

1 **Global miRNA expression profiling of domestic cat livers following acute**
2 ***Toxoplasma gondii* infection**

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19 **ABSTRACT**

20 Although microRNAs (miRNAs) play an important role in liver homeostasis, the extent to
21 which they can be altered by *Toxoplasma gondii* infection is unknown. Here, we utilized
22 small RNA sequencing and bioinformatic analyses to characterize miRNA expression profiles
23 in the liver of domestic cats at 7 days after oral infection with *T. gondii* (Type II) strain. A
24 total of 384 miRNAs were identified and 82 were differentially expressed, of which 39 were
25 up-regulated and 43 down-regulated. Also, 5690 predicted host gene targets for the
26 differentially expressed miRNAs were identified using the bioinformatic algorithm miRanda.
27 Gene ontology analysis revealed that the predicted gene targets of the dysregulated miRNAs
28 were significantly enriched in apoptosis. Kyoto Encyclopedia of Genes and Genomes analysis
29 showed that the predicted gene targets were involved in several pathways, including acute
30 myeloid leukemia, central carbon metabolism in cancer, choline metabolism in cancer,
31 estrogen signaling pathway, fatty acid degradation, lysosome, nucleotide excision repair,
32 progesterone-mediated oocyte maturation, and VEGF signaling pathway. The expression
33 level of 6 upregulated miRNAs (mmu-miR-21a-5p, mmu-miR-20a-5p, mmu-miR-17-5p,
34 mmu-miR-30e-3p, mmu-miR-142a-3p, and mmu-miR-106b-3p) was confirmed by stem-loop
35 quantitative reverse transcription PCR, which yielded results consistent with the sequencing
36 data. These findings expand our understanding of the regulatory mechanisms of miRNAs
37 underlying *T. gondii* pathogenesis and contribute new database information on cat miRNAs,
38 opening a new perspective on the prevention and treatment of *T. gondii* infection.

39
40 **Keywords:** *Toxoplasma gondii*, Domestic cat, Liver, Host-pathogen interaction, MircoRNA,
41 RNA-Seq

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43

44 INTRODUCTION

45 *Toxoplasma gondii* is a highly prevalent apicomplexan protozoan parasite, which can
46 cause serious clinical illnesses in humans and animals [1]. It has been reported to chronically
47 infect roughly one-third of the world's human population [2]. *T. gondii* acquired during
48 pregnancy may cause damage to the fetus and reactivation of latent infection can cause
49 life-threatening encephalitis in immune-compromised individuals [3]. This parasite has an
50 indirect two-host lifecycle, which is composed of asexual reproductive phase in the
51 intermediate host and sexual reproductive phase in the definitive host (members of the Felidae
52 family). The enteroepithelial sexual cycle of *T. gondii* is completed within 3 to 10 days after
53 ingestion of intermediate host tissue containing *T. gondii* cysts. *T. gondii* can also spread
54 throughout the cat's body and affect many organs [4-7]. Hence, cats are unique in respect of
55 their ability to accommodate both sexual and asexual reproductions of *T. gondii*, making cats
56 a significant source of infection to humans and animals [2, 8].

57 Besides the adverse clinical consequences on humans and other intermediate hosts *T.*
58 *gondii* can cause disseminated and fatal infection in cats [4-7, 9]. Although any organ in the
59 cat's body can be affected, clinical cases related to hepatic and pulmonary damage are
60 particularly important because they are associated with quicker mortality [6,10-12]. Also,
61 liver dysfunction, enlargement, icterus, cholangiohepatitis, vomiting, abdominal effusion,
62 and ascites are complications that frequently occur in *T. gondii*-infected cats. Therefore, with
63 the great need for the development of efficacious treatment interventions (due to the lack of a
64 vaccine and limited efficacies of current therapeutics), it is important to identify the molecular
65 mechanisms that underpin liver damage caused by *T. gondii* infection. However, information

66 about the molecular pathways that regulate the interaction between *T. gondii* and hepatic
67 tissues has been limited to a few studies [13,14].

