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Profiling kidney microRNAs from juvenile grass carp (*Ctenopharyngodon idella*) after 56 days of oral exposure to decabromodiphenyl ethane

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ABSTRACT

Grass carp (*Ctenopharyngodon idella*) is one of the most important species in China. Decabromodiphenyl ethane (DBDPE) is a brominated flame retardant that has been used widely in industry, and has been observed to accumulate in the tissues of fish from South China. Evidence has shown that DBDPE is toxic to aquatic animals, but the molecular response has been unclear. MicroRNAs (miRNAs) are small noncoding and negative regulatory RNAs that are 20–24 nucleotides in length, which are involved in a wide range of biological processes. We took advantage of deep-sequencing techniques to accurately and comprehensively profile the kidney miRNA expression of grass carp after 8 weeks of oral exposure to DBDPE. After mapping sequencing data to the genome and Expressed Sequence Tags (ESTs) of grass carp, we identified 493 miRNAs in the sequenced grass carp samples, which included 51 new miRNAs. The results indicated that 5 miRNAs were significantly down-regulated and 36 miRNAs were significantly up-regulated (FDR < 0.001, 1.5-fold change) after DBDPE exposure. Real-time quantitative PCR (RT-qPCR) was performed on 4 miRNAs from the two samples, and the sequencing and RT-qPCR data were consistent. This study provides the first comprehensive identification of grass carp miRNAs, and the first expression analysis of grass carp miRNAs following DBDPE exposure. The results indicated that miRNAs have potential for use as biomarkers.

Introduction

Decabromodiphenyl ethane (DBDPE) is a brominated flame retardant that has been on the market since the mid-1980s. The negligible water solubility and vapor pressure of DBDPE suggest that it has little potential for being released into the environment (Hardy et al., 2012). DBDPE had been widely used in industrial applications to decrease the risk of accidental fire. The production of DBDPE in China, for example, where most of the world's electrical equipment is manufactured, has

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been growing by 80% per year (Wang et al., 2010). Some research has identified DBDPE in the aquatic food web (Law et al., 2006). A rapid and increasing trend in DBDPE concentrations was observed by analyzing sediment cores from the Pearl River Delta (South China) (Zhang et al., 2009) and North China (Hu et al., 2010) over the past several years. Some investigators have shown that DBDPE can significantly accumulate in the tissues of fish from South China (He et al., 2012).

No evidence of maternal and developmental toxicity was found when rats or rabbits were exposed to DBDPE dosages as high as 1250 mg/kg-day (Hardy et al., 2010). The lack of DBDPE toxicity is likely related to the poor bioavailability, caused by its high molecular weight and low solubility (Hardy et al., 2002). However, a significant decrease in creatinine levels and aspartate aminotransferase as well as alkaline phosphatase activities was observed, and an increase in the total bile acid levels was noted when rats were orally exposed to DBDPE for 90 days (Wang et al., 2010). These findings indicated possible oxidative stress from the accumulation of DBDPE or its metabolites. DBDPE has cytotoxic and antiproliferation effects and can induce apoptosis in human HepG2 hepatoma cells (Sun et al., 2012). DBDPE was demonstrated to be acutely toxic to *Daphnia magna* and to have negative effects on the reproductive physiology of zebrafish; it also reduced the hatching rates of exposed zebra fish and significantly increased the mortality of hatched larvae (Nakari and Huhtala, 2010). Oxidative stress was triggered when *Carassius auratus* were exposed to DBDPE for longer periods (Feng et al., 2013).

MicroRNAs (miRNAs) are small noncoding RNA genes of 20–24 nucleotides (nt) in length that post-transcriptionally regulate the expression of many protein-coding genes (Ambros, 2003, 2004; Bartel, 2004). miRNA genes represent approximately 1%–2% of the known eukaryotic genomes and are involved in a wide range of biological processes (Bartel, 2004). Many varieties of miRNAs have been reported and are involved in several physiological or disease-associated cellular processes, such as cell differentiation and proliferation, immune responses, cancer, insulin secretion and metabolism (He and Hannon, 2004). Growing evidence has demonstrated that miRNA expression levels are associated with environmental chemical exposures (Hou et al., 2011); therefore, miRNAs should help to elucidate a variety of issues intrinsic to environmental toxicity. Brzuzan et al. (2012) observed that liver miRNA levels in whitefish changed rapidly following microcystin-LR exposure. A total of 18 muscle miRNAs from Atlantic salmon were differentially expressed to a significant degree when the fish were exposed to acidic, aluminum-rich water, with 14 up-regulated and 4 down-regulated miRNA genes (Kure et al., 2013). Although the relationship between toxicity and miRNA expression is poorly understood, chemical-specific miRNAs have the potential to be used as biomarkers of exposure-related diseases.

