miR-378 inhibits cell growth and enhances apoptosis in human myelodysplastic syndromes

XINGYI KUANG1, CHUNMEI WEI1, TAO ZHANG1, ZESONG YANG1, JIANXIANG CHI2 and LI WANG1

1Department of Hematology, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, P.R. China; 2The Center for the Study of Haematological Malignancies, 2032 Nicosia, Cyprus

Received April 16, 2016; Accepted July 13, 2016

DOI: 10.3892/ijo.2016.3689

Abstract. miR-378 has been proven to inhibit cell growth, migration and invasion in different types of cancers. In this study, we found that miR-378 was commonly downregulated in the bone marrow cells obtained from myelodysplastic syndrome (MDS) patients. We further investigated the role of miR-378 in the proliferation and apoptosis of SKM-1 cells, an acute myeloid leukemia cell line established in the leukemic phase during the progression of MDS to AML (MDS/AML). Results indicated that overexpression of miR-378 in SKM-1 cells interfered with proliferation via inducing apoptosis and G0/G1-phase cell cycle arrest, and suppressive effect of miR-378 on MDS/AML cells may be mediated partly through Bcl-w and CDC40. Moreover, apoptosis induced by miR-378 correlated with increased expression of Bax and activation of caspase-3, -8 and -9. Taken together, our data support a critical role for miR-378 in the pathogenesis of MDS and provide a novel therapeutic target in this complex disease.

Introduction

MDS is one of the most common blood cancers among the elderly, and ~33% patients will develop into acute myeloid leukemia (AML). In fact, MDS are a class of heterogeneous clonal disorders that derive from primitive CD34+ stem cells and present with diverse clinical symptoms and outcomes (1). Regarding therapy, the only way to cure this disease is haematopoietic stem cell transplantation (HSCT). Unfortunately, HSCT is only applicable for a small number of MDS patients owing to a variety of factors such as advanced age, various comorbidities, and donor availability (2). Moreover, prognoses are still bleak in MDS patients. The median survival ranges from 68.4 months in the low-risk group to 4.8 months in the high-risk group, and the prognosis is even worse in secondary AML (3). Current knowledge of the molecular mechanisms of MDS is still limited, even though haploinsufficiency, gene mutations, epigenetic deregulation, microenvironmental changes have been proven to be critical contributors in the pathophysiology of this disease (4). Therefore, it is important to clarify the molecular pathogenesis of MDS and to explore novel therapy for clinical use.

microRNAs (miRNAs) are short single-stranded RNAs that inhibit gene expression at the post-transcriptional level (5), and over half of miRNA genes are located in cancer-related genomic regions (6). In recent years, increasing studies have shown that miRNAs are implicated in normal hematopoiesis (7), and dysregulation of miRNA has been found in hematological malignancies including MDS (8-11). More importantly, deregulation of miRNA emerges as displaying a key role in the pathogenesis of MDS (12-15). For example, expression of miR-143 and miR-145 is diminished in MDS with del(5q) and knockdown of these two miRNAs in mice results in abnormal erythropoiesis and megakaryopoiesis, which are important features of 5q- syndrome (one subtype of MDS with isolate 5q-) (15). miR-22 is upregulated in MDS patients, and its downregulation in leukemic cells inhibits cell proliferation via targeting TET2 (14).

With the use of miRNA microarray or real-time PCR, aberrant expression of miR-378 has been detected in a number of cancers. Expression of miR-378 was downregulated in the specimens of colorectal cancer, gastric cancer, and cutaneous squamous cell carcinoma (16-18), whereas it is overexpressed in nasopharyngeal carcinoma, breast cancer, and ovarian cancer (19-21). More importantly, miR-378 has been reported to be implicated in the tumorigenesis of various solid cancers (16,17,19,22). In non-small cell lung cancer, miR-378 exerts oncogenic activity to promote cell migration, angiogenesis and tumor growth via SUFU-related and VEGF/MMP pathways (22). In contrast, miR-378 performs as a tumor suppressor in gastric cancer and colorectal cancer by targeting VEGF and vimentin, respectively (16,17). However, to date, the majority of functional studies of miR-378 focus on solid cancers,
only a few studies have investigated the physiological role of miR-378 in hematologic malignancies. In leukemia cells, miR-378 is upregulated during the differentiation induced by 4-hydroxyxenonenal (23), and miR-378 is related to prognosis of AML patients (24). A recent miRNA profiling study showed that miR-378 was the most potently downregulated miRNA in MDS patients (25), providing evidence that miR-378 may be of significance for the pathogenesis of MDS. However, the functional role of miR-378 has not been reported in MDS.