68 The feline genome already encodes roughly 3,182 microRNAs (miRNAs) homologues,
69 which can regulate the expression of signalling cascades that perform key cellular functions,
70 such as cell cycle regulation, proliferation, differentiation, apoptosis, and carcinogenesis.
71 miRNAs constitute a group of endogenous non-coding small RNAs (18 to 25 nucleotides [nt]
72 long) that regulate gene expression by binding to mRNA and inhibiting translation
73 [15-18]. miRNAs play an important role in liver homeostasis, and aberrant expression of
74 miRNAs has been associated with a variety of liver diseases, such as viral hepatitis,
75 hepatocellular carcinoma and fatty liver disease [19]. Alterations of host miRNA expression
76 have also been observed in some parasitic infections, such as *Cryptosporidium parvum*,
77 *Plasmodium falciparum* and *T. gondii* (reviewed in [20]), underscoring the potential role of
78 miRNAs in mediating the interaction between *T. gondii* and host cells. Despite the impact of *T.*
79 *gondii* infection on hepatic function the mechanisms underlying the alterations of hepatic
80 miRNAs expression following acute *T. gondii* infection remain poorly understood.

81 In this study, we hypothesized that *T. gondii* infection alters the expression of hepatic
82 miRNAs and that differentially expressed miRNAs mediate the interaction between *T. gondii*
83 and cat's liver. Here we use genome-wide, small RNA sequencing to characterize the global
84 miRNA transcriptional response of feline liver to infection with *T. gondii* (Type II) PRU strain.
85 Our study provides a full picture of the hepatic miRNA repertoire during acute *T. gondii*
86 infection in domestic cats, including novel miRNAs, involved in host cell cyclin, apoptosis and
87 anti-*T. gondii* defense.

88

89 **RESULTS**

90

91 **Confirmation of *T. gondii* infection in the cat livers**

92 Under the conditions we used positive PCR results were obtained, providing laboratory
93 confirmation of *T. gondii* infection in the livers of infected cats. RFLP analysis of the positive
94 PCR amplicons of *T. gondii* *B1* gene revealed a restriction fragment pattern characteristic to
95 *T. gondii* genotype II. The livers of control cats and negative PCR control yielded negative
96 PCR results.

97

98 **Analysis of miRNA expression**

99 miRNA libraries of livers from two *T. gondii*-infected or two control cat groups were
100 successfully sequenced and sequencing data is summarized in Table 1 and Table 2. Length
101 distributions of clean reads in the libraries were between 20-24 nt (Fig. 1A-D). A very high
102 intra-group correlation was detected between the two miRNA libraries of *T. gondii*-infected
103 liver samples ($R^2 = 0.989$) (Fig. 2A) and the two miRNA libraries of *T. gondii*-uninfected liver
104 samples ($R^2 = 0.99$) (Fig. 2B). The known and novel mature miRNAs in *T. gondii*-infected and
105 control groups were summarized in Table 3 and Table 4. Finally, through comparing *T.*
106 *gondii*-infected and uninfected sRNA libraries, 82 differentially expressed miRNAs were
107 identified, including 33 up-regulated and 49 down-regulated miRNAs (Table 5).

108

109 **Pathway analysis of miRNA targets**

110 A total of 5690 predicted host targets were identified (Supplementary Table 2). Based on the
111 predicted targets of the differentially expressed miRNAs GO enrichment analysis was
112 performed in order to identify the biological processes, molecular functions and cellular
113 components. The enriched GO terms of the biological processes, molecular functions and
114 cellular components are shown in Fig. 3A, respectively. KEGG enrichment analysis showed
115 that target genes were related to multiple pathways, including nucleotide excision repair,
116 lysosome, vascular endothelial growth factor (VEGF) signaling, estrogen signaling, acute
117 myeloid leukemia, central carbon metabolism in cancer, choline metabolism in cancer, fatty
118 acid degradation, progesterone-mediated oocyte maturation, and renal cell carcinoma. The top
119 20 KEGG enrichment pathways are shown in Fig. 3B.

