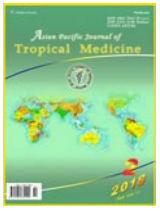

IF: 0.925

Asian Pacific Journal of Tropical Medicine

journal homepage: www.apjtm.org

doi: 10.4103/1995-7645.225021

Effect of RNA interference on WD101 gene of *Schistosoma japonicum*

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ARTICLE INFO

Article history:
Received 28 October 2017
Revision 20 November 2017
Accepted 23 December 2017
Available online 2 February 2018

Keywords:
Schistosoma japonicum
RNA interference
WD101

ABSTRACT

Objective: To study the effect of RNA interference (RNAi) on WD101 gene and its effect on the expression of WD101 mRNA and protein in *Schistosoma japonicum*. **Methods:** Double-stranded RNA (dsRNA) WD101 gene and control gene (lacZ) were generated by *in vitro* transcription and transfected into mechanically transformed schistosomula. The total RNA and protein were isolated simultaneously using TRIzol reagent. The expression levels of mRNA and the protein were determined by quantitative real-time PCR (qPCR) and Western blotting, respectively. After injected dsRNA-electroporated schistosomula into BALB/c mouse six weeks, the male and female reproductive organs were observed and measured under the confocal laser scanning microscope. **Results:** After 1, 3 and 5 d of RNAi, *WD101* mRNA level was decreased by 15%, 39%, and 58% in experiment group compared to that in control group; meanwhile, WD101 protein level was decreased by 11%, 28%, and 43% in experiment group compared to that in control group. There were significantly more sperms in testicular lobes in experiment group than that in control group, while there were no significant differences in terms of ovary and vitelline glands between two groups. **Conclusions:** The dsWD101-RNAi can effectively induce suppression of WD101 gene expression at both mRNA and protein levels. WD101 gene might be a reproduction-related gene in *Schistosoma japonicum*.

1. Introduction

Schistosomiasis is a public health problem throughout the world[1,2]. The disease afflicts approximately 240 million individuals globally, causing the loss of approximately 70 million disability-adjusted life years[3]. In China, schistosomiasis is caused by the continental strain of *Schistosoma japonicum* (*S. japonicum*). Despite the tremendous decrease of schistosomiasis incidence after almost 60 years of control, the schistosomiasis has been still increasing recently along the middle and downward of Yangtz River due to the fast changes of the biology, nature, society and economics. The continuous surveillance with a focus on schistosomiasis should be enhanced in potential risk areas in China[4,5]. The life cycle of schistosomes is complex. In intermediate host or mammalian definitive host, they become adult worms that are reproductive active for years. The eggs are the agent of schistosomiasis transmission and lead to granulomatous reaction. In order to prevent schistosomiasis, controlling sexual maturation, sexual dimorphism and labour division may be effective means.

The protein encoded by WD101 gene in *S. japonicum* has 6 WD-repeats function domain and 6 WD40 structure domain. The protein that has specific spatial structure HMM model can lead the interactions among the proteins[6,7]. Previous studies in laboratories have shown the significance role of WD protein family in adjusting cell information transduction, transcription, processing precursor mRNA[8], but the function of WD101 gene in reproductive system has not been verified in *S. japonicum*.

In this study, we aimed to identify the effect of RNA interference (RNAi) on WD101 gene and its effect on the expression of WD101 mRNA and protein in *S. japonicum*.

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Foundation project: This research was supported by Natural Science Foundation of Anhui Education Department (KJ2013B318).

How to cite this article: Peng Zhang, Gang Feng, Wei-Na Zhang, Ying-Ying Zhang, Chun-Sheng Liu. Effect of RNA interference on WD101 gene of *Schistosoma japonicum*. Asian Pac J Trop Med 2018; 11(2): 136-140.

