Correlation between hammerhead ribozyme-mediated eggshell protein gene cleavage and reproduction inhibition of *Schistosoma japonicum*

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Received October 25, 2011; Accepted December 27, 2011

DOI: 10.3892/mmr.2012.752

Abstract. *Schistosoma japonicum* (*S. japonicum*) is an extremely harmful pathogen, which infects humans and causes severe public health problems. To date, no effective therapeutic drugs for this pathogen are available. In this study, we designed and constructed three hammerhead ribozymes targeting the eggshell protein gene of *S. japonicum* (S*j*ESG). The cleavage activities of these three ribozymes were determined using cleavage experiments. The *in vitro* cleavage results showed that among the three synthesized ribozymes (Rz1, Rz2 and Rz3), Rz1 and Rz3 cleaved their target RNAs effectively. However, Rz2 did not cleave its target RNA detectably. The putative therapeutic roles of these three ribozymes to inhibit the reproduction of *S. japonicum* in mice were studied *in vivo*. Compared with the negative controls, Rz1 and Rz3 treatments resulted in increased levels of IFN-γ but decreased levels of IL-4 in mice. Rz2 affected levels of IFN-γ and IL-4 to degrees similar with those caused by the vector controls. In addition, Rz1 and Rz3 reduced the amounts of adult worms and eggs in the livers of mice more extensively than Rz2 and the vector controls. Altogether, these results suggest a correlation between the *in vitro* cleavage abilities of Rz1 and Rz3 and their roles in reproduction inhibition of *S. japonicum*.

Introduction

Schistosoma japonicum (*S. japonicum*) is an extremely harmful pathogen, which infects humans and causes severe public health problems. To date, effective therapeutic drugs for this pathogen are lacking. Scientists are diligently searching for anti-infectious vaccines against *S. japonicum;* however, all of the vaccine candidates reported are not effective enough to protect individuals from the infection of *S. japonicum*. Therefore, effective methods for curing *S. japonicum*-related diseases are needed.

S. japonicum causes disease mainly through its egg granuloma in hosts, which is followed by host hepatic and intestinal fibrosis (1). Therefore, a suitable strategy may be to target the maturity of eggs and reproduction of *S. japonicum*. The eggshell protein gene (S*j*ESG) of *S. japonicum* encodes the major component of its egg yolk (2). It is known that this gene is required for the maturity and egg deposition of female *S. japonicum* (3). Sequence analyses show that over 85% of the S*j*ESG sequences are conserved among members in the family (4).

Ribozymes, a class of RNA molecules with endonuclease activities, were found to be able to bind to their targeting mRNAs and digest them specifically (5). Ribozymes bind to target RNAs containing the NUH sequence (N stands for any base; H stands for C, U or A) via specific cleavage sites downstream of the NUH triplet. The ribozyme-mediated mRNA cleavage results in inhibition or blockage of expression of the target genes (6,7).

Hammerhead ribozymes have been found to control many types of diseases (8,9). In this study, we designed and synthesized 3 ribozymes targeting the S*j*ESG gene and evaluated their cleavage abilities *in vitro* and *in vivo*. We found that the *in vitro* cleavage abilities of two of these 3 designed ribozymes were correlated with their abilities to inhibit the maturity of eggs and reproduction of *S. japonicum* in mice.

Materials and methods

DNA preparation. Snails infected with *S. japonicum* cercarie were purchased from the Hunan Institute of Schistosomiasis Prevention and Treatment. Genomic DNA was isolated from the collected worms (*S. japonicum*) according to the methods described previously (10). The designed ribozyme DNA oligonucleotides were synthesized by Invitrogen and dissolved in TE buffer (pH 8.0). The oligonucleotides (A chain and

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Key words: Schistosoma japonicum, eggshell protein gene, hammerhead ribozyme, *in vitro* cleavage, anti-reproduction

Table I. Oligonucleotides annealed for making Rz1, Rz2 and Rz3.

