

miR-216b Downregulates Intestinal IL2RB to Inhibit the Invasion of *Echinococcus granulosus* Eggs in the Kazakh Sheep

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ABSTRACT

To study the early molecular immunity of *Echinococcus granulosus* infection in livestock, especially in sheep, the immunohistochemistry, in-situ hybridization was used to study the relationship and the distribution between miR-216b and IL2RB in the intestine tissue of kazakh sheep with resistant and non-resistant MHC haplotypes after oral infection with *E. granulosus* eggs. The results demonstrated that miR-216b negatively regulated IL2RB in the cystic echinococcosis intestinal tissues, and they were differentially expressed in the intestine tissue between the resistant sheep and non-resistant sheep, more widely distributed in the resistant group. These results illustrated that miR-216b and IL2RB activated stronger immune response in the resistant sheep, compared with the non-resistant sheep, and participating in the T cell immune response.

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Authors' Contribution

XW, XL and BJ designed the study and wrote the manuscript. FY, CCL and SJ performed experiments and analyzed the data.

Key words

microRNAs, Molecular immunity, Intestine tissue, Sheep, Cystic echinococcosis

INTRODUCTION

Cystic echinococcosis (CE) is a complex, chronic and difficult to manage, a globally distributed zoonotic parasitic disease, the liver is the most important place for the presence of the parasitic cyst, which life cycle has three stages, includes adult, eggs and larvae (Jindra *et al.*, 2010). Dogs and other canines are its definitive hosts. After the spores are eaten by the dog and then transported to its liver for colonization, in which they will develop into adults or ovulates and finally form cysts in livers of cattle or sheep. The health of people and livestock caused a great threat, and seriously affect the development of animal husbandry, resulting in significant economic losses.

Currently, the main methods of CE treatment include drug administration and surgery, which generally involves the administration of benzimidazole such as albendazole and mebendazole (Zhu *et al.*, 2016). The shortcomings of these methods is that they are expensive and particularly challenging in underdeveloped countries and remote areas. Therefore, it is necessary to develop a relatively cheap and efficient treatment at the same time. Livestock vaccination may provide an additional method for CE management. Studies have been reported for the protection of certain

animals, such as cattle, goats and sheep, because of *Echinococcus granulosus* cyst caused by CE (Larrieu *et al.*, 2013). Hydatid disease infection can induce the production of Th1 and Th2 cytokines. Th1 cytokines are associated with protective immunity of echinococcosis, however Th2 cytokines can induce susceptible diseases. A large number of cell-mediated immune responses were found in the early stages of hydatidosis (Hui *et al.*, 2015). However, the specific molecular immune mechanism of early infection is still unclear.

In our previous study, we examined the expression of differential genes of the sheep intestine tissue in response to the primary *E. granulosus* infection during the 4h infection and produced extensive gene expression in the sheep. Such as IL17RE, IL1RL1, IL1R2, IL13, IL2RB are up-regulated, and these genes are associated with T cells activation (Magambo *et al.*, 1995). Furthermore, microRNAs profile was conducted in the intestinal tissue of sheep with resistant and non-resistant MHC haplotypes after peroral infection with *E. granulosus* eggs, and the expression of a mass of microRNAs were significantly differentially, including up-regulated microRNAs (miR-101, miR-214, miR-182, miR-181, miR-184 etc.) and down-regulated microRNAs (miR-98, miR-128, miR-216b, miR-301 etc.), which are connected with the activation of T cells. microRNAs are important for the development and function of T cells, such as miR-101, miR-155, miR-214, miR-27 and miR-31, which are involved in the activation of T cells (Jindra

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et al., 2010; Magambo *et al.*, 1995; Chiang *et al.*, 2012; Fan *et al.*, 2012; Glasmacher *et al.*, 2010; Chen *et al.*, 2012; Yu *et al.*, 2007; Weitzel *et al.*, 2009). To explore the molecular immunity mechanism of *E. granulosus* infection in livestock, ISH, IHC were used to analyze the relationship between differential gene expression and miRNAs in a sheep model.