120

121 **miRNA expression validation by qRT-PCR**

122 Six miRNAs, including mmu-miR-21a-5p, mmu-miR-20a-5p, mmu-miR-17-5p,
123 mmu-miR-30e-3p, mmu-miR-142a-3p, and mmu-miR-106b-3p, were selected for
124 confirmation by real time PCR to verify the expression levels of the differentially expressed
125 miRNAs using miRNA specific primers (Supplementary Table 1). The results confirmed that
126 these selected miRNAs were differentially expressed between infected livers and uninfected
127 livers and were consistent with the results obtained by RNA-sequencing analysis
128 (Supplementary Fig 1). Data set from the RNA-sequencing experiment has been deposited in
129 the GEO database under accession number PRJNA356106.

130

131 **DISCUSSION**

132 Previous studies showed that *T. gondii* infection can alter the expression of host
133 miRNAs, indicating that miRNAs may be involved in the pathogenesis of *T. gondii* infection
134 [21-24]. Our results have also shown that acute *T. gondii* infection alters the level of miRNAs
135 in the liver of domestic cats. In the present study, we used small RNA sequencing to identify
136 cellular miRNAs and signaling pathways involved in the response of cats to *T. gondii*
137 infection. Specifically, we determined miRNAs that are differentially expressed by comparing
138 sham-infected to *T. gondii*-infected cat livers at 7 dpi. This analysis identified 82 differentially
139 expressed microRNAs, of which 39 were increased and 43 decreased. By using qRT-PCR, the
140 expression level of six up-regulated miRNAs (mmu-miR-21a-5p, mmu-miR-20a-5p,
141 mmu-miR-17-5p, mmu-miR-30e-3p, mmu-miR-142a-3p, and mmu-miR-106b-3p) were
142 consistent with the results obtained by sequencing analysis. Knowledge of molecular changes
143 in human liver during the acute phase of toxoplasmosis is lacking due to the mild and subtle
144 nature of this infection especially in immune-compromised individuals [1,2]. Hence, these
145 findings may serve as the basis for understanding the molecular mechanisms associated with
146 hepatic pathology during acute *T. gondii* infection.

147 Differentially expressed miRNAs were associated with signalling pathways involved
148 mainly in cell cycling, apoptosis, oncogenesis, and host defense. Among the differentially
149 expressed miRNAs, miR-21a-5p, miR-17-5p, miR-223-3p, miR-27a-5p, miR-126, and
150 miR-486 were significantly upregulated in *T. gondii*-infected livers compared to controls. Of
151 note, the level of miR-21a-5p expression was elevated in various cancer tissues, including
152 rectal, gastric and lung tissues [25-27] and has been suggested to play a role in tumor biology
153 [27]. The similarity between the expression of miR-21a-5p during *T. gondii* infection and

154 various forms of cancers is interesting. One striking finding was the correlative link between
155 upregulation of miR-17-92 in *T. gondii*-infected human foreskin fibroblasts [28] and in
156 human astrocytic glioma tissue [29]. The presence of RNA silencing machinery and small
157 silencing RNAs in *T. gondii* genome [30] indicates that this parasite has the ability to use its
158 own miRNAs to interrupt host cell functions in analogy to oncogenic viruses [31].

159 miR-17-5p, a key regulator of the G1/S phase cell cycle transition, was up-regulated in
160 our study in agreement with others who reported overexpression of miR-17-5p in human and
161 mouse spleen in response to *T. gondii* infection [21,31]. *T. gondii* can increase miR-17~92
162 and miR-106b~25 that play key roles in the regulation of mammalian cell cycle by
163 influencing the functionally intertwined pathways of apoptosis and G1/S cell cycle
164 progression [32]. miR-17-5p targets mouse Bcl2l11, Zmat3, Aifm1, and Capn2 to increase
165 host apoptotic process and targets mouse Ppp3r1 and Akt3 to promote cellular apoptosis
166 process [21]. Also, miR-17-5p may function as both a tumor suppressor [33] and as an
167 oncogenic activator [34] by targeting both pro- and anti-proliferative genes and by competing
168 with each other in different cellular contexts [35]. The effect of *T. gondii* infection on the
169 expression of miRNAs (miR-30c-1, miR-125b-2, miR-23b-27b-24-1, and miR-17~92 cluster
170 genes) that have anti-apoptotic activity has been reported [36]. Modulating these
171 apoptosis-related miRNAs with mimics or inhibitors can validate their roles in the
172 dysregulation of host cell apoptotic machinery during *T. gondii* infection.