2. Material and methods

2.1. Preparation of double-stranded RNA (dsRNA)

The WD101 gene template was generated by PCR from *S. japonicum* using gene-targeted primers (Table 1). The negative control group, pcDNA™1.2/V5-GW/lacZ plasmid was provided by the BLOCK-iT™ RNAi TOPO® Transcription Kit. The control group gene was also amplified by PCR using gene-targeted primers (Table 1). Then both of them were linked with T7 promoter. Then the genes that were linked with T7 promoter were amplified separately by PCR using each primer (WD101 or LacZ) and T7 promoter primer (Table 1). Each single-stranded RNA (ssRNA) was received through transcription of PCR production, and then ssRNA were purified and mingled correspondently. Finally ssRNA transformed into dsRNA. In the process of preparing dsRNA, the BLOCK-iT™ RNAi TOPO® Transcription Kit (Invitrogen, Carlsbad, CA) was used according to the manufacturer's instructions.

Table 1

The primers for WD101 and control gene, T7 promoter.

Primers	Sequence
WD101 forward primers	AAAGGATCCATGCCTGGGATTAATTGTCTCGATATC
WD101 reverse primers	TATCTCGAGTGTACCAAATACTTTAACAGAACGATC
LacZ forward primer	ACCAGAAGCGGTGCCGGAAA
LacZ reverse primer	CCACAGCGGATGGTTCGGAT
BLOCK-iT™ T7Primer	GATGACTCGTAATACGACTCACTA

2.2. Parasite preparation, eletroporation and culture in vivo/vitro

The continental strain of *S. japonicum* maintained in the Institution of Jiangxi Parasitic Disease Prevention was used for this study. After exposing two hundreds infect *Oncomelania* to light for 3 h (22-26 ℃), cercariae were collected in a centrifuge tube and placed on ice for 10 min. Next, the cercariae were collected by centrifugation at 1 500 rpm for 10 min and washed twice with RPMI 1640 (Invitrogen) containing 200 U/mL penicillin G sulfate, 200 μg/mL streptomycin sulfate and 500 ng/mL amphotericin B (Sangon). The cercarial tails were sheared off by passing the suspension through a 20-gauge needle 30 times. Schistosomula were isolated from the free tails by swirling and pipetting. All worms were incubated in 10 mL of the medium-841 containing 10.4 g/L RPMI 1640, 10% (v/v) rabbit serum (Invitrogen), 0.1% (w/v) lactalbumin hydrolysate (Hyclone), 10^{-6} M hydrocortisone (Sigma), 10^{-6} M 5-hydroxytryptamine (Sigma), 0.5×10^{-6} M hypoxanthine (Sigma), 0.2U/mL bovine insulin (Sigma), additionally with 100 U/mL Penicillin G sulfate, 100 μg/mL streptomycin sulfate, 250 ng/mL amphotericin B and 2.0 g sodium bicarbonate in an atmosphere of 37 ℃, 5% CO_2 for 3 h to prepare for electroporation. For electroporation, parasites were washed twice and resuspended at 20 000/mL in wash medium containing dsRNA of WD101 and LacZ (40 μg). The suspension of this type were transferred into 4 mm gap cuvettes (BTX) and incubated on ice for 10 min, and electroporated at 120 V for 20 ms square-wave pulse using an Electro Square Porator™ ECM830 (BTX), respectively. After electroporation, all parasites were divided into two groups. One group were transferred to pre-warmed 841 medium in 12 well plates, cultured as described above, and supplied with fresh medium every 2 d. Aliquots of parasites were harvested on day 1, 3, 5 after electroporation, respectively (1 500 parasites/well). Another group were injected into the rear thigh muscles of Balb/c mice (two injection sites and 500 parasites/site).

2.3. Isolation of nucleic acid and protein for analysis

Before collected, schistosomula were washed twice with PBS. Total RNA, DNA and protein were isolated using TRIzol reagent (Invitrogen, USA) according to the manufacturer's guidelines.