MATERIALS AND METHODS

Sheep intestinal tissue

All animals were in strict accordance with the the Animal Ethics Procedures and Guidelines of the People's Republic of China preparation and processing. The protocol was ratified by the Institutional Animal Protection Committee of Shihezi University. One resistance healthy kazakh sheep (RG) and a non-resistance healthy Kazakh sheep (NRG as control) were kept in parasite-free conditions, and divided into resistance and non-resistance group. All of sheep were negative for antibodies to hydatid cyst fluid (HCF) antigen, carried out as previously reported (Hui *et al.*, 2015). *E. granulosus* eggs were obtained from the gut content of three highly-infected dogs according to the method described (Veit *et al.*, 1995). Handling the eggs were followed by Strict safety procedures (Jiang *et al.*, 2016). Each sheep individual was infected perorally via a medical syringe with 8000 *E. granulosus* eggs suspended in 5 mL physiological saline. At 8 h post-infection, animals were sacrificed as previously reported (Glasmacher *et al.*, 2010). At the same time, about 1cm³ size of the intestinal tissue block were collected and fixed in the formalin prior to the immunohistochemistry (IHC) or in situ hybridization (ISH).

Immunohistochemistry (IHC)

The small intestine tissue of sheep from RG and NRG was fixed in 4% formaldehyde. The small intestine tissue was embedded in paraffin and cut into a thickness of 5 µm. The slides were dried at 65 °C for 7 h. The sections were dewaxed in Xylenes I and II for 5 min each and the sections were dehydrated with 100%, 95%, 90%, and 80% ethyl alcohol for 2 min one time each, and then washed with distilled water (twice, 5 min each wash) and 3% H₂O₂ to inactivate endogenous peroxidases (once, 5 min). Antigen retrieval was carried out in 20×Tris EDTA (pH 9.0, Tris base 12.1 g, EDTA 3.7g, distilled water 500ml, boiling) for 2 min. Sections were incubated with affinity purified anti-IL2RB (final concentration 0.5mg/ml) in PBS at 4 °C overnight and for 1 h at room temperature and then with biotinylated goat anti-rabbit IgG (OriGene Technologies, Inc., Beijing, China) for 30 min at 37 °C. Between each step, the slides were washed in PBS (twice, 5 min each

wash). The secondary antibody was visualized using DAB reagent (ZSGB-BIO. Inc. Beijing, China). The specimens were then rinsed in distilled water, air dried, mounted with Neutral balsam (Shanghai Yiyang Instrument CO., LTD., Shanghai, China) and observed under Leica DM1000 LED microscope (Leica, Germany).

The fish of miR-216b in the small intestine tissue

Oar-miR-216b detection probe, scrambled probe and the G-type miRNA in situ hybridization assay kit were purchased from Focobio Corporation (Guangzhou, China). The sections were prepared like IHC and dewaxed in xylenes I, II and III for 5 min each. Then the slides were put in 100%, 85%, 70% ethanol for 5 min each. After washed once with PBS for 5 min, and the slides were incubated in solution A for 20 minutes at room temperature, then the solution A was removed and treated with solution B at room temperature for 15 min. After that, the slides were washed in PBS for 5 min and fixed in 4% formaldehyde in PBS for 15 min. The slides were washed in PBS for 10 minutes before addition of 200µl of C solution at 37°C for prehybridization. The C solution was removed, 10µl of a hybridization solution containing 1.5-2.0 µM mRR-216b probe was added and the slides were crossed overnight in an incubator at 40-42 °C. On the next day, the probe was removed, the slide was washed washing 6 buffer I for 15 min at room temperature, and then rinsed twice in wash buffer II for 15 minutes each time. The slides were washed in 75%, 100% ethanol for 2 min, air dried for 10 min, and then 10 µl of DAPI was added for 10 min. The slides could be analyzed by Leica DMI8 fluorescence microscope (Leica, Germany).

miRNA target prediction

To predict the targets of miR-216b during the process of CE, miRNA-IL2RB pairs were selected using algorithms from RNAhybrid (<https://bibiserv.cebitec.uni-bielefeld.de/Rnahybrid>).