173 The miR-223-3p has been implicated in the regulation of inflammatory response [37]
174 and granulocyte production and function [38], and can function as a tumor suppresser in
175 osteosarcoma by regulating the osteosarcoma cell cycle progression and proliferation [Xu et

176 al 2013]. The level of miR-223-3p was significantly increased in infected samples, suggesting
177 that *T. gondii* infection of feline liver stimulates the production of miR-223-3p, which plays a
178 role in the activation of inflammatory response elicited in response to *T. gondii* infection. This
179 is concordant with a previous study showing that miRNAs, such as miR-146a and miR-155,
180 known to activate immune and inflammatory responses can influence host response to *T.*
181 *gondii* infection [39-42]. Also, the upregulation of miR-27a-5p (a regulator of lipid
182 metabolism-related genes) and miR-21-5p in the infected liver samples suggests that both
183 miR-27a-5p and miR-21-5p play a role in host response to infection. This assumption is
184 supported by the association between inhibition of miR-21 and increased *Cryptosporidium*
185 *parvum* burden [29].

186 miR-126 is associated with tumorigenesis and has recently been found to modulate the
187 survival and function of Plasmacytoid dendritic cells (pDCs) via positive regulation of the
188 vascular endothelial growth factor (VEGF) signaling pathway (Ferretti and La Cava, 2014).
189 miR-126 upregulation may activate Toll-like receptor (TLR)/MyD88 signalling in pDCs
190 to secrete large amounts of type I interferons (IFNs), which is essential for host resistance to
191 *T. gondii* infection. Also, the biological significance of *T. gondii*-induced upregulation of
192 miR-486 may lie in its ability to augment the host defense mechanisms. miR-486 has been
193 shown to activate nuclear factor (NF)- κ B signaling pathway [Song et al., 2013], which leads
194 to the production of proinflammatory cytokines, thereby providing a protection against *T.*
195 *gondii* infection. Of note, both miR-486 and the NF- κ B signaling pathway have oncogenic
196 roles of in human cancers, such as glioma progression [Song et al., 2013].

197 The let-7 family is a key regulator of the innate immune response. The level of let-7i
198 during protozoal infection with *C. parvum* infection was found reduced together with increase
199 of TLR4 in biliary epithelial cells, contributing to cholangiocyte's defense responses [43]. In
200 line with this study our results revealed significant downregulation of mmu-let-7f-5p,
201 mmu-let-7i-5p, mmu-let-7a-5p, mmu-let-7g-5p, mmu-let-7e-5p, and mmu-let-7c-5p in *T.*
202 *gondii*-infected cat's livers compared with controls. Finally, the fact that miRNAs are host-,
203 tissue-, and strain-specific [44] explains why the expression of some miRNA (e.g.
204 miR-712-3p, miR-511-5p and miR-217-5p) that are dysregulated during *T. gondii* infection
205 [45] was not altered in our study.

206 In conclusion, these findings provided new insight regarding the ability of *T. gondii* to
207 alter the expression of 82 microRNAs in cat's livers. Our study revealed that through
208 reprogramming of hepatic miRNAs expression *T. gondii* influences the cellular
209 microenvironment and host anti-*T. gondii* response, which are likely to play roles in the parasite
210 pathogenesis. GO analysis revealed that the predicted targets of the differentially expressed
211 miRNAs were involved in the regulation of cell cycle and most of the identified KEGG
212 pathways were related to cancer. Considering the immunoregulatory effects of miRNAs and
213 their ability to modulate crucial host cellular targets needed for *T. gondii* replication, miRNAs
214 may hold promise as biomarkers for prediction of disease progression. Finally, miRNA
215 pathways that are stimulated during infection may offer potential targets for therapeutic control
216 of toxoplasmosis.

217

218 **MATERIALS AND METHODS**

219 **Ethics Statement**

220 This study was performed in strict accordance with the recommendations set forth in the
221 Animal Ethics Procedures and Guidelines of the People's Republic of China. All animal
222 experiments were reviewed and approved by the Animal Ethics Committee of Lanzhou
223 Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Approval No.
224 LVRIAEC2014-009). Liver tissue collection was performed as a terminal procedure under
225 isoflurane anesthesia and all efforts were made to minimize suffering.