2.4. Quantitative real-time PCR analysis

The cDNAs were synthesized using a reverse transcription kit (Promega, USA) according to the manufacturer's protocols. Then the cDNAs were experimented for qPCR on a ABI Prism 7500 Sequence Detector (Applied Biosystem, USA). The probes and primers designed by our group using Primer Premier 5.0 software (Table 2) and synthesized by Shanghai Sangon Company (China). A final volume of 50 μL containing Premix Ex Taq™ 2× (Takara), 10 μM forward primer, 10 μM reverse primer, ROC×Reference Dye Ⅱ 50× (Takara), 2 μL probe, 15 μL sterile water and 5 μL template DNA (cDNA diluted 1:5). Each sample was performed in triplicate. As the following was the cycling conditions: 95 ℃, 45 s; 58 ℃, 45 s; 72 ℃, 45 s for 40 cycles. According to the manufacture's protocol, GAPDH which served as standard curves indicating the template quantity and Ct value was obtained. R^2>0.99 was determined using linear equations. The qPCR results were analyzed with ABI Prism 7500 SDS software based on a relative standard curve. The experiment was repeated three times.

Table 2

The primers and TaqMan probes for real-time qPCR.

Gene	Primers and probes	Sequence
WD101	Forward primer	TGTCTCCTGAGAGAATTGTT
	Reverse primer	GCTACAGCTGTGACTGGTTG
	Probe	TTCACCGGTTCTCCCGACACATCTCTAAGG
GAPDH	Forward primer	CCTTGATTTGATCGTAGGTAGCT
	Reverse primer	CATCCCTGCATTAAACGGAA
	Probe	CTGAAACATTCGCTGTAGGCACCCG

2.5. Western blotting analysis

Total protein from WD101 dsRNA-containing schistosomula were quantfied with Lowry method. The samples heated at 100 ℃ for 10 min after mixing the equal volume of 2×SDS loading buffer.

Then the protein were separated with 12% SDS-PAGE (15 μg/well), transferred to a nitrocellulose membrane. Then the membrane containing WD101 protein was incubated with the anti-SjWD101 antibody diluted 1:2000 for 1 h at room temperature with gentle shaking, while the membrane containing an endogenous control protein β-actin was incubated with rabbit anti-actin antibody (Bioss, China) diluted 1:2000 for 1 h at room temperature with gentle shaking. After washed with PBST, the membranes were incubated in HRP-conjugated goat anti-mouse and anti-rabbit IgG (Zhongshan, China) in PBS buffer at a 1:100000 dilution for 1 h at room temperature with gentle shaking separately. After washed with PBST, the protein-antibody complexes were visualized using chemiluminescence (SuperSignal® West Femto Maximum Sensitivity Substrate, Pierce). The results were analyzed using densitometry (Jieda image analysis system). The experiment was repeated three times.

2.6. Observation of parasite morphology change

Adult parasites of schistosome were obtained from the portal vasculature of the mice. Ten male and nine female parasites were obtained from test group, while eighteen male and sixteen female parasites from control group. All these parasites were fixed in FAA (10% formaldehyde, 2% acetic acid, 48% alcohol), stained with acetic acid carmine at 37 ℃ for 16 h, dehydrated in a series of alcohols (70%, 90%, 100%), clarified with methyl salicylate mixed with an equal amount of alcohol for 0.5 h and clarified again with methyl salicylate for 0.5 h, and whole-mounted on glass slides with neutral gum. Then observed the morphology of the parasites by confocal laser scanning microscopy (Leica).

2.7. Statistic analysis

All data were analyzed using statistical software SPSS 21.0. Independent sample *t*-tests were used to determine differences of WD101 mRNA and protein between different groups in both *in vivo* and *in vitro* experiments at various times of post-dsRNA treatment. Differences were considered significant at a level of $P < 0.05$.

3. Results

3.1. Reduction of WD101 mRNA levels after dsRNA interference

After 1, 3 and 5 d of RNAi, WD101 mRNA level was decreased by 15%, 39% and 58% respectively, compared to that in control group (Table 3). The mRNA levels of endogenous GAPDH gene in two groups showed no significant differences ($P>0.05$).

Table 3

The percentage of WD101 mRNA level compared with control (LacZ) at day 1, 3, and 5 after RNAi (%).