Rzl	A chain: 5'AGCTTCACACCCTGATGAGTCCGTGAGGACGAAACCTCCG3'
	B chain: 5'GATCCGGAGGTTTCGTCCTCACGGACTCATCAGGGTGTGA3'
Rz2	A chain: 5'AGCTTCACCATCTGATGAGTCCGTGAGGACGAAACCGCG3'
	B chain: 5'GATCCGCGGTTTCGTCCTCACGGACTCATCAGATGGTCA3'
Rz3	A chain: 5'AGCTTTCACCATCTGATGAGTCCGTGAGGACGAAACCACCG3'
	B chain: 5'GATCCAGTGGTTTCGTCCTCACGGACTCATCAGATGGTGAA3'

The conserved sequences of the ribozymes are underlined.

B chain) (Table I) for each ribozyme were mixed equivalently and annealed by denaturation for 5 min at 95˚C, followed by slow cooling to room temperature.

Plasmid constructs. The S*j*ESG gene fragment was amplified from the genomic DNA of *S. japonicum* by PCR, using primers (5'-CCAAGCTTATGTACCCACCATCATCC-3' and 5'-CGGGATCCTCAATAATAGGAGGGTGCA-3'). For constructing the plasmid pcDNA3.1(+)/S*j*ESG, the S*j*ESG PCR products were cloned into the *Hin*dIII and *Bam*HI sites of the vector pcDNA3.1(+), which were purchased from Promega. The annealed ribozyme dsDNAs (Rz1, Rz2, Rz3) were cloned into the sites of *Hin*dIII and *Bam*HI of pcDNA3.1(+), resulting in plasmids pcDNA3.1(+)/Rz1, pcDNA3.1(+)/Rz2 and pcDNA3.1(+)/Rz3. The ligation products were transformed into *E. coli* JM109, and positive colonies were selected.

In vitro transcription. The plasmids pcDNA3.1(+)/S*j*ESG, pcDNA3.1(+)/Rz1, pcDNA3.1(+)/Rz2 and pcDNA3.1(+)/Rz3 were linearized with *Bam*HI and purified with a gel purification kit (catalogue no. DV805A; Takara Co.). The *in vitro* transcription was performed by using a transcription kit (catalogue no. L1170; Promega), according to the manufacturer's instructions. Briefly, DNA constructs were linearized and used as templates in a 20-μl *in vitro* transcription reaction containing 4 μl of 5X buffer, 2 μl of DTT, 1 μl of RNase inhibitor, with or without 2 μl of digoxin labeling mix, 1 μl of the DNA template, 1 μl of T7 RNA polymerase, and H_2O. The reactions were performed at 37˚C for 2 h, followed by addition of stop buffer. The RNAs labeled with or without digoxin were purified and used for ribozyme cleavage experiments.

RNA blotting. RNAs were separated on formaldehyde denaturation agarose gels, transferred to a nylon membrane by a transblotting system, and then detected by a DIG nucleic acid detection kit (catalogue no. 11175025910; Roche).

RNA cleavage by ribozymes. The 3 ribozymes (Rz1, Rz2 and Rz3) were respectively mixed with the substrate (S*j*ESG mRNA, labeled with a digoxin marker) in a ratio of 1:1 in a 10-μl reaction system, containing 50 mM of Tris-HCl (pH 7.5), 20 mM of $MgCl_2$, 20 mM of NaCl and 1 μl of RNasin (11). The mixture was incubated at 95˚C for 1 min, rapidly transferred to ice, incubated at 37˚C for 2 h, and followed by addition of the stop buffer (formamide 960 mM, EDTA 20 mM). The cleaved products were detected using RNA blotting with a digoxin marker. The bands were scanned and analyzed with the software AlphaImager 2200 (Beckman). Cleavage efficiency was calculated with the following formula: CE = [P/(S + P)] x 100%; S, substrate; P, digested product; CE, cleavage efficiency.

Animal experiments. Forty age-matched (4-6 weeks of age) BALB/c female mice were infected with *S. japonicum* cercarie via the vena caudalis. The infected mice were randomly divided into 5 groups and injected i.v. with PBS, vector, or the ribozyme constructs (Rz1, Rz2 or Rz3) as previously described (12). The constructs were diluted to 0.25 μg/μl with PBS to construct a plasmid DNA solution for the mouse treatments. Each mouse was injected with 200 μl of plasmid DNA solution containing DNA 50 μg or PBS at the schedule of 14, 21 and 28 days post-infection with *S. japonicum*.