226

227 **Animals, parasite infection and sample collection**

228 Twelve, 3 month-old, domestic cats (*Felis catus*) of the Chinese Li Hua breed were
229 purchased from a local breeder and were housed in a controlled environment. The cats
230 belonged to two litters, six cats per litter. These 12 cats were randomly allocated to four
231 groups (two infected and two control) with three cats per group. Before the experiment, all
232 cats were confirmed to be free from *T. gondii* using the modified agglutination test and free of
233 major viral infections (e.g. feline calicivirus and coronavirus, feline immunodeficiency virus,
234 feline leukemia virus, and feline parvovirus) based on serological examination. Cats were
235 maintained on commercial cat diets (Royal Canine Inc., St. Charles, MO, USA) and water *ad*
236 *libitum* during the 3 weeks prior to experimentation in order to allow cats to acclimate and to
237 minimize any potential dietary influence on the study results. During the experiment cats were
238 individually fed once daily based on daily energy requirements and water was available *ad*
239 *libitum*.

240 *Toxoplasma gondii* strain used in this study was the PRU strain (Genotype II), which is
241 maintained in our laboratory by passage through Kunming mice as described previously [46].
242 *T. gondii* type II was used in this study because it seems to be the predominant genotype
243 circulating in cats [Herrmann et al. 2010; Maksimov P et al. 2013; Brennan et al., 2016].
244 Also, the PRU strain is able to produce brain tissue cysts in mouse and oocysts in the gut of
245 cats and is thus a suitable candidate for a standardized challenge model in cats. The number of
246 *T. gondii* cysts was determined using an optical microscope and was adjusted to 100 cysts
247 mL⁻¹ in phosphate buffered saline (PBS), pH 7.4. Each cat was infected by intragastric
248 inoculation with 100 cysts in 1 mL PBS. Control cats were sham-infected with PBS only.
249 Livers were harvested 7 days after infection in order to allow sufficient time for the infection
250 to be established in the liver [1]. Collected livers were rinsed extensively in saline, flash
251 frozen in liquid nitrogen, and stored at -80°C until processing.

252

253 **Detection of infection in the liver**

254 Genomic DNA was extracted from the collected liver samples using TIANamp Genomic
255 DNA kit according to the manufacturer's recommendations (TianGen™, Beijing, China).
256 Then, a semi-nested PCR targeting *T. gondii* *B1* gene was performed to detect *T. gondii*
257 infection [47]. DNA samples giving positive *B1* amplification were genotyped using
258 PCR-restriction fragment length polymorphism analysis as described previously [48].

259

260 **RNA extraction and qualification**

261 Total RNA was prepared individually from the cryo-preserved liver tissues of the cats
262 using TRIzol Reagent according to the manufacturer's instructions (Invitrogen Co. Ltd). RNA
263 degradation and contamination was checked on 1% agarose gels. RNA purity was evaluated
264 using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA concentration
265 was determined using Qubit® RNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies,
266 CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent
267 Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

268

269 **RNA Sequencing library preparation and transcriptomic analysis**

270 RNA samples from *T. gondii*-infected and non-infected livers collected at 7 dpi were sent to
271 Beijing Novogene Bioinformatics Institute for Illumina sequencing. To analyze miRNAs by
272 sequencing, a total of 3 µg RNAs of three pooled samples from each group were used for the
273 construction of four small RNA (sRNA) libraries, which were subjected to sequencing on a
274 Hi-seq 2500 platform. Raw data (raw reads) of fastq format were firstly processed through
275 custom perl and python scripts. In this step, clean reads were obtained by removing reads
276 containing ploy-N, with 5' adapter contaminants, without 3' adapter or the insert tag,
277 containing ploy A or T or G or C and low quality reads from raw data. At the same time, Q20,
278 Q30 and GC-content of the raw data were calculated. Then, we chose a certain range of length
279 from clean reads to do all downstream analyses. Next, the small RNA tags were mapped to
280 the feline reference genome sequence using Bowtie software [49]. The following parameters
281 were used: -k [valid alignments per read], 1; -m [number of possible alignments], 10; -l
282 [seed length], 25; --best [optimal alignments]).