Plasmid construction and luciferase reporter assay

The dual-luciferase reporter vectors construct was generated by cloning the total 3'UTR or the mutant3'UTR of IL2RB into pmiR-GLO vector (Promega, Madison, USA) at the site of NheI and XbaI enzyme digestion. The IL2RB 3'UTR fragment was cloned using PCR. Based on the predicted interaction sites, miR-216b had 8 seed bases paired with IL2RB 3'UTR. Eight of them were mutated by PCR and enzymatic digestion to construct Mut-IL2RB-3'UTR. The Firefly luciferase vector as internal reference. The mutated 3'UTR of IL2RB (Mut-IL2RB) was constructed as a negative control. Fifty ng of pmiR-GLO-IL2RB 3'UTR (IL2RB-W) or pmiR-GLO-Mut-IL2RB

Table 1: PCR primers.

Gene	Forward primer	Reverse primer	Annealing temp (°C)	Product Size (bp)
IL2RB	GCACAGAGGAGCTTAATG	TGAGGAGACTCGGAGAGA	53	144
Mut-IL2RB	GCACAGAGGAGCGATTGA	TGAGGAGACTCGGAGAGA	53	147

3'UTR (IL2RB-M) and miR-216b mimics or miR-216b inhibitor were co-transfected into 293T cells in a 6-well plate using Lipofectamine 2000 (Invitrogen). After 48 h, all the target validation assays were performed with the dual-luciferase reporter system (Vigorous Biotechnology, Beijing) according to the manufacturer's instructions (Yang *et al.*, 2015). The activity was measured by the BHP9504 Optical Analysis Reader (Hamamatsu, Japan). The fluorescence signal was collected each 0.5 °C for 20 s. The double $\Delta\Delta C_t$ method was used to measure the expression alteration.

qRT-PCR

Total RNA was extracted with RNA simple Total RNA kit (Tiangen, China) from 293t cells. Single strand cDNAs were prepared from 1 μ g RNA using a reverse transcription Kit (Takara). The qRT-PCR reactions were set up in 20 μ l reaction mixtures containing 10 μ l lightCycle® 480 SYBR Green I Master (Roche), 0.4 μ l sense primers, 0.4 μ l antisense primers, 7.2 μ l distilled water, 2 μ l template. Reaction conditions were as follows: 95 °C for 5 min, and then 35 cycles 95 °C for 10 s, 53 °C for 30 s, and 72 °C for 30 s. The qRT-PCR primers are listed in Table 1.

Statistical analysis

All images represent at least three independent experiments. Every qRT-PCR, luciferase reporter assay and cell viability assay were repeated in triplicate. The results are expressed as mean $\pm$ SEM. P-values were analyzed by the two-tailed Student's t-test using GraphPad Prism 5 software (IBM), and $p < 0.05$ was considered significant.

RESULTS

To assess the expression and distribution of IL2RB in the intestinal tissue, we performed immunohistochemistry (IHC) analysis. IHC analysis showed that IL2RB protein was highly expressed in the intestinal mucosa of RG sheep compared to NRG at 8 h after infection, especially in T cells of the epithelial layer (Fig. 1A). In addition, IL2RB expression intensity at sites with immobilized IL2RB antibodies was significantly higher (Fig. 1A and 1B) and was strongly expressed in the duodenum and jejunum. Interestingly, IL2RB is mainly expressed in the intestinal mucosa of RG (Fig. 1C), as well as increasingly expressed

from intestinal mucosa to muscle layer in the duodenum and jejunum in the NRG (Fig. 1D). This disparity suggests that the RG sheep had a stronger resistance compared to the NRG sheep during the early of infection with *E. granulosus* eggs. In the former work of our laboratory, the

