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Full Length Article

Transcriptomic Analysis Provides Insights into the Molecular Mechanisms of Epigallocatechin-3-Gallate to Attenuate Schistosomiasis Hepatic Fibrosis in Mice

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Abstract

Epigallocatechin-3-gallate (EGCG) was shown to possess anti-inflammatory, anti-infection, and anti-fibrotic effects. Our previous study showed EGCG could attenuate *Schistosoma japonicum* hepatic fibrosis, however, its underlying molecular mechanisms are still elusive. In this work, RNA-sequencing (RNA-seq) and bioinformatics analyses were used to detect the gene expression alterations in *S. japonicum* hepatic fibrosis mouse model following EGCG treatment. Our results showed 106 common differentially expressed genes (DEGs) were upregulated in EGCG_LF_*vs*._Mod_LF (EGCG treatment group relative to *S. japonicum* infected model group) and downregulated in Mod_LF_*vs*._Nor_LF (model group relative to normal group), and they are enriched in cytochrome and metabolic genes involving in the metabolism pathways, implying EGCG could improve the *S. japonicum* egg induced hepatic injury and recover the metabolic functions of the liver. The 410 common DEGs, downregulated in EGCG_LF_*vs*._Mod_LF and upregulated in Mod_LF_*vs*._Nor_LF, are associated primarily with inflammation and immunology. Among them, proinflammatory cytokines, chemokines to activate the hepatic stellate cells (HSCs), profibrotic genes, and extracellular matrix (ECM) production and accumulation genes, together with part components of TLR2 and NF-κB signaling pathways, were upregulated in Mod_LF_*vs*._Nor_LF and downregulated in EGCG_LF_*vs*._Mod_LF. These results indicate that EGCG treatment could ameliorate *S. japonicum* egg induced hepatic fibrosis progression via inhibiting the HSCs activation and reducing ECM production and accumulation, which may in part result from suppressing the TLR2 and NF-κB signaling pathways. Our results reveal the molecular mechanisms of EGCG to be used as a potential drug to regress *S. japonicum* egg induced hepatic fibrosis.

Keywords: EGCG; Hepatic fibrosis; Mechanism; RNA-Seq; *Schistosoma japonicum*

Introduction

Human schistosomiasis is still considered to be one of the most prevalent and important parasitic diseases causing severe morbidity and mortality in infected individuals in the worldwide. The World Health Organization (WHO) reported that about 800 million people are at risk of schistosomiasis, and approximately 260 million individuals afflicted in over 70 countries and territories in the worldwide (Engels *et al.*, 2002; WHO, 2016). In China, *Schistosoma japonicum* is mainly prevalent and endemic in the south of China, which gives rise to a serious public health problem with the intestinal and hepatosplenic schistosomiasis (Engels *et al.*, 2002). By the end of 2015, 77,194 cases of schistosomiasis were calculated, and 30,843 cases of advanced schistosomiasis were reported in China (Zhang *et al*., 2016).

In human schistosomiasis, the pathology is mostly caused by the eggs trapped in the hepatic and intestinal tissues of host. The toxic egg material damages cells in host tissues, and the host tissues lodged with eggs generate persistent antigenic stimulation. Subsequently, the inflammatory and immune cells were recruited to the infected locates, which induce the production of egg granulomas and progressively replaced by chronic and advanced fibrosis in some infected cases (Chuah *et al.*, 2014). The most severe consequence of *S. japonicum* infection is given rise to hepatic fibrosis, which primarily induced by hepatic stellate cells (HSCs) to remodel the extracellular matrix (ECM) and deposit the collagens (Carson *et al.*, 2018). The persistent hepatic fibrosis of schistosomiasis usually links with liver-related mortality (Takemura *et al.*, 1998). Therefore, effective drug treatments to control and regress hepatic fibrosis of schistosomiasis are urgently needed.

Currently, praziquantel (PZQ) is still chosen as the first-line anti-schistosome drug for chemotherapy of schistosomiasis (Gryseels, 2012). However, the effect of

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PZQ on *Schistosoma* egg induced hepatic fibrosis is still a controversial issue. Previous studies reported that the PZQ had anti-fibrotic effects on mice in infected with *S. japonicum* (Liang *et al.*, 2011) and *S. mansoni* (El-Lakkany *et al.*, 2012). However, other study revealed that the hepatic fibrosis persists to develop even after the PZQ treatment (Abdel-Hafeez *et al.*, 2012). Thus, no clear consensus has been reached on anti-fibrotic effects of PZQ on mice which were infected with *Schistosoma*. Furthermore, PZQ induces considerable side effects in clinical treatments (Pinlaor *et al.*, 2008). Hence, developing high efficiency and low side-effect anti-fibrosis drugs to control and regress hepatic fibrosis is imperious demands for the schistosomiasis.

Green tea polyphenols have antioxidant activity, which results in the potential promise of using them as dietary agents to prevent and treat several human chronic diseases (Khan and Mukhtar, 2007). Among the mixture of biologically-active green tea polyphenols, one of the amplest constituent is the Epigallocatechin-3-gallate (EGCG) (Steinmann *et al.*, 2013). EGCG has been shown to possess antioxidant, anti-viral, anti-bacterial, anti-fungal, anti-inflammatory and anti-fibrotic functions, and neuroprotective properties in various diseased individuals (Dona *et al.*, 2003; Chakrawarti *et al.*, 2016; Ying *et al.*, 2017; Muhammed *et al.*, 2018). Numerous previous reports showed that EGCG is vital in anti-fibrotic effect by suppressing the production of collagen and regulating the collagenase activity in HSCs (Yasuda *et al.*, 2009; Tipoe *et al.*, 2010; Sriram *et al.*, 2015; Hsieh *et al.*, 2017). More importantly, we previously revealed that EGCG intervention on mice infected with *S. japonicum* can decrease the egg granulomas and collagen deposition significantly, and furtherly alleviated the *S. japonicum* egg induced hepatic fibrosis (Yuan *et al.*, 2016). However, the underlying molecular mechanisms of EGCG to ameliorate the *S. japonicum* egg induced hepatic fibrosis are still elusive.

