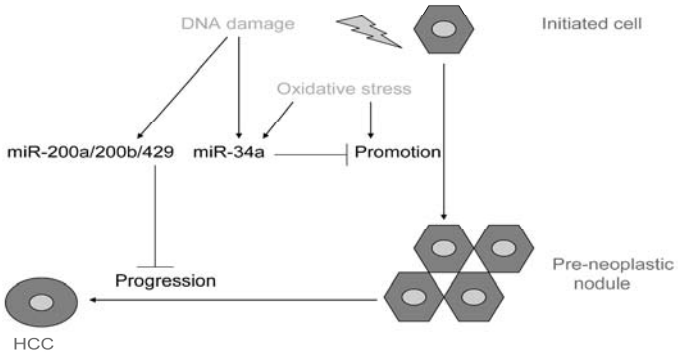


Fig.6

Figure 6. Proposed model of the adaptive functions of hepatic miR-34a and miR-200a/200b/429 during the early stages of chemical hepatocarcinogenesis
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