Grass carp (*Ctenopharyngodon idella*) has a long history in aquaculture and is one of the most important species cultured in inland water bodies in China. It constitutes 7.18% of the world aquaculture production (FAO, 2010), which is common in China. DBDPE can significantly accumulate in the tissues of carp from South China (He et al., 2012). DBDPE was detected in the muscle, liver, and kidneys of birds, with relatively higher concentrations of DBDPE found in the kidneys than in muscles and livers (Shi et al., 2009). Therefore, in this study, grass carp were orally exposed to DBDPE to identify the kidney miRNA response, which may be useful to provide basic data for the control and management of these chemicals.

1. Materials and methods

1.1. Fish exposure

Grass carp juveniles from our facilities with individual weights of 4.13 ± 0.02 g were used. Before oral exposure, the fish were acclimated to the experimental conditions for 2 weeks and fed to satiation with a commercial diet containing 32% protein and 4% lipids. 180 healthy fish were randomly distributed into 6 experimental fiberglass tanks (with water volumes of 400 L) connected to a recirculation system. During the exposure period, the diurnal photoperiod was 12 hr light/12 hr dark. The water temperature was 28.9 ± 1.5°C, the pH was 7.12 ± 0.02, the dissolved oxygen was 6.01 ± 0.08 mg/L, and the total ammonia-nitrogen was 0.01 ± 0.02 mg/L. The experimental feed was prepared by adding DBDPE at concentrations of 0 and 3000 mg/kg dry diet as previously described by Gan et al. (2012). The fish were fed manually three times per day at 6% of their body weight for 8 weeks. After oral exposure, all fish from each tank were anesthetized with tricaine methane sulfonate (MS222, 50 mg/L) for kidney collection. Each kidney was dissected and divided into two equal parts for sequencing and RT-qPCR confirmation, which were then frozen immediately in liquid nitrogen and stored at –80°C until use.

1.2. Sequencing small RNAs

Thirty kidney tissue samples from each treatment (10 mg each in weight) were pooled together and homogenized. Approximately 100 μg of total RNA was extracted from the pooled samples with Trizol (Invitrogen) reagent according to the manufacturer's instructions. The total RNA was subsequently used for Solexa sequencing and RT-qPCR analysis. The quantity and quality of RNA were determined by 1.5% agarose gel electrophoresis and a BioPhotometer 6131 (Eppendorf).

Twenty micrograms of total RNA from pooled grass carp samples was separated by size on a 15% denaturing polyacrylamide gel, and all of the 18- to 25-nt small RNA was ligated with the selected 5′ and 3′ terminus adaptors. Subsequently, the resulting 18- to 25-nt small RNA was used as a template for cDNA synthesis and amplified by using adaptor primers for 17 cycles. The amplified products were isolated from agarose gels, and the purified DNA was sequenced with a Solexa (Illumina) machine. The image files produced by the sequencer were then processed to generate data in digital format. To explore the differentially expressed kidney miRNAs after DBDPE exposure, two small RNA libraries were constructed from control or orally exposed grass carp juveniles, and each was analyzed using Solexa high-throughput sequencing technology.

1.3. Data analysis

1.3.1. Bioinformatics analysis of sRNA library

Low quality sequence reads were removed according to the criteria of Solexa/Illumina. After removing the reads without

Table 1 – MicroRNA primers for qRT-PCR validation.

Gene	Primer
miR-155 STLP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACCCCTAT
miR-155F	TTAATGCTAATCGTGATAGGGGT
miR-205 STLP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAGACTC
miR-205F	GCAGACTCCTTCATTCCACCG
novel-13 STLP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGGTTGAT
novel-13F	GAAAAGTGTCGCAATCAACCT
miR-276 STLP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGAGCACG
miR-276F	TAGGAACTTCATACCGTGCTCT
β-Actin-F	GCAACACGCAGCTCGTTGTA
β-Actin-R	TGACGAGGCTCAGAGCAAGA

the adaptor sequences, polyA reads and 5 adaptor contaminants, the remaining 19- to 31-nt reads were regarded as clean reads and were used for further analysis. The clean reads that first mapped to small RNAs of known rRNAs, tRNAs, snoRNAs and other non-coding RNAs were deposited in the Rfam database (ftp://selab.janelia.org/pub/Rfam/) and NCBI database (http://www.ncbi.nlm.nih.gov/).