283 Mapped small RNA tags were used to look for known miRNA. miRBase20.0 was used
284 as reference, and modified software mirdeep2 [50] and srna-tools-cli were used to obtain the
285 potential miRNA and draw the secondary structures. Custom scripts were used to obtain the
286 miRNA counts and base bias on the first position of identified miRNA with certain length and
287 on each position of all identified miRNA, respectively. To remove tags originating from
288 protein-coding genes, repeat sequences, rRNA, tRNA, snRNA, and snoRNA, small RNA tags
289 were mapped to RepeatMasker, Rfam database or those types of data from the specified
290 species itself.

291 The available software miREvo [51] and mirdeep2 [50] were integrated to predict novel
292 miRNA through exploring the secondary structure, the dicer cleavage site and the minimum
293 free energy of the small RNA tags unannotated in the former steps. miRNA expression levels
294 were estimated with TPM (transcript per million) units [52]. Differential expression analysis
295 of infected versus control groups was performed using the DESeq R package (1.8.3). The
296 *P*-values was adjusted using the Benjamini & Hochberg method. Corrected *P*-value of 0.05
297 was set as the threshold for significantly differential expression by default.

298 Predicting the target gene of miRNA was performed by psRobot_tar in miRanda [53].
299 Gene Ontology (GO) enrichment analysis was used to categorize the target genes of the
300 differentially expressed miRNAs. Goseq based Wallenius non-central hyper-geometric
301 distribution [54], which could adjust for gene length bias, was implemented for GO
302 enrichment analysis. The enrichment of target genes in KEGG pathways were tested by the
303 software KOBAS [55].

304

305 **Validation of miRNA expression**

306 The stem-loop quantitative reverse transcription PCR was used to validate the results of
307 miRNA expression analysis as described previously [21,56]. Stem-loop RT-PCR was
308 performed on ABI PRISM® 7500 Sequence Detection System using SYBR Green
309 qPCRSuperMix according to the manufacturer's protocol (Invitrogen). All qRT-PCR reactions
310 were performed in three replicates. Gene expressions were calculated by $2^{-\Delta\Delta CT}$ relative
311 expression method as previously described [57]. snRNA U6 was used as normalization
312 control in qRT-PCR.

313

314 **CONFLICTS OF INTEREST**

315 The authors declare that they have no competing interests.

316

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321

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497

498 **Table 1: Summary of small RNA sequencing data obtained in the present study.**

Library type	Reads	Bases	Error rate	Q20	Q30	GC content
Infected liver-1	10665548	0.533G	0.01%	96.21%	92.13%	49.65%
Infected liver-2	12569494	0.628G	0.01%	96.16%	92.13%	49.98%
Uninfected liver-1	11727706	0.586G	0.01%	96.24%	92.33%	49.56%
Uninfected liver-2	10273236	0.514G	0.01%	96.44%	92.60%	49.72%

499

500 **Table 2: Clean process overview.**

Library	Infected groups		Uninfected groups	
	Infected liver Group 1	Infected liver Group 2	Uninfected liver Group 1	Uninfected liver- Group 2
Total reads	10665548 (100.00%)	12569494 (100.00%)	11727706 (100.00%)	10273236 (100.00%)
N%>10%	531 (0.00%)	636 (0.01%)	606 (0.01%)	540 (0.01%)
Low quality	40551 (0.38%)	47641 (0.38%)	42076 (0.36%)	32984 (0.32%)
5_adapter_contaminate	1247 (0.01%)	1565 (0.01%)	1026 (0.01%)	915 (0.01%)
3_adapter_null or insert_null	174157 (1.63%)	176650 (1.41%)	223708 (1.91%)	195007 (1.90%)
With ployA/T/G/C	10772 (0.10%)	12621 (0.10%)	7991 (0.07%)	6939 (0.07%)
Clean reads	10438290 (97.87%)	12330381 (98.10%)	11452299 (97.65%)	10036851 (97.70%)