Measurement of IFN-γ and IL-4 levels in the serum of the treated mice. Serum samples were obtained from the mice by cutting the vena caudalis prior to treatment, 2 days after the first treatment and 2 weeks after the third treatment, respectively. IFN-γ and IL-4 levels in the serum were measured by ELISA and analyzed using SPSS software as described (13).

Measurement of quantities of worms and eggs in the treated mice. The mice were sacrificed by extracting the eyeballs 45 days after the third treatment of ribozymes. Adult worms were collected by flushing the portal of vein. Livers were extracted and incubated in 5% of KOH solution for 20 h at 37˚C, and then the worms and eggs in these tissues were counted under a microscope.

Results

Design of ribozymes targeting the SjESG mRNA. Computer software (14) was used to analyze the computer-predicted secondary structure of S*j*ESG mRNA. Six potential hammerhead ribozyme sites were found in the mRNA (Table II). After further analyses, the 76th, 283rd and 160th sites were chosen for ribozyme cleavage, since they have a lower ΔEr than the sequences on other sites (Table II). In addition, the sequences on these 3 sites (76th, 283rd and 160th) and the sequences around them were conserved among all members of the S*j*ESG gene family (GenBank nos. M32280, M32281, M59318, DQ225185 and AB017096).

The ribozymes targeting the 76, 283 and 160 sites were named Rz1, Rz2 and Rz3, respectively. The sequence of the

Table II. Parameters of the hammerhead ribozymes.

Rz	5'ΔE (kcal/mol)	3'ΔE (kcal/mol)	ΔEs (kcal/mol)	ΔEr (kcal/mol)
Rzl (76th site)	-11.9	-10.7	1.2	4.2
Rz2 (283rd site)	-10.4	-9.5	2.7	2.6
Rz3 (160th site)	-11.8	-11.8	0.0	1.6
Rz4 (35th site)	-11.8	-11.6	0.0	5.5
Rz5 (67th site)	-11.9	-11.0	1.2	4.2
Rz6 (109th site)	-11.9	-12.4	0.0	4.2

5'ΔE, the required energy when the 5' side sequence of ribozyme binds to the substrate; 3'ΔE, the required energy when the 3' side sequence of the ribozyme binds to the substrate; ΔEs, the required energy when the substrate transforms from the most steady state to the loosened state; ΔEr, the required energy when the ribozyme transforms from the most steady state to the loosened state.

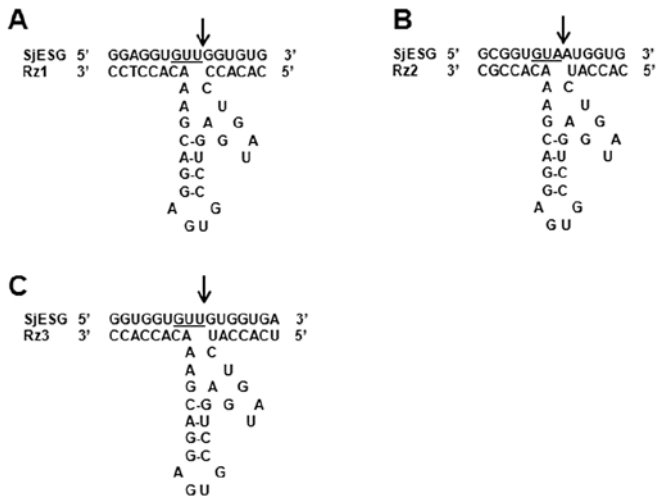

Figure 1. Ribozymes and their targets on the S*j*ESG gene of *S. japonicum*. (A) Rz1 and its target sequence on the S*j*ESG gene. (B) Rz2 and its target sequence on the S*j*ESG gene. (C) Rz3 and its target sequence on the S*j*ESG gene.

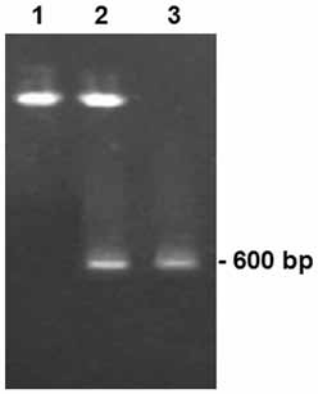

Figure 2. Constructs digested with *Hin*dIII and *Bam*HI. Lane 1, pcDNA3.1(+) digested by *Hin*dIII and *Bam*HI; lane 2, pcDNA3.1(+)/S*j*ESG digested by *Hin*dIII and *Bam*HI; lane 3, PCR products of S*j*ESG from pcDNA3.1(+)/S*j*ESG.