Reads matched with those small RNAs were excluded, and the retained reads were mapped to the known miRNA database: miRbase (version 20.0). The maps allow 1 mismatch, using a bowtie with the following parameter: bowtie–f–v1. The sequences that overlapped with known miRNAs with reads ≥10 were defined as conserved miRNAs, and the sequences that did not overlap with any of these annotations were classified as "un-annotated".

1.3.2. Conserved miRNAs and novel miRNA prediction

Only the "un-annotated" sequences that mapped perfectly onto the draft genome and ESTs were further left for the discovery of novel miRNA candidates. To identify potential miRNA genes, we first pooled the "un-annotated" sequences, and aligned them to the grass carp genomic sequence and EST sequences. The EST sequences of grass carp were obtained from dbEST (http://www.ncbi.nlm.nih.gov/dbEST/). The CAP3 program (http://pbil.univlyon1.fr/cap3.php) was used for EST sequence assembly and redundancy removal. To analyze whether a matched sequence could form a suitable hairpin (the secondary structure of the small RNA precursor), the sequences surrounding the matched sequence were extracted. The secondary structure was predicted by miRDeep2.0 (Friedländer et al., 2011). We removed the novel miRNA candidates with sequenced reads smaller than 10 and excluded the protein-coding sequence by the Basic Local Alignment Search Tooling (BLAST) potential miRNA sequences against the protein database, as described above. Ultimately, these potential novel miRNA precursors were then BLASTed online (http://blast. ncbi.nlm.nih.gov/Blast.cgi) to search against the NCBI protein database with the following parameter to remove the protein coding sequences: blastall–p blastx–F F–e 0.01.

1.3.3. Comparison of the miRNA expression

The relative expression levels of individual miRNA genes used the RPM (reads per million) value to determine the expression preferences in these two libraries. The program winflat (http://www.igs.cnrs-mrs.fr/SpipInternet/IMG/tgz/winflat.tgz) was used to compute the probability of differential miRNA. The false discovery rate (FDR) of BH was estimated, and the miRNA genes with FDR ≤ 0.001 and fold-change ≥1.5 were chosen as significantly differentially expressed. The Pearson's correlation coefficient R was used to measure the product–moment coefficient of the correlation between the quantitative variables. All of the data were normalized by the log2 (Read number + 1) transformation. All statistical analyses were performed with the R program.

1.4. Real-time quantitative PCR (RT-qPCR)

The primers were designed on the basis of sequenced miRNA using Premier 5.0 (Table 1). RT-qPCR was used to evaluate four randomly selected miRNAs that were differentially expressed, as revealed by Solex sequencing. Four samples contained in the pooled samples were used for each primer set. According to the manufacturer's protocol, the RT-qPCR assay was conducted to qualify the selected miRNAs after total RNA extraction using Trizol (Invitrogen) reagent. In brief, 1 μg of total RNA from each sample was reverse-transcribed into cDNA with M-MLV reverse transcriptase (Promega) using a looped antisense primer. After incubation at 42°C for 1 hr and deactivation at 75°C for 10 min, the mixture was used as a PCR template. RT-qPCR was performed

Table 2 – Statistics of small RNA sequences from the grass carp library.

	Control group		Exposure group	
	Redundant reads	Unique reads	Redundant reads	Unique reads
Raw reads	8,089,457	101,383	9,983,454	245,549
Clean reads(removed the low-quality reads and sequences <18 nt reads)	8,010,298	88,696	9,850,556	218,338
Non-coding RNA exact matches (mapped_to_Rfam)	231,914	3402	655,226	6993
Sequences matching the library and genome	16,670	5271	43,544	23,022
Match known miRNAs	6,044,186	1571	6,843,515	1591

nt: nucleotides.

Table 3 – sRNA read distribution.