To elucidate the underlying molecular mechanisms of EGCG to attenuate the *S. japonicum* egg induced hepatic fibrosis, we focused on bioinformatics analyses of RNA-Seq sequence data by using differentially expressed gene (DEG) analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, and gene ontology (GO) enrichment analysis to identify the genes and pathway altered, which provide insight into gene expression alternations in EGCG administrated *Schistosoma*-infected mouse livers after the formation of hepatic fibrosis. Our results revealed that EGCG significantly ameliorates *S. japonicum* egg induced hepatic fibrosis by suppressing the toll-like-receptors 2 (TLR2) and NF-κB signaling pathway to repress the activation of HSCs and decrease the accumulation of ECM. Our results reveal the molecular mechanisms of EGCG to be used as a potential drug to treat and regress *S. japonicum* egg induced hepatic fibrosis.

Materials and Methods

Ethics Statement

Experimental mice were kept and cared under the guide-lines of the Jianghan University Ethics Committee for using experimental animals. Mice experiments were carried out under the international accepted regulations and with the approval of Jianghan University Ethics Committee. To minimize the suffering, sodium pentobarbital anesthesia were carried out for all surgery.

Mice and Parasites

The male BALB/c mice weighing 20–25 g and aged 7–8 weeks were provided by the Hubei Provincial Center for Disease Control and Prevention (Wuhan, China). The animals were fed with the standard pelleted food and water *ad libitum* in an automatically controlled animal house with at 23–25°C room temperature.

The *S. japonicum* infected *Oncomelania hupensis* snails was supplied by the Jiangsu Institute of Schistosomiasis Control (Jiangsu, China). The cercariae used for modeling mice were shed from the infected *O. hupensis* snails in dechlorinated water with artificial light at 28°C.

Experimental Design and Sample Preparation

Mice were randomly divided into normal control (Nor_LF), *Schistosoma* model (Mod_LF) and *Schistosoma* + EGCG (400 mg/kg) (EGCG_LF) groups with ten mice in each group. Following the methods used in previous studies (Liu *et al.*, 2011), 25±2 cercariae of *S. japonicum* were put on the shaved abdominal skin of mice to infect them percutaneously in both the Mod_LF group and EGCG_LF group. At the same time, treatments were also performed on mice in the Nor_LF group but with no cercariae infection. The infected mice were orally administered PZQ (500 mg/kg) on week 7 for 2 days to eliminate the adult worms. Then, mice in the EGCG_LF group were treated intragastrically with EGCG (400 mg/kg) daily from week 7 to week 11 after infection. During the same time, mice in both the Nor_LF group and Mod_LF group were treated intragastrically with sterile water daily. All animals were sacrificed after intraperitoneal injection with 0.75% sodium pentobarbital at 24 h after the last oral gavage. Then they were immediately dissected, and the right lobe of the livers of the animals were removed, frozen and stored at −80°C until further processing.

RNA Sequencing and Analysis

Trizol reagent (Invitrogen, USA) was used to extract the total RNA individually from the cryopreserved mice liver samples, and the DNase I was used to treat the extracted total RNA. Subsequently, poly-T oligo-attached magnetic

beads were used to purify mRNA from total RNA. The mRNA was fragmented into short fragments, which were used as templates to synthesize the cDNA. After purifying and resolving the short fragments and connecting with adaptors, the 150~200 bp fragments were used as templates to perform PCR amplification. The quantification and qualification of the library was assessed by using the Agilent Bioanalyzer 2100 system. The library was paired-end sequenced on an Illumina Hiseq2000 platform to produce 150 bp read.

Raw reads were subjected to removing low quality reads, reads with unknown nucleotides larger than 5% and reads containing adaptors to obtain clean reads. The low quality sequencing reads were filtered with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) . Then the high quality clean reads were mapped to the reference genome. Both the reference mouse genome and gene model annotation files were obtained from genome website (ftp://ftp.ensembl.org/pub/release-87/fasta/mus_musculus/dna/). Bowtie v2.2.3 was used to build index of the reference genome and TopHat v2.0.12 was used to align the paired-end clean reads to the reference genome.

Screening DEGs and Functional Analysis

The read numbers mapped to each gene were counted by HTSeq v0.6.1. The FPKM (fragments per kb per million reads) methods were used to measure the gene expression level of each gene (Trapnell *et al.*, 2010). DESeq R package (1.18.0) was used to conduct the differential expression analysis. Two-fold change (log2 fold-chang $\geqslant$1 or $\leqslant$-1) was used to screen the DEGs of each biological replicate, with referring to the significance of digital gene expression profiles for statistical test (Audic and Claverie, 1997) and a 0.001 Benjamini and Hochberg False Discovery Rate (FDR) (Benjamini and Hochberg, 1995) corrected P-value cut-off. The DEGs were identified with a corrected P-value less than 0.05.

GO functional enrichment and KEGG pathway analyses were implemented with a hypergeometric distribution model to reveal the functions of the DEGs. The GOseq R package was used to perform GO enrichment analysis of differentially expressed genes. The corrected P-value 0.05 was used as cut-off to assign the significantly enriched GO terms. The KEGG pathway analysis was performed by using the KEGG database (http://www.genome.jp/kegg/) (Kanehisa and Goto, 2000), and the pathways enrichment analysis was conducted by the KOBAS software (Xie *et al.*, 2011).

Results

Gene Expression Analysis of the RNA-seq

We assessed the correlations of three biological replicates of liver samples from each group. We found the correlation among the nine samples was from 0.85 to 0.99, and among the three biological replicates of each group was more than 0.96 (Fig. 1). In the Nor_LF group, Mod_LF group, and EGCG_LF group, hierarchical clustering heat-map of the significant DEGs were drawn by Genespring software (Fig. 2). Two major expression patterns (upregulation and downregulation in infected mice compared to control uninfected mice) are shown by the hierarchical clustering. These expression data showed that the greatest number of DEGs were present in mice between the Nor_LF group and the Mod_LF group, and significant differences of DEGs were also identified in mice between the Nor_LF group and EGCG_LF group or between the Mod_LF group and EGCG_LF group, illustrating that the intragastric administration of EGCG affects the liver gene expression in *Schistosoma*-infected mice (Fig. 2).