catalytic center was designed according to the hammerhead structure model introduced by Symons *et al* (15), and the sequences on both sides of the ribozyme were complementary to corresponding substrate (16). Restricted enzyme sequences were added to the 5' side and 3' side for easy cloning. The

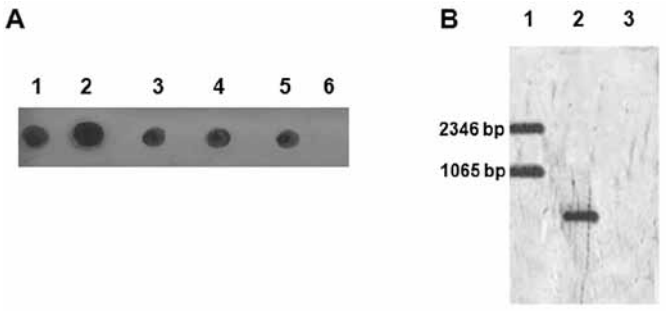

Figure 3. RNA blots of digoxin-labeled RNAs synthesized in the *in vitro* transcription. (A) Lane 1, S*j*ESG-mRNA (1 μl); lane 2, S*j*ESG-mRNA (2 μl); lane 3, Rz1 (1 μl); lane 4, Rz2 (1 μl); lane 5, Rz3 (1 μl); lane 6, H_2O (1 μl). (B) Lane 1, marker; lane 2, S*j*ESG RNAs; lane 3, vector.

sequences of Rz1, Rz2 and Rz3 targeting the 76, 283 and 160-bp sites, respectively, are shown in Fig. 1.

Confirmation of the plasmid construction. To confirm whether the plasmid constructs were cloned correctly, the pcDNA3.1(+)/S*j*ESG plasmid was digested with *Hin*dIII and *Bam*HI. The digested products were separated on agarose gels along with the PCR product of the S*j*ESG gene. As shown in Fig. 2, a DNA fragment ~600 bp was dropped off from the pcDNA3.1(+)/S*j*ESG, but not from the vector plasmid. The dropped fragments had a similar size with the PCR product amplified from the S*j*ESG gene. In addition, the 3 ribozyme constructs [pcDNA3.1(+)/Rz1, pcDNA3.1(+)/Rz2 and pcDNA3.1(+)/Rz3] were confirmed by DNA sequencing. Therefore, these results suggest that the constructs were constructed correctly.

In vitro transcription of SjESG mRNA and ribozyme RNAs. To obtain the S*j*ESG mRNAs and the 3 ribozyme RNAs, *in vitro* transcription was performed using the enzyme-linearized pcDNA3.1(+)/S*j*ESG, pcDNA3.1(+)/Rz1, pcDNA3.1(+)/Rz2, or pcDNA3.1(+)/Rz3, respectively, as templates. After *in vitro* transcription reactions were completed, the DNA templates in the reaction system were digested using DNase, and the synthesized S*j*ESG mRNA and the ribozyme RNA were gel purified. The digoxin-labeled S*j*ESG mRNA and ribozyme RNAs were confirmed using RNA blotting (Fig. 3A). The synthesized S*j*ESG mRNA products, which were labeled with a digoxin marker, were also confirmed by the RNA blotting

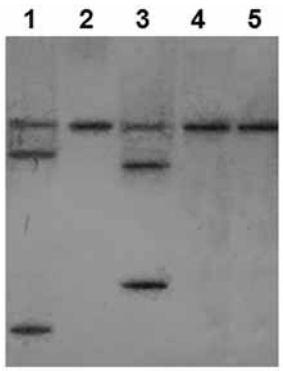

Figure 4. *In vitro* cleavage of S*j*ESG mRNA mediated by ribozymes. Lane 1, Rz1; lane 2, Rz2; lane 3, Rz3; lane 4, cleavage buffer; lane 5, H_2O.

as shown in Fig. 3B. The S*j*ESG mRNA and ribozyme RNAs labeled with or without digoxin were prepared and used for the following *in vitro* cleavage experiments.