Mapped_to_Rfam	Control group				Exposure group			
	Redundant reads	Percentage (%)	Unique reads	Percentage (%)	Redundant reads	Percentage (%)	Unique reads	Percentage (%)
snoRNA	390	0.00	138	0.14	899	0.01	276	0.11
rRNA	17,074	0.21	1013	1.00	32,635	0.33	2082	0.85
tRNA	213,985	2.67	2041	2.01	612,937	6.14	3759	1.53
Spliceosomal RNA	401	0.01	184	0.18	8435	0.08	781	0.32
other type small RNA	64	0.00	26	0.03	320	0.00	95	0.04
Known miRNA	6,044,186	75.46	1571	1.55	6,843,515	68.55	1591	0.65
Un-annotated	1,734,198	21.65	96,410	95.09	2,484,713	24.89	236,965	96.50
Total	8,010,298	100.00	101,383	100.00	9,983,454	100.00	245,549	100.00

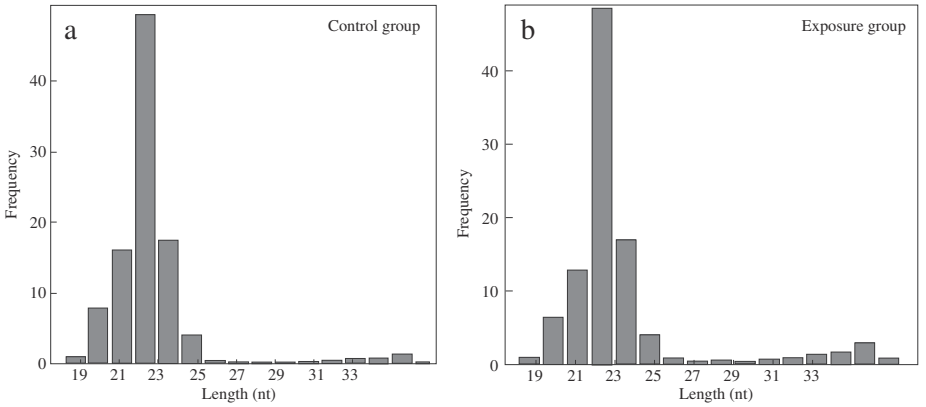

Fig. 1 – Size distribution of sequenced sRNA reads in samples from the control and exposed groups. nt: nucleotides.

using standard protocols for the Mx3005P sequence detection system (Stratagene). In each assay, 1 μl of cDNA (1:10 dilution) was added to 19 μl of mix containing 10 μl 2× SYBR green PCR master mix and 1.5 μm of each primer. The reaction was amplified in a 96-well optical plate at 95°C for 1 min, followed by 35 cycles of 95°C, 56°C and 72°C for 15 sec, 15 sec and 40 sec, respectively. All reactions were run in triplicate, and each run included reactions with no template as a negative control for each gene. The cycle threshold was collected from each reaction, and the relative amount of each miRNA to β-actin RNA was described by using the equation $2^{-(CT\text{miRNA}-CT\beta\text{-Actin RNA})}$.

2. Results

2.1. MicroRNA analysis

To survey the miRNA transcriptome in grass carp after oral exposure to DBDPE, we used the Illumina/Solexa deep sequencing technology on small RNA libraries from the control and the orally exposed kidney samples. We obtained 8,230,974 raw reads from the control kidney library and 9,983,454 raw reads from the oral exposure kidney library. We refined our sequencing data by discarding empty adaptor sequences, low-quality sequences, sequences shorter than 15 nt and longer than 26 nt, and low three-read sequences (Table 2). Furthermore, we excluded certain known types of RNA sequences for mRNA, rRNA, tRNA, snRNA, and snoRNA as well as repetitive sequence elements (Table 3). After these exclusions, 8,010,298 and 9,850,556 high-quality reads remained for the control and oral exposure samples, respectively, for miRNA analysis. The length distribution peak of the reads was 22 nt in all of the samples (Fig. 1). These results are consistent with the common size of miRNAs.

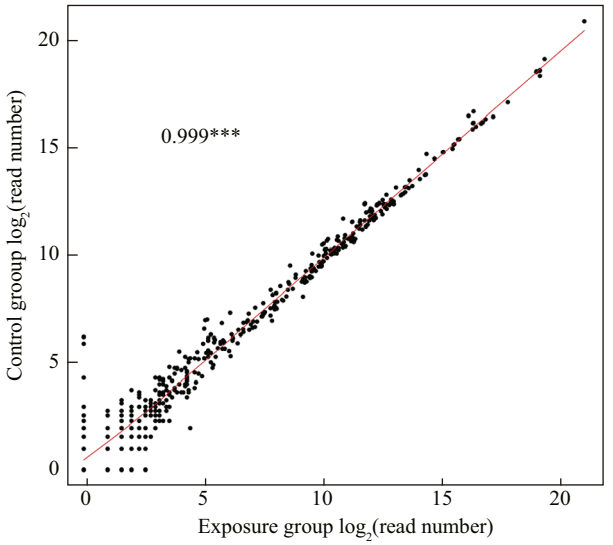

Fig. 2 – Pearson correlation of the RNA-sequence data between the control and the exposure group.