Gene	Day 1	Day 3	Day 5
WD101 mRNA	85	61	42
GAPDH mRNA	100	100	100

3.2. WD101 protein expression levels after dsRNA interference

After 1, 3 and 5 d of RNAi, WD101 protein level was decreased by 11%, 28% and 43% respectively compared to that in control group (Table 4). The protein levels of endogenous β-actin gene in two groups showed no differences($P>0.05$).

Table 4

The percentage of WD101 protein level compared with control (LacZ) at day 1, 3, and 5 after RNAi (%).

Gene	Day 1	Day 3	Day 5
WD101 protein	89	72	57
β-actin protein	100	100	100

3.3. Morphological change after dsRNA interference

The characteristics variation statistics of worms in two groups are summarized in Table 5. There were more sperms in testicular lobes in experiment group than that in control group, while there were no differences in ovary and vitelline glands between two groups (Figure 1).

Table 5

The characteristic variation statistics of worms in two groups.

Group	Male (n=28)						Female (n=25)					
	n	Body length (μm)	Body width (μm)	Testicular lobes number	Length (μm)	Width (μm)	Area (μm^2)	n	Body length (μm)	Body width (μm)	Ovary (μm)	Length (μm)	Width (μm)
Experiment	10	8056±587	236±28	6±1	95±19	72±16	5758±1221	9	12256±1352	142±19	439±69	136±18	55627±13265
Control	18	8516±869	402±55	7±1	559±48	163±25	80079±10256	16	12750±836	206±29	546±41	186±38	83526±15329
P		>0.05	<0.05	>0.05	<0.05	<0.05	<0.05		>0.05	<0.05	<0.05	<0.05	<0.05

$P < 0.05$ stands for significant difference; $P > 0.05$ stands for no significant difference.

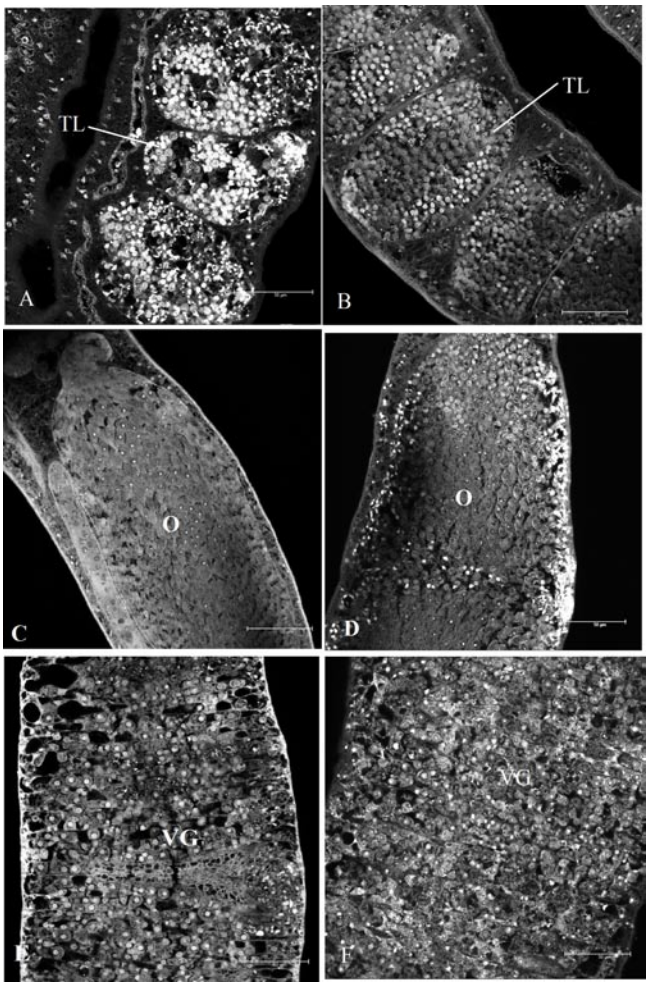

Figure 1. Confocal scanning laser microscope images of *S. Japonicum* isolated from experiment group and control group (bar: 50 μm).
The images of testicular lobe in experiment group (A) and control group (B). The images of ovary in experiment group (C) and control group (D). The images of vitelline gland in experiment group (E) and control group (F).