Comparisons of DEGs Among different Groups

Between any two groups of mouse livers, genes which were upregulated or downregulated with at least two-fold changes in expression were assigned as DEGs. The venn diagrams of upregulated and downregulated DEGs among different groups are shown in Fig. 3a and b, respectively. The number of shared DEGs between groups is shown in the overlapping part, and the number of unique DEGs between groups is presented in the non-overlapping part. We found 2066 and 1322 genes were specifically upregulated and downregulated in livers of mice in the Mod_LF group relative to those in the Nor_LF group, respectively. Furthermore, in mouse livers of the EGCG_LF group relative to those of the Nor_LF group, the specifically upregulated and downregulated gene numbers were ten and two, respectively. Compared the Mod_LF group with the EGCG_LF group, numbers of the specifically upregulated and downregulated genes of the mouse livers were 119 and 431, respectively. As compared with those of the Nor_LF group, 22 common upregulated and 76 common downregulated genes were found in mouse livers of both infected groups.

Functional Enrichment Analysis of DEGs

DEGs between the Nor_LF group and the Mod_LF group: There were 3,486 DEGs between the Nor_LF group and the Mod_LF group. Compared with the samples from the Nor_LF group, 2,088 upregulated and 1,398 downregulated DEGs in the Mod_LF group, respectively (Fig. S1). GO terms of the upregulated DEGs in the Mod_LF group were focused on immune system process, regulation of response to stimulus, positive regulation of biological process, immune response and inflammatory response, which are mainly related to inflammatory and immune response (Fig. S2a), while GO terms of the downregulated DEGs were focused on metabolism, such as oxidation-reduction process, small molecule metabolic process, carboxylic acid metabolic process, fatty acid metabolic process, and lipid metabolic process (Fig. S2b).

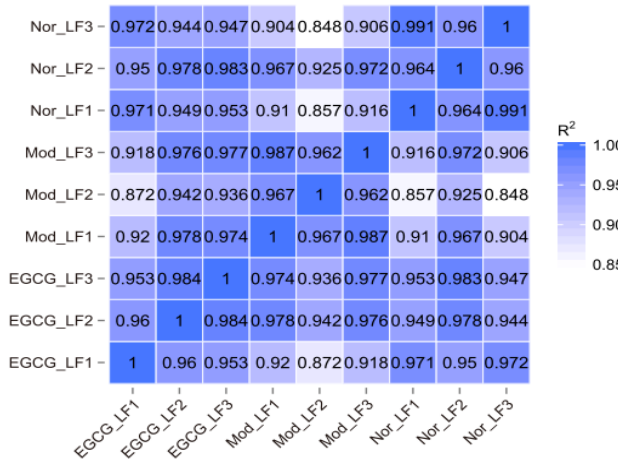

Fig. 1: Correlation analysis for all samples. Nor_LF1, Nor_LF2, and Nor_LF3 represent three biological replicates of samples from the Nor_LF group; Mod_LF1, Mod_LF2, and Mod_LF3 represent three biological replicates of samples from the Mod_LF group; EGCG_LF1, EGCG_LF2, and EGCG_LF3 represent three biological replicates of samples from the EGCG_LF group

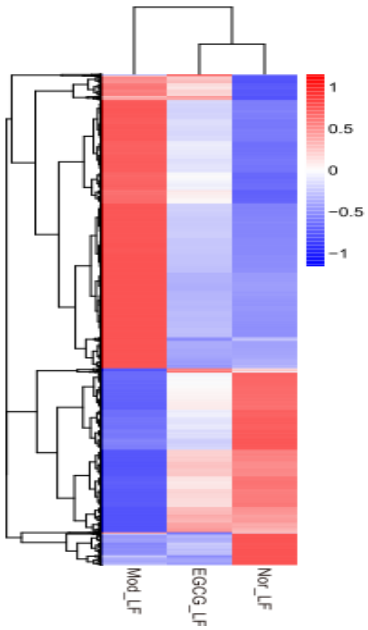

Fig. 2: Cluster analysis for DEGs of the Nor_LF, Mod_LF, and EGCG_LF groups ($p < 0.01$). Gene expression is represented as a heat map with relatively unchanged genes coloured white, upregulated genes coloured red, and downregulated genes coloured blue. The increasing color gradient of the legend indicates increasing expression levels of genes

Results of the KEGG pathway enrichment analysis show that the upregulated DEGs in the Mod_LF group were predominantly related to infection and immune pathways, including chemokine signaling pathway, cytokine-cytokine receptor interaction, focal adhesion, NF-kB signaling pathway, ECM-receptor interaction, platelet activation, and

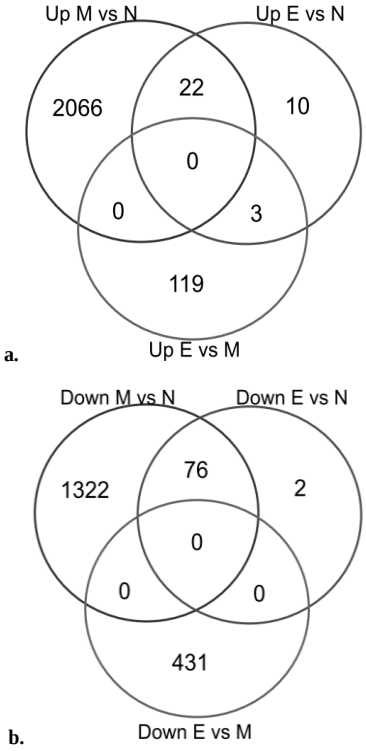

Fig. 3: Analysis of differentially expressed genes (DEGs) in pairwise groups by Venn diagrams. (**a**) Venn diagram of upregulated DEGs of livers between any two groups. (**b**) Venn diagram of downregulated DEGs of livers between any two groups. Upregulated DEGs with fold changes of ≥ 2 between groups were named Up M *vs.* N, Up E vs N, and Up E *vs.* M. Downregulated DEGs with fold changes of ≥2 were named Down M *vs.* N, Down E *vs.* N, and Down E vs M. M, N, and E represent the Mod_LF group, the Nor_LF group, and the EGCG_LF group, respectively. Finally, the Venn diagram was obtained by importing DEGs into Calculate and draw custom Venn diagrams (http://bioinformatics.psb.ugent.be/webtools/Venn/)