After mapping to the reference genome and ESTs of grass carp, a total of 493 miRNAs were identified (Supplementary Tables S1 and S2), and 51 new miRNAs were included (Supplementary Table S2). The Pearson correlation of the expression between the control and the exposure group was approximately 0.999 (Fig. 2). A total of 41 miRNAs were differentially regulated in a significant manner (FDR < 0.001, 1.5-fold change) between the exposed and control group, with 5 down-regulated and 36 up-regulated (Table 4).

2.2. Confirming the differential expression in miRNAs

RT-qPCR was used to validate Solexa sequencing results. Four miRNAs were selected randomly, and the RT-qPCR analysis yielded similar results to those of Solexa sequencing and thus confirmed that kidney miR-155, miR-205, miR-276 and novel-13 were expressed at higher levels after oral exposure to DBDPE. There was a general consistency between the quantitative assay and deep sequencing analysis for the 4 miRNAs in terms of the directions of regulation and their significance (Fig. 3).

3. Discussion and conclusions

Characterizing kidney miRNA transcriptomes and identifying novel miRNAs is an important step toward understanding the toxic molecular mechanisms that occur after oral exposure to DBDPE. The present study provided the first survey of miRNAs in grass carp kidneys through Solexa sequencing. Solexa sequencing is based on the massive parallel sequencing of millions of fragments; this novel approach ensures high accuracy and true base-by-base sequencing, which has been used to study cancer and toxicity (Kure et al., 2013) and to discover miRNAs (Li et al., 2012).

Most recent studies have indicated that using high throughput sequencing technologies will contribute to a better understanding of individual genotype relations to phenotypes and transcriptomic responses under varying types of environmental pollution (Mehinto et al., 2012). Altered miRNA expression profiles may lead to widespread gene expression changes and have been implicated in pathophysiological processes, such

Table 4 – Different expression levels of microRNAs after oral exposure to DBDPE (1.5-fold).

Gene	Control reads	Exposure reads	*p* value	False discovery rate	Fold change
Bantam	0	80	1.68E−23	1.55E−22	73.75963
bantam-3p	0	80	1.68E−23	1.55E−22	73.75963
miR-81a	0	64	5.29E−19	4.37E−18	59.18982
miR-276	0	20	1.25E−06	6.26E−06	19.12287
miR-276a	0	20	1.25E−06	6.26E−06	19.12287
miR-276-3p	0	20	1.25E−06	6.26E−06	19.12287
miR-276a-3p	0	20	1.25E−06	6.26E−06	19.12287
novel-13	33	140	2.82E−15	2.08E−14	3.776364
novel-21	35	143	4.65E−15	3.39E−14	3.642451
miR-205-5p	32	105	2.67E−09	1.76E−08	2.924998
miR-205a	32	105	2.67E−09	1.76E−08	2.924998
miR-10a-3p	15	49	3.91E−05	0.000177424	2.845665
miR-205	69	180	7.02E−11	4.83E−10	2.354584
miR-205a-5p	69	179	9.74E−11	6.66E−10	2.341575
miR-34a	54	127	5.78E−07	3.08E−06	2.119244
miR-34a-5p	39	87	7.57E−05	0.0003297	2.003348
novel-23	1857	4074	1.23E−144	3.72E−143	1.997173
miR-34	39	86	0.000101214	0.000438427	1.980583
miR-155-5p	398	860	4.45E−31	5.34E−30	1.965006
miR-155	398	860	4.45E−31	5.34E−30	1.965006
novel-37	36	77	0.00035472	0.001423761	1.91967
novel-7	31	64	0.00161432	0.006088491	1.849682
miR-96-5p	36	71	0.0017886	0.006659681	1.772003
miR-96	36	71	0.0017886	0.006659681	1.772003
miR-182	3504	6715	5.89E−165	2.06E−163	1.744843
miR-182-5p	3459	6581	3.61E−158	1.17E−156	1.73227
miR-10-5p	70,633	121,864	0	0	1.571082
miR-10a-5p	81,747	140,929	0	0	1.569857
miR-10a	81,769	140,965	0	0	1.569835
miR-10	81,766	140,951	0	0	1.569737
novel-24	1198	2062	1.63E−36	2.16E−35	1.566801
miR-10b	759	1286	3.53E−22	3.12E−21	1.542051
miR-10c-5p	226	382	9.55E−08	5.50E−07	1.536408
miR-10b-5p	1012	1708	2.11E−28	2.26E−27	1.536266
miR-10d	1157	1946	1.17E−31	1.47E−30	1.531056
miR-10d-5p	1056	1773	8.11E−29	8.76E−28	1.528313
miR-144-3p	225	163	2.33E−05	0.000107894	0.660799
novel-34	70	42	0.00083782	0.003272734	0.551498
novel-4	235	137	9.67E−10	6.51E−09	0.532477
miR-101b	579	307	1.64E−26	1.58E−25	0.483567
novel-38	21	3	3.09E−05	0.000142351	0.165566