4. Discussion

The eggs produced by the female worms are the main cause of the granulomatous, as it can lead to pathology damage and spread diseases[9]. Thus the molecular level research on the development of the female worms could provide a new means in prevention and treatment of schistosomiasis. Previous study shown that there are high homologous among WD101 gene and GTP binding-protein gene, mRNA precursor processing factor gene of other species[10]. The WD101 protein has an important role in cell development, movement and information interaction. But in process of reproductive system development, the role of WD101 gene has not been verified.

dsRNA-WD101 which was synthesized by chemical method was electroporated into the body of *S. japonicum* to inhibit gene specifically[11-14]. In this experiment, the results showed there were significant changes in gene, protein and morphology in WD101 dsRNA interference group. In order to improve the purity, re-combined plasmids of WD101 and GAPDH as standard curves were used. If the coefficient correlation is above 0.99, it demonstrates the quantitation of standard curves is accuracy and the result is high-reliablility. The results showed that WD101 mRNA levels were decreased in the dsRNA-WD101 transfected group. It indicated that dsRNA of WD101 could silence the expression of WD101 gene transcription specifically. Correspondingly, accompanied by the decreases in its mRNA levels, WD101 protein expression levels were also decreased in the dsRNA-WD101 transfected group. It indicated that dsRNA-WD101 could reduce the protein expression of WD101 gene specifically. After 6 weeks, the difference in morphology of the *S. japonicum* between test and control groups were observed, the length, width of worms and the length, width, area of testicular lobes and the length, width, area of ovary were tested. The results showed there were more specimen in the dsRNA-WD101 group than that in control group, while there were no significant differences in ovary and vitelline gland between two groups. The width of worms and the length, width, area of testicular lobes and ovary have significant differences between two groups. From the research, it is concluded the WD101 gene had tight correlation to reproductive development.

RNAi could been used widely in many fields because of the degradation of homologous mRNA triggered by RNAi[15,16]. In schistosoma, Zhao used short hairpin RNA (shRNA) to inhibit the Mago nashi gene expression[17], Ren and Zhang used dsRNA to inhibit the Tsunagi gene expression[18]. Tran and colleagues soaked 3-hour-old larval parasites in 1 mg/mL of dsRNA against tetraspanin 1 or 2 (Sm-tsp-1 or Sm-tsp-2) and cultured *in vitro* at 37 ℃ for 7, 14, and 21 d, with fresh changes of media, blood, and dsRNAs every second day. As a result, transcript levels decreased to less than 33% compared to control and schistosomula displayed a significantly thinner and more vacuolated tegument[19]. Compared these methods, shRNA need vector to transducted into cell and take advantage of Enzyme digestion mechanism to obtain its function, the process was complicated and has many influencing factors, while dsRNA which was synthesized *in vitro* and could silence target gene through many ways directly.

The study of *Schistosoma* species and schistosomiasis has undergone a dramatic change in recent years[20]. RNAi has significantly helped the advancement in the study of non-model species. Since RNAi is a process which apparently depends on total complementarity between siRNA and target RNA, more specific drugs may arise from this technology.

In this research, dsRNA was applied to interfere the target gene and the results of this experiment was satisfactory. Our study has several possible limitations. We only tested RNAi for a short time because of the culture condition limitation and the few samples of worms,

a part of schistosomula died after electroporation, thus the results could not reflect the total real status, the transfection method need improvement in the future. Considering the large amount of genomic data that might be available in the future, it is clear that there are a number of important biological questions that could be addressed using this type of gene silencing technology.

In conclusion, the dsWD101-RNAi can induce effective suppression of WD101 gene expression at both mRNA and protein levels. WD101 gene might be a reproduction-related gene in *Schistosoma japonicum*.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Acknowledgements

This research was supported by Natural Science Foundation of Anhui Education Department (KJ2013B318).

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