so on (Fig. S3a), while the downregulated DEGs were mainly associated with drug metabolism, metabolic pathways, retinol metabolism, and oxidative phosphorylation (Fig. S3b). To sum up, compared with the Nor_LF group, the upregulated genes in the Mod_LF group are predominantly related to the inflammatory and immune processes, while the downregulated genes are primarily associated with the metabolism.

DEGs between the EGCG_LF group and the Mod_LF group: There were 553 DEGs between the EGCG_LF group and the Mod_LF group. As compared the EGCG_LF group with the Mod_LF group, 122 upregulated DEGs and 431 downregulated DEGs were found (Fig. S4). The GO function analysis of the upregulated DEGs in the EGCG_LF group showed that the primarily enriched GO terms are associated with metabolism, such as small molecule metabolic process, carboxylic acid metabolic process, lipid metabolic process, and fatty acid metabolic process (Fig. 4a). While the downregulated DEGs in the EGCG_LF

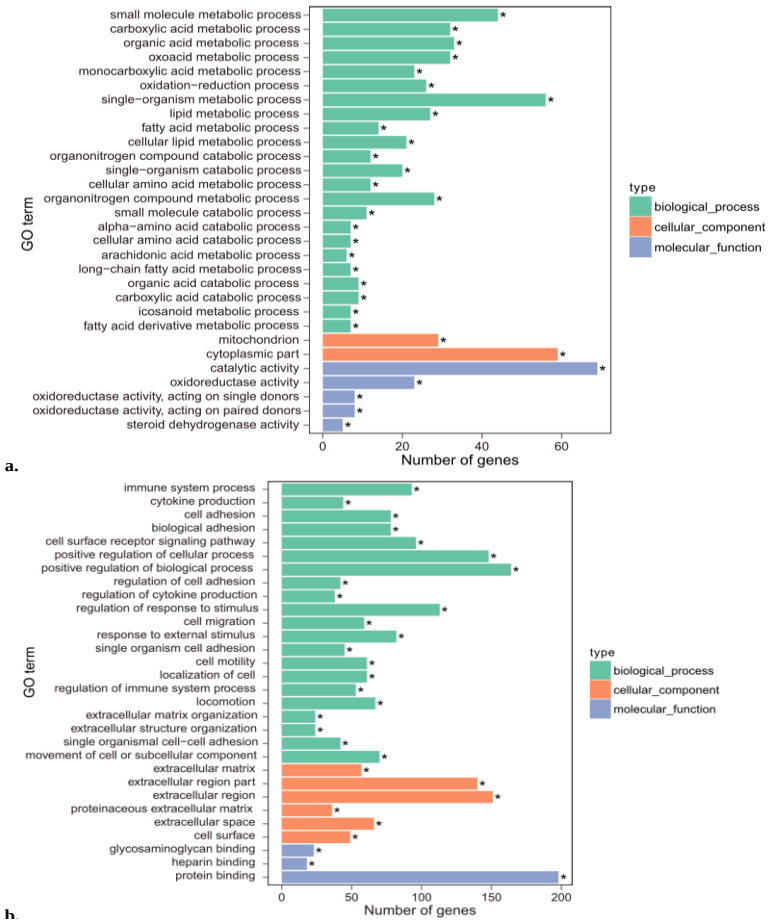

Fig. 4: GO functional enrichment of differentially expressed genes (DEGs) of livers between the Mod_LF group and the EGCG_LF group. **a**. GO functional enrichment of upregulated DEGs of livers between the two groups. **b**. GO functional enrichment of downregulated DEGs of livers between the two groups. * represents significantly enriched

group were mainly correlated with inflammatory and immune response, such as immune system process, cytokine production, cell adhesion, biological adhesion, regulation of cell adhesion and cytokine production (Fig. 4b). Further pathway analysis revealed that the upregulated DEGs are focused on the metabolism pathways, metabolism of xenobiotics by cytochrome P450, steroid hormone biosynthesis, and retinol metabolism (Fig. 5a), while the downregulated DEGs are focused on the cytokine-cytokine receptor interaction, ECM-receptor interaction, rheumatoid arthritis, malaria, and NF-kappa B signaling pathway (Fig. 5b). Altogether, relative to the Mod_LF group, the downregulated genes in the EGCG_LF group are predominantly related to the inflammatory and immune processes, while the upregulated genes are primarily associated with the metabolism. Compared these results of DEGs between the EGCG_LF group and Mod_LF group (EGCG_LF_*vs*._Mod_LF) with those of DEGs between the Mod_LF group and Nor_LF group (Mod_LF_*vs*._Nor_LF), we found that the GO functional and KEGG pathway enrichment results between them are just the reverse.

Comparisons of the DEGs between the EGCG_LF_*vs*._Mod_LF and Mod_LF_*vs*._Nor_LF: The above results revealed that the GO functional enrichment and KEGG pathways of the up-/down-regulated DEGs in the EGCG_LF_*vs*._Mod_LF are almost contrary to those in the Mod_LF_*vs*._Nor_LF. Therefore, we further compared the DEGs between them in detail. We found 106 common genes are shared by the 122 upregulated DEGs in the EGCG_LF_*vs*._Mod_LF and 1398 downregulated DEGs in the Mod_LF_*vs*._Nor_LF (Table. S1). The 106 common genes are involved predominantly in metabolic processes (Fig. S5a) and metabolism related pathways (Fig. S5b), consistent with the results of the upregulated DEGs in EGCG_LF group compared with the Mod_LF group. Notably, among them, expression levels of five cytochrome genes were shown different significantly between the 122 upregulated DEGs in EGCG_LF_*vs*._Mod_LF and 1398 downregulated DEGs in Mod_LF_*vs*._Nor_LF. Cytochromes 2c29 (Cyp2c29), Cyp4f14, Cyp2j5, Cyp2f2, and Cyp2c67 were significantly upregulated in the EGCG_LF group relative to the Mod_LF group, while

downregulated in the Mod_LF group compared with the Nor_LF group (Table 1.).