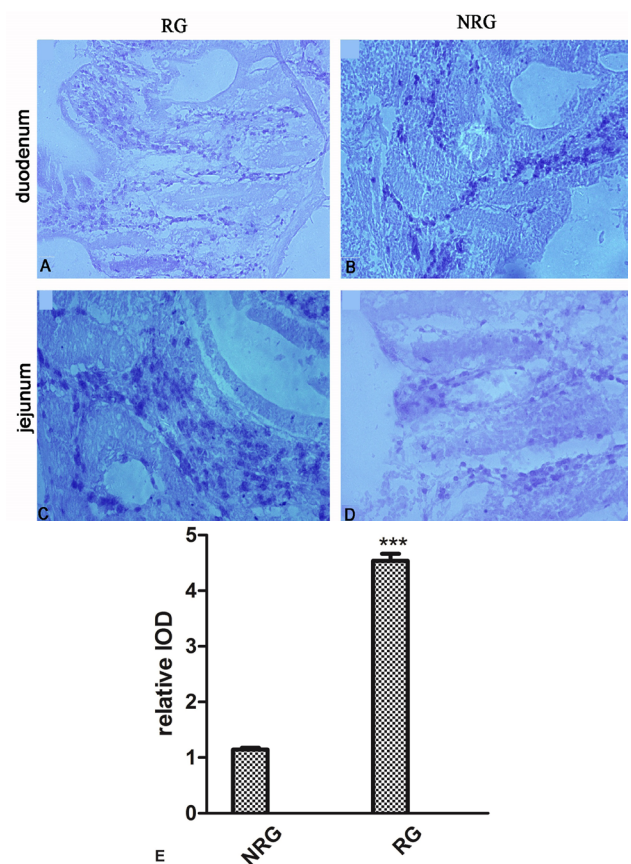


Fig. 1. The distribution of IL2RB in the small intestine of sheep. Intestinal IL2RB expression in NRG and RG sheep was determined by immunohistochemical staining. A, C: Strong IL2RB positive expression was observed in T cells of mucosal layer and muscular layer. B, D: The positive signal of IL2RB was only detected in mucosal layer, especially in the lamina propria and epithelium. E: The average IOD was obtained by analysing IL2RB IHC in four random fields of each slide. Upper panel: representative image; lower panel: quantitative analysis. Results are presented as the mean $\pm$ SEM (magnification, 200 $\times$, scale bars= 100 μ m).

IL2RB mRNA level was also upregulated (Hui *et al.*, 2015). These results may indicate some microRNAs regulate the expression level of IL2RB mRNA and protein, and then we performed microRNAs (miR-216b) and targets verification.

Compared with their targets, miRNAs usually show the opposite expression pattern. Therefore, we investigated whether miR-216b was negatively correlated with IL2RB in CE intestinal tissue. Previous study carried out high-throughput sequencing, we found that the expression of miR-216b in the intestinal mucosa of RG CE sheep was significantly lower than that of NRG sheep samples (Fig. 2A). Using miRNA *in situ* hybridization, we examined miR-216b expression in intestinal mucosa and muscular layer T cells (Fig. 2). Animal miRNAs are thought to prevent translation without affecting the level of transcriptions. Our results suggest that IL2RB expression is regulated by a typical miRNA-mediated post-transcriptional mechanism. According to the calculations in active CEs and the inverse correlations of miR-216b and IL2RB protein levels, these data indicate that IL2RB is a miR-216b target.

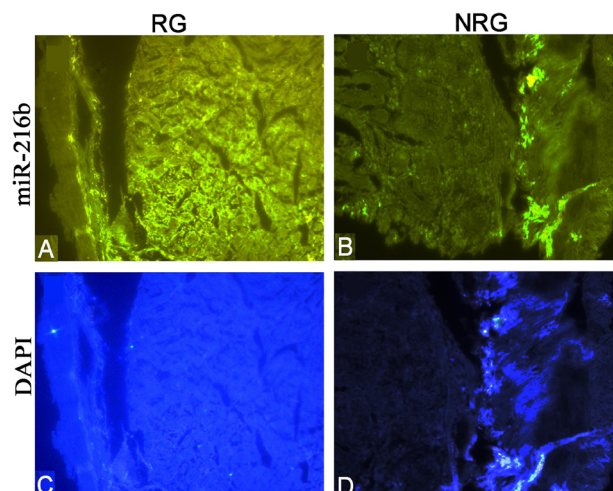


Fig. 2. miR-216b and IL2RB protein levels are inversely correlated in RG and NRG sheep intestinal tissues. miRNA *in situ* hybridization for miR-216b was performed on intestinal sections from RG and NRG controls (green, miR-216b; blue, DAPI nuclear staining). (Magnification 200 $\times$, scale bar= 200 μ m).