501

502

503 **Table 3: Known miRNA mapping.**

Criteria	Infected groups		Uninfected groups	
	Infected liver Group 1	Infected liver Group 2	Uninfected liver Group 1	Uninfected liver Group 2
Mapped mature	358	360	348	356
Mapped hairpin	273	274	266	268
Mapped uniq sRNA	2531	2604	2411	2409
Mapped total sRNA	3086174	3665542	3484600	3037510

504

505

506 **Table 4: Novel miRNA mapping.**

Types	Infected liver-1	Infected liver-2	Uninfected liver-1	Uninfected liver-2
Novel mature	69	76	75	73
Novel star	10	14	12	10
Novel hairpin	72	78	80	77
Mapped uniq sRNA	186	187	161	161
Mapped total sRNA	12381	12103	13702	7981

507

508

509 **Table 5:** Differentially expressed miRNAs.

Type	miRNA	Fold change	<i>p</i> -value	<i>p</i> -adjustment
Up-regulated	mmu-miR-21a-5p	2.564	1.32E-17	3.15E-15
	mmu-miR-20a-5p	1.610	3.26E-09	3.88E-07
	mmu-miR-339-5p	1.839	5.42E-06	0.0002
	mmu-miR-101a-3p	1.855	8.22E-06	0.0002
	mmu-miR-320-3p	1.507	9.27E-06	0.0002
	mmu-miR-195a-5p	1.805	1.33E-05	0.0003
	mmu-miR-126a-3p	1.486	2.05E-05	0.0003
	mmu-miR-23a-3p	1.585	2.13E-05	0.0003
	mmu-miR-140-3p	1.701	2.45E-05	0.0003
	mmu-miR-28a-5p	1.500	0.0002	0.0018
	mmu-miR-223-3p	1.917	0.0002	0.0023
	mmu-miR-30f	1.504	0.0002	0.0023
	mmu-miR-126a-5p	1.437	0.0003	0.0025
	mmu-miR-126b-3p	1.435	0.0003	0.0025
	mmu-miR-17-5p	1.436	0.0005	0.0036
	mmu-miR-19b-3p	1.485	0.0007	0.0048
	mmu-miR-27a-5p	1.741	0.0011	0.0076
	mmu-miR-486b-3p	1.593	0.0011	0.0076
	mmu-miR-151-3p	1.338	0.0017	0.0093
	mmu-miR-27a-3p	1.389	0.0017	0.0093
	mmu-miR-486a-3p	1.571	0.0017	0.0093
	mmu-miR-99b-5p	1.359	0.0021	0.0106
	novel_1	1.998	0.0030	0.0134
	mmu-miR-3074-5p	1.315	0.0049	0.0202
	mmu-miR-30e-3p	1.325	0.0052	0.0211
	mmu-miR-24-3p	1.306	0.0055	0.0212
	mmu-miR-101c	1.0519	0.0069	0.0252
	mmu-miR-378c	1.528	0.0069	0.0252
	mmu-miR-486a-5p	1.494	0.0075	0.0271
	mmu-miR-361-3p	1.379	0.0101	0.0342
	mmu-miR-106b-3p	1.255	0.0153	0.0451
	mmu-miR-142a-3p	1.690	0.0152	0.0451
	mmu-miR-142b	1.690	0.0152	0.0451
Down-regulated	mmu-let-7f-5p	-1.546	1.73E-07	1.38E-05
	mmu-let-7i-5p	-1.705	1.37E-06	6.52E-05
	mmu-miR-365-3p	-1.532	1.24E-06	6.52E-05
	mmu-miR-148a-3p	-1.662	6.17E-06	0.0002
	mmu-miR-381-3p	-2.264	4.87E-06	0.0002
	mmu-miR-370-3p	-2.876	8.54E-06	0.0002
	mmu-miR-3071-5p	-2.007	1.62E-05	0.0003