The ribozymes efficiently cleave SjESG mRNA. To determine whether the 3 ribozymes (Rz1, Rz2 and Rz3) were able to cleave the S*j*ESG mRNA, *in vitro* cleavage experiments using digoxin-labeled S*j*ESG mRNA and unlabeled ribozyme RNAs were performed. The cleaved, digoxin-labeled S*j*ESG mRNA products were detected using RNA blotting (the unlabeled ribozymes were undetectable on the blots). As shown in Fig. 4, Rz1 cleaved the S*j*ESG mRNA into two smaller fragments: 561 and 76 bp. Rz3 cleaved the S*j*ESG mRNA into 477- and 160-bp fragments. However, no Rz2-mediated cleavage products were detectable. Densitometry analyses indicated that Rz1 and Rz3 cleaved the S*j*ESG mRNAs with an efficiency of 68.9 and 69.6%, respectively.

IFN-γ and IL-4 levels in the serum of mice. Since the *in vitro* ribozyme cleavage results found that two of the 3 designed ribozymes cleaved S*j*ESG mRNA efficiently, these ribozymes were investigated *in vivo*. Mice infected with *S. japonicum* received injections of Rz1, Rz2 or Rz3 in PBS at several time points. The serum samples were collected from mice to measure the levels of two important cytokines, IFN-γ and IL4, using ELISA. As shown in Fig. 5A, after the 1st treatment, the levels of IFN-γ in all 5 groups (treated with PBS, vector, Rz1, Rz2, or Rz3) increased very slightly. After the 3rd treatment, IFN-γ levels in the 3 groups (treated with PBS, vector, and Rz2) still increased slightly, while IFN-γ levels in the groups treated with Rz1 and Rz3 increased by up to 4-fold, when compared to the levels in the groups treated with PBS and vector. Notably, when compared with the highly up-regulated IL-4 levels (Fig. 5B) in the groups treated with PBS, vector, and Rz2, the IL-4 levels in the groups treated with Rz1 and Rz3 increased much less. These results suggest that Rz1 and Rz3 treatments induce similar effects on IFN-γ and IL-4 levels in mice, which is different from the groups treated with PBS, vector, or Rz2.