It is believed that miRNAs regulate the IL2RB expression. The computer-aided algorithms RNAhybrid were used to predict miRNAs that could possibly target IL2RB. Using this approach, seven miRNAs (miR-16a/b, miR-15a/b, miR-216b, miR-424, miR-497) indentified that could possibly bind to the sequences of IL2RB 3'-UTR. We

observed miR-216b-IL2RB 3'UTR hybrid; the minimum free energy (ΔG) of miR-216b-IL2RB hybridization was -21.5 kcal/mol, as shown in Figure 3A. In addition, there was a perfect base pairing between the seed region and the homologous target. Moreover, the binding sequence of miR-216b in IL2RB 3'-UTR is highly conserved among species (Fig. 3B). Thus, sheep IL2RB may be a direct miR-216b target. We examined the association between miR-216b and IL2RB by altering the level of miR-216b and examining the effect of IL2RB expression on 293T cells. We first mimic miR-216b precursors by pre-miR-216b transfection 293T cells to overexpress miR-216b, a synthetic RNA oligonucleotide duplex.

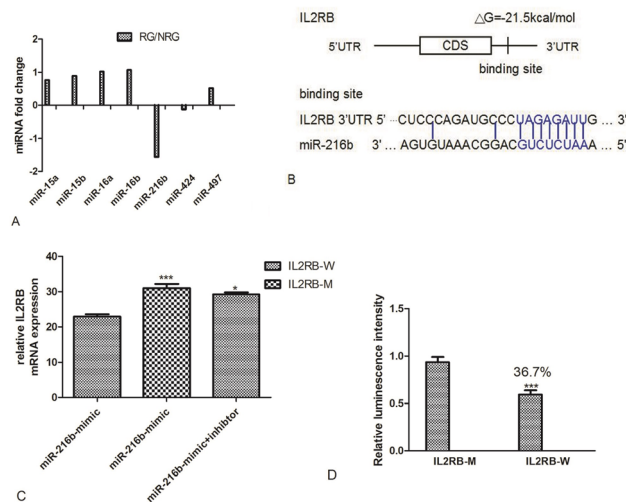


Fig. 3. The IL2RB 3'-UTR contains conserved miR-216b target sites. A: The fold-changes of seven miRNAs predicted by three algorithms in RG intestinal tissues compared to NRG (control), as analysed by high-throughput sequencing. B: Schematic illustration of conserved duplexes formed by IL2RB 3'UTR and miR-216b interactions. The predicted free energy of the hybrid is noted. Paired bases are marked by a blue line. The complementary seed sites are marked in blue. C: Quantitative real-time PCR analysis of IL2RB expression in 293T cells transfected with miR-216b mimics, miR-216b inhibitor, IL2RB-W or IL2RB-M. Data are presented as the mean $\pm$ SEM from three independent experiments (293T group: *** $p=0.000092$, IL2RB-M vs. IL2RB-W). D: Relative luminescence intensity detected by a Hamamatsu optical analyze reader after miR-216b mimics and dual-luciferase vectors were co-transfected into 293T cells (** $P<0.01$).

It was validated that they did have interaction analyzed by luciferase reporter assay. To further investigate the effects of miR-216b on 293T cells, negative control small RNAs, miR-216b mimic, miR-216b inhibitor, and in

combination with miR-216b mimic and its inhibitor were transfected into 293T cells, qRT-PCR results manifested that miR-216b were transfected efficiently into 293T cells (Fig. 3C). Each reporter construct was separately co-transfected with the miR-216b mimics into 293T cells. Compared to the mut-IL2RB-3'UTR control, the luciferase activity declined by about 36.7 % after transfection with miR-216b mimics and IL2RB-3'UTR reporter vector (Fig. 3D). The luciferase analysis showed that ectopic overexpression of miR-216b reduced IL2RB expression via directly binding to IL2RB 3'UTR, indicating that IL2RB is one target of miR-216b.