mmu-miR-136-3p	-1.987	2.23E-05	0.0003
mmu-miR-30c-2-3p	-1.683	6.94E-05	0.0009
mmu-miR-128-3p	-1.487	7.45E-05	0.0009
mmu-miR-30d-5p	-1.358	0.0002	0.0018
mmu-let-7a-5p	-1.426	0.0002	0.0023
mmu-miR-340-5p	-1.570	0.0002	0.0023
mmu-let-7g-5p	-1.349	0.0003	0.0023
mmu-miR-493-5p	-2.565	0.0003	0.0023
mmu-miR-218-5p	-1.618	0.0003	0.0025
mmu-miR-127-3p	-1.517	0.0003	0.0026
mmu-let-7e-5p	-1.380	0.0006	0.0042
mmu-miR-98-5p	-1.452	0.0012	0.0077
mmu-miR-409-3p	-1.919	0.0013	0.0083
mmu-let-7c-5p	-1.572	0.0015	0.0089
mmu-miR-139-5p	-1.418	0.0015	0.0089
mmu-miR-129b-3p	-1.526	0.0017	0.0093
mmu-miR-129-5p	-1.526	0.0018	0.0095
mmu-miR-382-3p	-2.192	0.0019	0.0096
mmu-miR-193b-3p	-1.554	0.0020	0.0102
mmu-miR-429-3p	-1.738	0.0021	0.0102
mmu-miR-181b-5p	-1.485	0.0023	0.0109
mmu-miR-1b-5p	-1.752	0.0024	0.0110
mmu-miR-1a-3p	-1.751	0.0024	0.0111
mmu-miR-450a-5p	-1.536	0.0025	0.0112
mmu-miR-423-3p	-1.296	0.0034	0.0148
mmu-miR-328-3p	-1.452	0.0035	0.0148
mmu-miR-532-5p	-1.316	0.0048	0.0200
mmu-miR-148a-5p	-1.521	0.0055	0.0212
mmu-miR-499-5p	-1.473	0.0055	0.0212
novel_115	-2.137	0.0059	0.0223
mmu-miR-425-5p	-1.490	0.0080	0.0283
mmu-miR-299a-3p	-1.481	0.0091	0.0319
mmu-miR-200a-3p	-1.500	0.0099	0.0342
mmu-miR-30b-5p	-1.305	0.0104	0.0350
mmu-miR-30e-5p	-1.486	0.0108	0.0356
mmu-miR-200a-5p	-1.410	0.0110	0.0358
mmu-miR-26a-5p	-1.260	0.0113	0.0363
mmu-miR-99a-5p	-1.379	0.0125	0.0396
mmu-miR-409-5p	-1.705	0.0127	0.0398
mmu-miR-92a-3p	-1.350	0.0132	0.0409
mmu-miR-129-1-3p	-1.769	0.0146	0.0446
mmu-miR-148b-3p	-1.277	0.0167	0.0484

511

512 **Figure legends:**

513

514 **Figure 1: Length distribution of the small RNA expressions in the liver of domestic cats**
515 **infected with *Toxoplasma gondii* compared to control cats.** (A and B) Group 1 and group 2
516 of *T. gondii*-infected domestic cats; (C and D) Group 1 and group 2 of *T. gondii*-uninfected,
517 control, domestic cats.

518

519 **Figure 2: Correlation analysis of the small RNA expressions in the livers of *T.***
520 ***gondii*-infected and uninfected cats.** (A) Correlation analysis of small RNA expression
521 levels between *T. gondii*-infected cats of group 1 and group 2; (B) Correlation analysis of
522 small RNA expression levels between *T. gondii*-uninfected cats of group 1 and group 2.

523

524 **Figure 3: GO enrichment analysis of predicted gene targets of differentially expressed**
525 **miRNAs. Abbreviations:** IL vs UL: infected liver vs uninfected liver, BP: biological process,
526 MF: molecular function, CC: cellular component.

527

528 **Figure 4: The top 20 enriched pathways of the predicted host targets of the differentially**
529 **expressed miRNAs.**

530

531 **Supplementary materials**

532

533 **Supplementary Figure 1: Validation of the differential expression of 6 miRNAs**

534 **identified in the sequencing analysis using stem-loop qRT-PCR. U6 was used as a**

535 **reference gene.**

536

537 **Supplementary Table 1: Primers for stem-loop qRT-PCR.**

538

539 **Supplementary Table 2: Predicted gene targets of differentially expressed miRNAs.**