There are 410 common genes shared by the 431 downregulated DEGs in the EGCG_LF_*vs*._Mod_LF and 2088 upregulated DEGs in the Mod_LF_*vs*._Nor_LF (Table S2). The significant difference between the expression patterns of DEGs in EGCG_LF_*vs*._Mod_LF and Mod_LF_*vs*._Nor_LF was the opposite expression profiles. The opposite expressed genes were predominantly focused on the biological process including immune system process, cytokine production, cell adhesion, biological adhesion, cell surface receptor signaling pathway, regulation of cytokine production, and collagen metabolic process (Fig. S6a). Prominent genes of interest were related to neutrophils, HSC activation and hepatic fibrosis (Table 1).

Three genes related to neutrophils, the neutrophilic granule protein (NGP), myeloperoxidase (MPO), and cathepsin G (CTSG), were upregulated significantly in Mod_*vs*._Nor_LF, while they were downregulated in EGCG_*vs*._Mod_LF (Table 1). Two chemotactic molecules for neutrophils, S100A8 and S100A9, were also significantly upregulated in Mod_*vs*._Nor_LF and downregulated in EGCG_*vs*._Mod_LF (Table 1). Activation of toll-like-receptors (TLRs) facilitates neutrophil recruitment and it was reported that TLR2 and TLR4 have increased expressions and important functions in liver disease (Seki *et al.*, 2011). Here, we found TLR2 and TLR4 are significantly upregulated in Mod_*vs*._Nor_LF (Table 1), and TLR2 is significantly downregulated in EGCG_*vs*._Mod_LF.

Activated HSCs produce ECM to progress the hepatic fibrosis. In this study, we revealed that many genes implicated in the ECM production and accumulation were upregulated significantly in Mod_*vs*._Nor_LF, such as the laminin (Lamb1 and Lama2), integrin (Itga4 and Itgb7), thrombospondinc (Thbs1, Thbs2 and Thbs3), and profibrotic cytokines (Il17ra, Ccl12, Ccl21 and Ccl6) (Table 1). However, they were significantly downregulated in EGCG_*vs*._Mod_LF. Furthermore, we detected that NF-κB2 and RelB were significantly upregulated in Mod_*vs*._Nor_LF and downregulated in EGCG_vs_Mod_LF (Table 1), which is in accordance with the expression patterns of genes implicated in the ECM production and accumulation. Previous study reported that NF-κB plays a vital role in fibrosis and regulates multiple essential functions of HSCs (Luedde and Schwabe, 2011). Therefore, our results imply that NF-κB may be involved in regulating the activation of HSCs with EGCG treatment.

Our previous study detected more severe hepatic fibrosis in livers of schistosoma-infected model group mice than those of EGCG treatment group mice (Yuan *et al.*, 2016). Thus, hepatic fibrosis associated genes were inspected in the two comparisons. Tissue inhibitor of metalloproteinase 1 and 2 (TIMP1, 2) and matrix metalloproteinases 2, 3, 8, 9, 11 and 23 (MMP2, MMP3, MMP8, MMP9, MMP11, MMP23) were significantly upregulated in Mod_*vs*._Nor_LF, while they are downregulated in EGCG_*vs*._Mod_LF (Table 1). Moreover, the profibrogenic genes, platelet-derived growth factor receptor-β (PDGFR-β), transforming growth factor-β (TGF-β), TNFα, Col11a1 and Col6a5, showed significantly upregulated in Mod_*vs*._Nor_LF, while they are significantly downregulated in EGCG_*vs*._Mod_LF (Table 1).

KEGG pathway enrichment analysis showed that the 410 common genes shared by the 431 downregulated DEGs in the EGCG_LF_*vs*._Mod_LF and 2088 upregulated DEGs in the Mod_LF_*vs*._Nor_LF are predominantly involving in four pathways (Fig S6b), including cytokine-cytokine receptor interaction and ECM-receptor interaction, indicating that EGCG intervention altered the ECM deposition.

Discussion

EGCG, the amplest and most active polyphenolic catechin found in green tea, has attracted considerable attentions (Steinmann *et al.*, 2013). Numerous beneficial biological effects of EGCG consumption have been revealed as regards physiological and pharmacological health benefits. The anti-inflammatory mechanisms of EGCG have been partly attributed to decrease the pro-inflammatory response (Tipoe *et al.*, 2010) or suppress the activation of TLR-4/NFκB p65 signal pathway (Marinovic *et al.*, 2015). Moreover, EGCG has been shown anti-fibrotic effect *via* different mechanisms in various diseases. Sriram had demonstrated that EGCG can suppress TGF-β1 signaling in pulmonary fibrosis to suppress the activation of fibroblast and reduce the collagen accumulation (Sriram *et al.*, 2015). Lin found that EGCG attenuated the myocardial fibrosis via inhibiting the inflammatory effect through JNK/AP-1 pathway (Lin *et al.*, 2016). Wang revealed that EGCG attenuating renal interstitial fibrosis in mice might be associated with its inhibition effects on inflammatory responses and TGF-β/Smad signaling pathway (Wang *et al.*, 2015). Previous studies have demonstrated that EGCG attenuates hepatic fibrosis in rat by regulating the TGF/SMAD, PI3 K/Akt/FoxO1 and NF-κB pathways (Xiao *et al.*, 2014) and suppressing the expression of the PDGFR and insulin-like growth factor receptor (IGF-1R) (Yasuda *et al.*, 2009).