It was demonstrated that they did analyze the interaction by luciferase reporter assay. In order to further study, the effect of miR-216b on 293T cells, miR-216b mimic, miR-216b inhibitor, negative control small RNA were transfected into 293T cells, qRT-PCR results indicated that miR-216b was efficiently transfected into 293T cells (Fig. 3C). Each reporter construct was co-transfected into 293T cells with miR-216b mimics, respectively. Compared to the mut-IL2RB-3'UTR control, the luciferase activity was reduced by about 36.7% after miR-216b mimic and IL2RB-3'UTR reporter vectors were transfected (Fig. 3D). Luciferase analysis suggested that overexpression of miR-216b reduced IL2RB expression by direct binding to IL2RB 3'UTR, manifested that IL2RB is a target of miR-216b.

DISCUSSION

In the present study, we demonstrated a miR-216b binding site in the sheep IL2RB mRNA 3'UTR. We measured miR-216b and IL2RB expression levels in sheep pathological intestinal tissue and normal intestinal tissue and found an opposite correlation between the miR-216b level and IL2RB mRNA level. This correlation was also demonstrated by luciferase reporter assay, the result showed direct binding between miR-216b and the IL2RB 3'-UTR. We further confirmed that miR-216b directly inhibited IL2RB post-transcriptional expression by over-expressing or inhibition miR-216b in 293 cells. The results showed a clear opposite relationship between the cellular miR-216b level and IL2RB mRNA level in this cell lines. We also demonstrated that miR-216b was located in both intestinal mucosa and muscle layer in intestine tissue by miRNA ISH in RG, compared to the NRG, only in mucosa. Together, these results indicate that miR-216b is a negative regulator of IL2RB expression in intestinal tissue.

However, there are many problems that cannot be ignored in the prevention and clinical treatment of hydatid disease in today's world. In the field of prevention and

control of hydatid disease, there are problems such as the control of transmission source, difficult to cut off the transmission route, making the prevalence of hydatid disease is difficult to cure at the source. In the field of clinical diagnosis and treatment, the diagnosis of hydatid disease mainly rely on B ultrasound, CT and MRI, and PET-CT, imaging diagnosis there is a certain lag. And immunization kit in the early diagnosis of specificity and sensitivity is more difficult. On the other hand, the pathogenesis of hydatid disease has not been recognized, to the early diagnosis and clinical treatment of hydatidosis brought many uncertain factors. Although evidence from sheep models has proved that the parasite infection caused liver and lung damage and the intestinal immune response (Jiang *et al.*, 2016). The intestinal cell immune response (such as T or B cells) is considered an important component of the mucosal immune system. In this study, we reported a direct and important role for miR-216b in regulating IL2RB expression in the early of CE. miRNAs are related to the innate and adaptive immune system and have made significant efforts to determine the expression of miRNAs in autoimmune diseases and their role in the diagnosis and treatment of some diseases, including CE. Nevertheless, most of CE studies focused primarily on the late stages of CE infection, and the early stage of CE was not clear.

IL2RB-related miRNAs (miR-15a/b, miR-16a/b, miR-424 and miR-497) are upregulated in intestine tissues in the sheep CE infection model (Yang *et al.*, 2015). Li *et al.* (2013) reported that miR-216b negatively regulates KRAS in nasopharyngeal carcinoma, and our study demonstrated its role in intestine tissue of sheep.

Indeed, T activation contributes to the expression of IL2RB during parasite infection (Satoh *et al.*, 2002), and then promote the secretion of IL-2, suggesting that IL2RB may be a useful target for CE clinical monitoring and early mucosal response. Our results also demonstrate that IL2RB expression is upregulated in intestinal mucosa and muscle layer of CE, which is consistent with previous reports.

Furthermore, the expression of IL2RB was inhibited in 293T cells after treatment with pre-miR-216b. Together, these results suggest that miR-216b may decrease T cells activation, due to miR-216b's targeted regulation of IL2RB. In short, miR-216b downregulation and IL2RB protein upregulation may play a role in molecular signature of CE. It is worth note that miR-216b may have additional function, prevention parasite eggs invading animal.

The results of miRNA from in situ hybridization shows that miR-216b is expressed in both IECs and T cells in intestinal tissue; thus, both miR-216b-IL2RB signals may be important for CE development. In our

results indicated that miR-216b as a suppressor of T cells activation in active CE, the association between miR-216b and T cells requires further investigation.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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