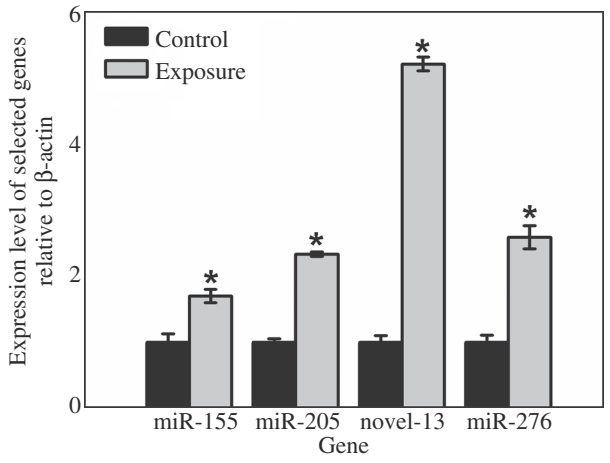

Fig. 3 – Relative change of miR-155, miR-205, miR-276 and novel-13 after oral exposure, as validated by qRT-PCR.

as inflammation and cancer (Sonkoly and Pivarcsi, 2011). Most environmental pollution, such as cigarette smoke (Izzotti et al., 2009) and diesel exhaust (Jardim et al., 2009), has been shown to change the miRNA expression profile, which strongly suggests that miRNAs could be involved in the adverse health effects of these exposures.

In our study, 493 miRNAs were identified through Solexa sequencing. The Solexa sequencing results indicated that 5 kidney miRNAs were significantly down-regulated and 36 kidney miRNAs were significantly up-regulated after DBDPE exposure. We verified the results by RT-qPCR, which was consistent with the Solexa sequencing results. Although little is known about miRNA function in grass carp, there are indications that some of the most differentially regulated miRNAs may play a role in various processes. In the present study, miR-155, miR-205 and most miR-10 family members were significantly up-regulated after DBDPE exposure. Some research has demonstrated that oxidative stress was triggered in fish exposed to DBDPE (Feng et al., 2013). Interestingly, miR-155 has been shown to regulate the immune response (Thai et al., 2007). Excessive vitamin E supplementation increased the expression of miR-155 in tilapia livers, which was related to oxidative stress and immune responses (Tang et al., 2013). Mice deficient in miR-155 have inhibited immune responses, whereas miR-155-overexpressing mice have a myeloproliferation that resembles chronic inflammation and hematopoietic cancers (Costinean et al., 2006; O'Connell et al., 2008, 2010). Neal et al. (2010) found that miR-155 expression was significantly increased in renal cancer relative to adjacent normal tissues. Some studies have found that intrarenal miR-205 is up-regulated in immunoglobulin A nephropathy, and its level is inversely correlated with the glomerular filtration rate and positively correlated with tubulointerstitial scarring (Wang et al., 2009). MiR-10 family members are de-regulated in several types of cancer (Lund, 2009). Our results suggest the alteration of numerous physiological responses after grass carp is orally exposed to DBDPE. Some investigations have also demonstrated that DBDPE was acutely toxic to aquatic organisms (Nakari and Huhtala, 2010).

We conclude that there was indeed an alteration in the kidney miRNA expression profile of grass carp after 8 weeks of oral exposure to DBDPE. The miRNAs identified in the present study are promising, and some may have potential as molecular markers for monitoring toxic responses in exposed animals.

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Supplementary data

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