More importantly, we have found that EGCG could alleviate the hepatic fibrosis induced by *S. japonicum* egg in mice (Yuan *et al.*, 2016). However, the mechanistic basis for EGCG to improve the *S. japonicum* egg induced hepatic fibrosis are still elusive. In order to explore the vital differential genes and pathways in *S. japonicum* egg induced hepatic fibrosis following EGCG intervention, in this work, based on the RNA-Seq sequence data, the differentially expressed genes and pathways were identified by using bioinformatics analyses such as the DEG analysis, KEGG pathway analysis and GO enrichment analysis. Our aim is to reveal the gene expression alternations in the *S.*

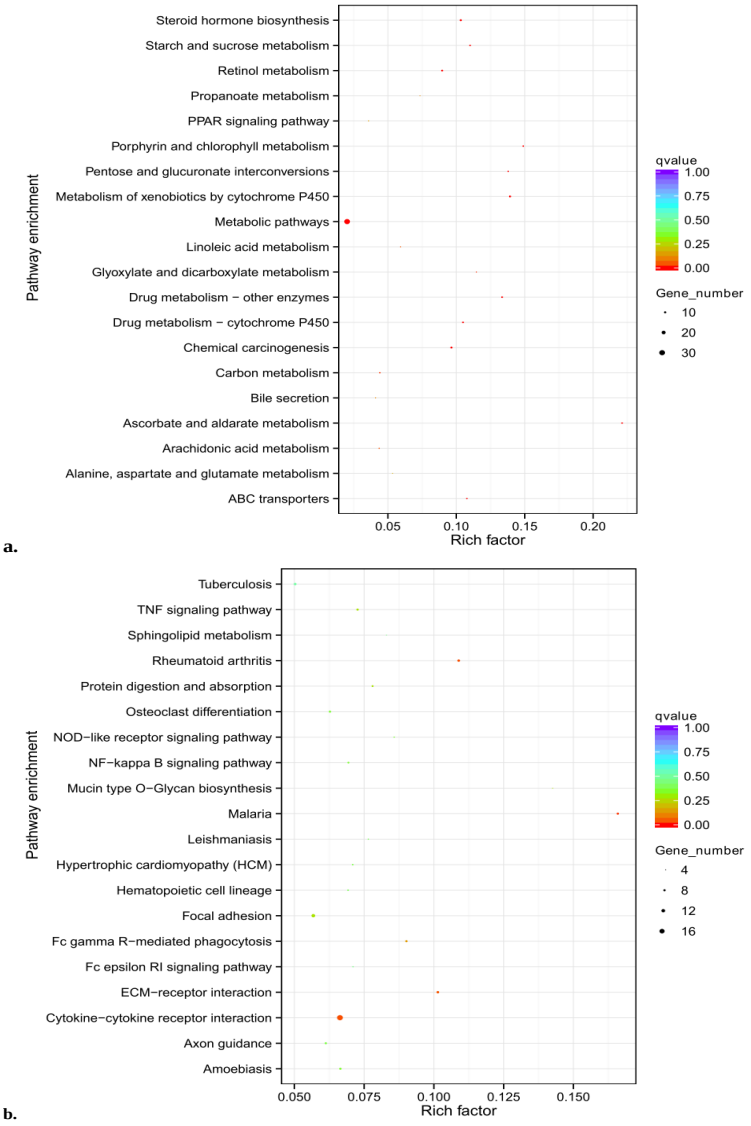

Fig. 5: KEGG pathway enrichment of differentially expressed genes (DEGs) of livers between the Mod_LF group and the EGCG_LF group. **a**. KEGG pathway enrichment of upregulated DEGs of livers between the two groups. **b**. KEGG pathway enrichment of downregulated DEGs of livers between the two groups

japonicum egg induced hepatic fibrosis mouse model following the EGCG intervention.

In previous study, we observed moderate granulofibrotic pathology in EGCG treatment group mice and severe one in the *S. japonicum* infected model group mice (Yuan *et al*., 2016). Here, the 106 common DEGs which are upregulated in EGCG_LF_*vs*._Mod_LF and downregulated in Mod_LF_*vs*._Nor_LF are primarily involved in the metabolic pathways, metabolism of xenobiotics by cytochrome P450 and drug metabolism - cytochrome P450 (Fig. S5).

The extensive downregulation of numerous genes involved in the metabolic pathways in the livers of mice infected with *S. japonicum* suggested that infection with *S. japonicum* could reduce the metabolic functions of the mice liver. These results are in accordance with the results from *S. japonicum* (Perry *et al*., 2011) and *S. mansoni* (Harvie *et al*., 2007) infections, indicating the growing damage of liver function is related to the progressive liver injury following *Schistosome* infection. However, with EGCG intervention, numerous cytochrome and metabolic genes were upregulated, implying that EGCG could decrease the *S. japonicum* egg induced hepatic injury and recover the metabolic functions of the liver.

More importantly, the 410 common DEGs downregulated in EGCG_LF_*vs*._Mod_LF and upregulated in Mod_LF_*vs*._Nor_LF play a vital role in attenuating the *S. japonicum* egg induced hepatic fibrosis following EGCG

Table 1: Expression patterns for genes of interest between the EGCG_LF_*vs.*_Mod_LF and Mod_LF_*vs.*_Nor_LF