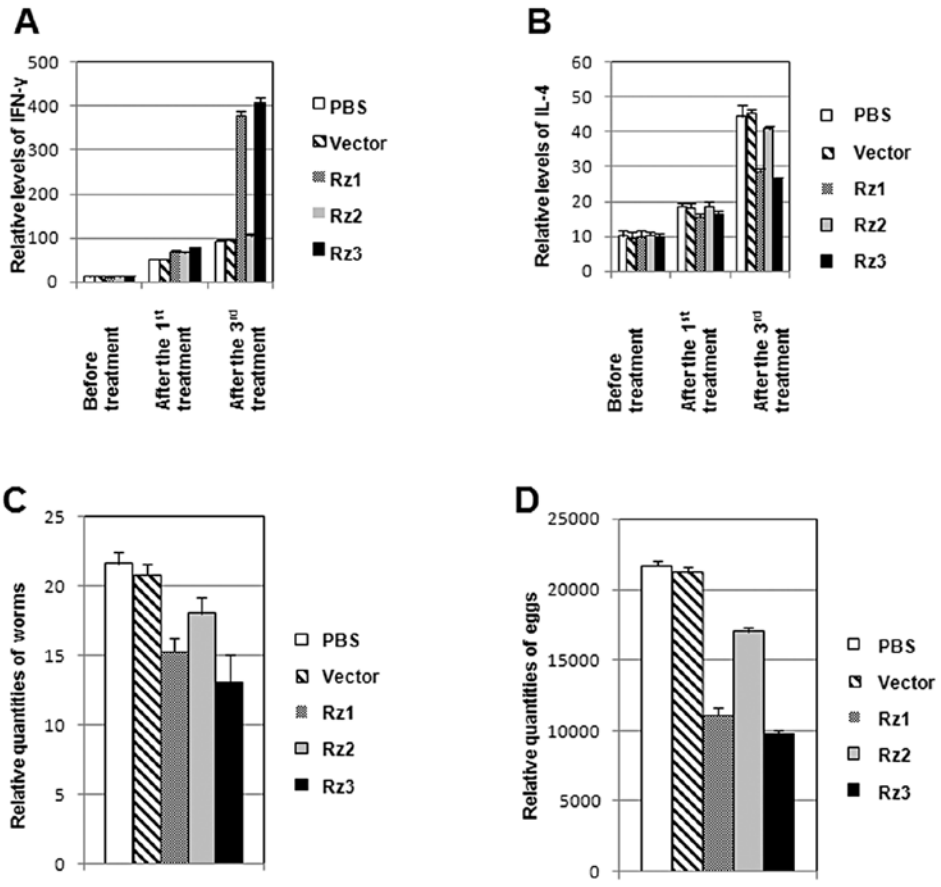

Figure 5. Effects of the ribozymes on mice infected with *S. japonicum*. (A) Levels of IFN-γ. (B) Levels of IL-4. (C) Relative quantities of worms. (D) Relative quantities of eggs.

Anti-reproduction contribution of the ribozymes. In order to investigate whether the ribozyme treatments affect the amount of worms and eggs, the infected mice in the animal experiments (Fig. 5A and B) were sacrificed 45 days after the 3rd treatment of ribozymes. Adult worms were collected by flushing portal of vein and the amounts of the worm eggs in the mouse livers were counted under a microscope. As shown in Fig. 5C, Rz1 and Rz3 caused more marked decreases in the amounts of adult worms when compared with the groups treated with PBS, vector, or Rz2. Similarly, Rz1 and Rz3 also decreased the amounts of eggs more effectively than Rz2 and the vector controls (Fig. 5D). Rz3 was more effective than Rz1, resulting in a 39.82% reduction in amounts of worms and a 54.95% decrease in the egg reduction rate in the liver. Altogether, these results suggest the *in vitro* S*j*ESG RNA cleavage mediated by Rz1 and Rz3 may be related to the regulation of the levels of the cytokines, IFN-γ and IL-4, consequently decreasing the amounts of adult worms and eggs.

Discussion

S. japonicum infects humans, livestock and snails, resulting in severe diseases. No effective therapeutic strategies are available to date. In this study, we designed 3 hammerhead ribozymes targeting the S*j*ESG gene of *S. japonicum*. We studied their cleavage activity *in vitro* and their roles in inhibiting the reproduction of *S. japonicum*. Our results suggest that there is a correlation between the *in vitro* cleavage abilities of Rz1 and Rz3 and their roles in reproduction inhibition of *S. japonicum*. To our knowledge, this is the first preliminary study of specific hammerhead ribozymes targeting S*j*ESG as a possible method to treat *S. japonicum*-related diseases.

After *in vitro* mRNA transcription of S*j*ESG and ribozymes from the constructs, we conducted *in vitro* cleavage experiments. The results showed that Rz1 and Rz3 cleaved their targeted mRNAs at specific sites, but Rz2 did not. These results indicated that the interaction between ribozyme and it's substrate mRNA as predicted by computer analyses may not be real. In addition, the cleavage efficiency between the different ribozymes was varied.

Since RNA is degraded easily *in vivo*, in the animal experiments we used the corresponding expression vectors to express RNA rather than the synthesized ribozyme RNAs. Secondly, we injected ribozymes frequently through the vena caudalis to maintain the concentration of ribozymes in the mice. In addition, the secondary structure of the ribozymes and the substrates had features to avoid degradation.

Notably, the IFN-γ level in the serum of the Rz groups was higher than that of the control groups, while the IL-4 level in the serum of the Rz groups was lower. One reasonable explanation may be that the Th1 cytokines of IFN-γ are correlated with *S. japonicum* granuloma formation and vigor, and the Th2 cytokines of IL-4 play an important role in down-regulating egg granuloma reaction at chronic schistosomiasis by inhibiting the Th1 cytokines.

The results in this study demonstrated that the hammerhead ribozymes for S*j*ESG play a significant role in the inhibition of the reproduction of *S. japonicum* by up to 39.82% in worm reduction in mice and 54.95% in egg reduction in the mouse liver. Further modification of hammerhead ribozymes for S*j*ESG and the screening of more ribozyme candidates for other genes of *S. japonicum* appears to be a promising novel therapeutic strategy in this field.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (no. 30972576), the Scientific Research Fund of Hunan Provincial Scientific and Technological Department (no. 2010SK3038), and supported by Hunan Provincial Innovation Foundation for Postgraduate (no. CX2009B190).

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