Gene ID		EGCG_LF_*vs.*_Mod_LF		Mod_LF_*vs.*_Nor_LF	
	Gene name	Fold change*	p-value	Fold change*	p-value
Cytochrome genes					
ENSMUSG00000003053	Cyp2c29	6.32E-05	0.008455	-2.1598	2.06E-07
ENSMUSG00000024292	Cyp4f14	6.90E-08	5.26E-05	-2.9585	3.36E-07
ENSMUSG00000052520	Cyp2j5	1.53E-05	0.003332	-2.1532	3.52E-08
ENSMUSG00000052974	Cyp2f2	2.95E-11	6.21E-08	-3.9564	4.19E-23
ENSMUSG00000062624	Cyp2c67	3.18E-07	0.000208	-2.8415	1.95E-23
Neutrophils associated genes					
ENSMUSG00000032484	Ngp	-3.5148	0.010505	3.9474	0.004792
ENSMUSG00000009350	Mpo	-3.301	0.000292	2.8154	0.001762
ENSMUSG00000040314	Ctsg	-3.2096	0.000882	3.3951	0.000501
ENSMUSG00000056054	S100a8	-3.0405	0.009204	4.2333	0.000867
ENSMUSG00000056071	S100a9	-3.0584	0.006572	3.9836	0.00091
Proinflammatory and profibrotic cytokines					
ENSMUSG00000035352	Ccl12	-2.0122	3.67E-05	2.5237	5.69E-06
ENSMUSG00000094686	Ccl21a	-1.8549	1.60E-05	1.7987	0.000115
ENSMUSG00000018927	Ccl6	-1.2982	0.000459	2.0402	6.09E-06
ENSMUSG00000002897	Il17ra	-0.99067	0.000897	1.1119	4.73E-05
ENSMUSG00000018930	Ccl4	-1.0858	0.096068	1.9461	0.002485
ENSMUSG00000029380	Cxcl1	-1.6904	0.023227	3.1712	0.00012
ENSMUSG00000058427	Cxcl2	-1.5885	0.002309	2.5045	2.00E-05
Profibrotic genes					
ENSMUSG00000021253	Tgfb	-2.019	9.96E-05	2.6624	9.23E-07
ENSMUSG00000024620	Pdgfrb	-1.3311	0.000105	2.0469	1.22E-12
ENSMUSG00000024401	TNFa	-1.6162	0.00054	2.1717	1.03E-05
ECM production and accumulation genes					
ENSMUSG00000028047	Thbs3	-1.5237	0.000324	2.2963	2.31E-07
ENSMUSG00000027966	Col11a1	-1.812	8.35E-06	3.1623	4.32E-13
ENSMUSG00000091345	Col6a5	-1.8203	0.000262	2.1575	0.000558
ENSMUSG00000040152	Thbs1	-1.7614	1.05E-05	2.2046	5.28E-06
ENSMUSG00000027009	Itga4	-1.2669	0.000171	1.2835	0.000189
ENSMUSG00000019899	Lama2	-1.6918	2.52E-05	2.196	4.45E-07
ENSMUSG00000023885	Thbs2	-2.3503	1.16E-07	2.1787	6.91E-06
ENSMUSG00000001281	Itgb7	-1.4902	8.51E-06	1.2404	0.001573
ENSMUSG00000002900	Lamb1	-1.0795	0.000509	1.3004	4.59E-06
ENSMUSG00000001131	Timp1	-0.77641	0.22762	2.5699	1.22E-07
ENSMUSG00000017466	Timp2	-1.7415	0.000232	2.0495	2.37E-05
ENSMUSG00000031740	Mmp2	-2.2208	0.000375	2.8911	2.11E-05
ENSMUSG00000043613	Mmp3	-2.4964	4.78E-07	4.2242	4.00E-13
ENSMUSG00000005800	Mmp8	-3.3155	0.035166	4.9207	0.005421
ENSMUSG00000017737	Mmp9	-2.1634	0.015543	3.7401	0.000265
ENSMUSG00000000901	Mmp11	-1.5068	0.000877	1.7745	9.22E-05
ENSMUSG00000029061	Mmp23	-1.5906	1.12E-05	2.2141	2.70E-07
NF-κB signaling pathway					
ENSMUSG00000002983	Relb	-1.493	2.16E-05	1.6694	5.02E-05
ENSMUSG00000025225	Nfkb2	-0.89742	0.014487	1.0483	0.002796
Toll-like receptor signaling pathway					
ENSMUSG00000027995	Tlr2	-1.3487	5.89E-05	1.981	2.12E-10
ENSMUSG00000039005	Tlr4	-0.93365	0.003266	1.3873	3.17E-06

*Expression is presented by value of log2FoldChange. Negative values represent down-regulation, and positive values represent up-regulation

treatment. The pathology of schistosomiasis is the T-helper (Th) cell induced fibro-granulomatous inflammatory response to eggs deposited in host tissues and the subsequent fibrogenesis (Lewis and Tucker, 2014). Thus, the inflammation response plays a vital role in exacerbating liver injury and promoting the subsequent fibrogenesis, and reducing the levels of proinflammatory cytokines may be favourable for hepatic fibrosis. In the present study, the expression levels of proinflammatory cytokines, such as Ccl4, Cxcl1, and Cxcl2, were significantly upregulated in Mod_LF_*vs.*_Nor_LF, while they were downregulated in EGCG_LF_*vs.*_Mod_LF, suggesting that EGCG treatment ameliorates liver injury, possibly due to reduced expression levels of inflammatory cytokines. Moreover, the neutrophil markers *NGP*, *MPO* and *CTSG* were significantly upregulated in Mod_LF_*vs.*_Nor_LF and downregulated in EGCG_LF_*vs.*_Mod_LF, indicating EGCG treatment ameliorates *S. japonicum* egg induced hepatic fibrosis might be associated with neutrophils.

In *Schistosome* infected livers, the activated HSCs are the major cells to produce the profibrogenic molecules and collagens (Bartley *et al.*, 2006). The profibrotic cytokines CCL21 and CCL12 have been found to be chemoactive for HSCs or myofibroblasts and have been involved in the

pathogenesis of various fibrotic diseases (Bonacchi *et al.*, 2003; Moore *et al.*, 2006). Here, the chemokines CCL12 and CCL21 were significantly upregulated in Mod_LF_*vs.*_Nor_LF and significantly downregulated in EGCG_LF_*vs.*_Mod_LF. Coincidentally, the expression patterns of profibrotic genes TGF-β, TNFα, and PDGFR presented a striking relevance to the expression patterns of the chemokines CCL21 and CCL12. TGF-β is considered as key profibrotic gene in the progress of hepatic fibrosis (Seki *et al.*, 2007). TNFα signaling elicits necroptosis in activated HSCs to ameliorate hepatic fibrosis (Chang *et al.*, 2015). PDGF is a potent mitogen to mediate the HSC proliferation during liver injury (Kinnman *et al.*, 2001). Moreover, studies revealed that EGCG might inhibit HSC proliferation and activation by interfering with PDGF/PDGFRβ signaling (Chen and Zhang, 2003; Yasuda *et al.*, 2009). Thus, our results indicate that EGCG treatment may be contributed to improve the HSC-associated fibrosis by regulating the expression of the chemokines of fibrogenic effector cells and the profibrotic genes.

Hepatic fibrosis results from the excessive accumulation of collagens and other ECM constituents. In liver pathology, activated HSCs are the primary source of MMPs, TIMPs, and various ECM constituents, such as collagen, laminin, fibronectin, proteoglycan and adhesive glycoproteins (Hemmann *et al.*, 2007; Seki and Brenner, 2015). We revealed that genes involved in ECM production and accumulation (Lamb1, Lama2, Itga4, Itgb7, Thbs1, Thbs2 and Thbs3), collagen deposition (Col11a1 and Col6a5), and profibrotic cytokines (Il17ra, Ccl12, Ccl21 and Ccl6) were significantly upregulated in Mod_LF_*vs.*_Nor_LF, but they were all significantly downregulated in EGCG_LF_*vs.*_Mod_LF (Table 1). Furthermore, our result showed that the expression levels of multiple MMPs (MMP-2, -3, -9, -11, -23) and TIMPs (TIMP-1, -2) were upregulated significantly in the Mod_LF_*vs.*_Nor_LF (Table 1), which is in accordance with the studies that multiple MMPs and TIMPs are upregulated in *S. japonicum* egg induced hepatic fibrosis (Burke *et al.*, 2010). However, they were downregulated in the EGCG_LF_*vs.*_Mod_LF (Table 1). The altered expressions of MMPs and TIMPs in EGCG_LF_*vs.*_Mod_LF and Mod_LF_*vs.*_Nor_LF indicated that the wound healing responses are different between livers of the *S. japonicum* infected mice and EGCG intervention ones, which may partly account for the less severe of hepatic fibrosis observed in the EGCG treated mice. Collectively, all these results imply that EGCG might exhibit the anti-fibrosis property to *S. japonicum* egg induced hepatic fibrosis *via* inhibiting the HSCs activation and subsequent ECM deposition. To confirm the conclusion, we will isolate the primary HSCs of EGCG-treatment mice for checking the mRNA expressions in the future.

To further investigate the underlying mechanism of EGCG attenuating the hepatic fibrosis, we examined the activation of the NF-κB signaling pathway, which had been reported to regulate the hepatic fibrogenesis mainly via modulating the activation of HSCs and hepatocyte injury (Luedde and Schwabe, 2011). Herein, NF-κB2 and Relb were significantly upregulated in Mod_LF_vs_Nor_LF, implying *S. japonicum* egg induced hepatic fibrosis significantly increased NF-κB signaling activation. Interestingly, EGCG treatment significantly decreased *S. japonicum* egg induced NF-κB activation as evidenced by the downregulation of NF-κB2 and Relb in EGCG_LF_*vs.*_Mod_LF. Taken together, these results suggest that EGCG treatment alleviates the *S. japonicum* egg induced hepatic fibrosis by reducing HSC activation, possible through inhibiting the NF-κB signaling pathway.

TLRs are implicated in the modulation of inflammation and fibrosis in liver (Mencin *et al.*, 2009). TLR2 has been reported to promote hepatic inflammation and fibrogenesis (Miura *et al.*, 2013), and TLR2 deficiency alleviates hepatic fibrosis *via* inhibiting NF-κB signaling pathway (Ji *et al.*, 2014). Here, the transcriptional levels of TLR2 and 4 were significantly upregulated in Mod_LF_*vs.*_Nor_LF, consistent with the previous report that TLR2 and 4 were important and expressed increasingly in acute and chronic liver disease (Seki *et al.*, 2011). However, we revealed that TLR2 was significantly downregulated in EGCG_LF_*vs.*_Mod_LF. Interestingly, the downregulation of TLR2 in EGCG_LF_*vs.*_Mod_LF was accompanied with the downregulation of the proinflammatory and profibrotic genes including Ccl4, Cxcl1, Cxcl2, PDGFR, TNFα, TGFβ, MMPs and TIMPs in EGCG_LF_*vs.*_Mod_LF (Table 1). TLR2 is highly expressed in the Kupffer cells and HSCs (Seki and Brenner, 2008), which are known as the primary cells to produce the profibrotic molecules and ECM components (Coenen *et al.*, 2011). Thus, the downregulated TLR2 in EGCG_LF_*vs.*_Mod_LF might be contributed to alleviate *S. japonicum* egg induced hepatic fibrosis with EGCG treatment, indicating EGCG alleviates *S. japonicum* egg induced hepatic fibrosis may be associated with the downregulation of TLR2.

To check the reliability of the RNA-Seq data analysis, qRT-PCR analysis is needed to check expression profiles of genes produced by Illumina. The main limitation of the study is lacking of experimental confirmation in mouse. However, our previous studies revealed that EGCG intervention on mice infected with *S. japonicum* can decrease the expression level of TIMP1 (Yuan *et al.*, 2016), which is consistent with the result from the RNA-Seq in this work, suggesting the RNA-Seq data were reliable.

Conclusion

Our studies indicate that EGCG intervention could ameliorate *S. japonicum* egg induced hepatic fibrosis progression, which might be result from the inhibition of HSC activation and subsequently decreased expression of procollagen and profibrotic genes to reduce ECM

production and accumulation, at least partly by suppressing the TLR2 and NF-κB signaling pathways. Our results reveal the molecular mechanisms of EGCG to be used as a potential drug to reverse *S. japonicum* egg induced hepatic fibrosis. However, at the present time, EGCG has only been used to treat *S. japonicum* egg induced hepatic fibrosis in mice. Therefore, in order to use EGCG as clinical agent to treat hepatic fibrosis of human schistosomiasis, the clinical trial and data to favor the present results of EGCG are needed in the future.

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