In this study, we detected the expression of miR-378 in bone marrow mononuclear cells (BM-MNCs) of 20 MDS patients and 13 healthy controls. The functional effect of miR-378 was further explored through cell growth, apoptosis and cell cycle analyses in MDS-derived cell line SKM-1 (26). Moreover, xenograft experiments in NOD/SCID mice were operated to investigate the potential role of miR-378 in MDS tumorigenesis in vivo. Finally, we went onto detail the mechanisms that mediated the suppressive effect of miR-378 on MDS/AML cells.

Materials and methods

Patients and specimens. For miR-378 expression analysis, bone marrow samples were obtained from 20 newly diagnosed MDS patients at The First Affiliated Hospital of Chongqing Medical University. Mononuclear cells were separated from bone marrow by Ficoll gradient centrifugation. The diagnosis of MDS was established according to 2008 WHO criteria. 5 patients had refractory anemia, 3 had refractory anemia with ringed sideroblasts, 2 had refractory anemia with multilineage dysplasia, 5 patients with refractory anemia with excess blasts, 1 with 5q- syndrome, 1 patient had secondary AML, and 3 with MDS unclassified. Among these MDS patients, male to female ratio was 12:8 and average age was 58.1 years (range 39-80). Non-malignant bone marrow samples were collected from 13 randomly selected healthy people, 6 men and 7 women, with a mean age of 60.2 years (range 40-83). This study was approved by the institutional review board of The First Affiliated Hospital of Chongqing Medical University and all patients provided informed consent.

Cell culture. SKM-1 cell line was kindly provided by Professor Jianfeng Zhou from Tongji Medical College of Huazhong University of Science and Technology. The cell line was cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Beyotime, Shanghai, China) at 37°C/5% CO₂.

Lentiviral vector construction and lentivirus transfection. To obtain SKM-1 cells with miR-378 overexpression, the pre-miRNA of miR-378 was cloned into the GV209 lentiviral vector (Genechem, Shanghai, China). The primers for cloning pre-miR-378 were: forward sequence 5'-CGGGTACCCGTTAC AGCTGGAGAAAGGCTG-3' and reverse sequence 5'-CGGAATTCAAAAAATTCTCTTGCGGCACCAC-3'. The production, purification, and titration of lentivirus were performed as described by Tiscornia and colleagues (27). Recombinant lentivirus carrying miR-378 was named LV-miR-378, and the lentivirus expressing empty vector was called LV-control. At 30-50% confluence, typically 24 h after plating, SKM-1 cells were transfected with LV-miR-378 and LV-control using 5 µg/ml polybrene (Genechem). After 6 days, the transfection efficiency was evaluated using fluorescence microscopy and flow cytometry, and the cells stably overexpressing miR-378 were confirmed by real-time PCR.

Cell counting kit (CCK-8) proliferation assay. Cell proliferation was evaluated using a CCK-8 assay after 5-day lentivirus infection. SKM-1 cells were seeded into 96-well plates at a density of 5x10³ cells/well with 3 replicate wells of each condition. For the CCK-8 assay, 10 µl of CCK-8 solution (Beyotime) was added to each well and incubated at 37°C for 2 h. Absorbance values at 450 nm were measured using the SpectraMax M2 Multi-Mode Microplate Readers (Molecular Devices, Silicon Valley, CA, USA) daily for four consecutive days.

Apoptosis detected by Hoechst 33258 staining. Cells were fixed and washed twice in PBS. Then, the fixed cells were stained with 500 µl Hoechst 33258 solution (Beyotime) in dark for 5 min. Then the cells were observed under a fluorescence microscope.

Annexin V-PE/7-AAD staining for apoptosis analysis. Approximately 10⁶ cells were harvested and incubated for 15 min in staining solution containing 500 µl of binding buffer, 1 µl of Annexin V-PE and 5 µl of 7-AAD (KeyGen Biotech, Shanghai, China). After that, apoptosis rate of cells were detected by flow cytometry using CellQuest software (BD Biosciences, San Jose, CA, USA).

Cell cycle analysis. Cells were collected and fixed in 75% ethanol at 4°C overnight, then incubated in staining cocktail containing 50 µg/ml propidium iodide (PI) and 50 µg/ml RNase for 30 min at 37°C. DNA content of samples were analyzed with the use of FACSVantage flow cytometer (BD Biosciences) and Multicycle software.

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Quantitative RT-PCR was carried out to validate the expression level of miR-378. Total RNA was extracted from cells using RNAiso Plus (Takara, Dalian, China), digested with DNase I and reversely transcribed with the use of Prime Script™ RT reagent kit (Takara). Each reverse transcription reaction was performed in 10 µl reaction volume containing 0.1 µg of total RNAs, 2 µl of 5X PrimeScript buffer, 250 fmol special stem-loop RT primer (Novland, Shanghai, China), and 0.5 µl of PrimeScript RT Enzyme Mix I. Reverse transcription conditions were as follows: 16°C for 30 min, 42°C for 45 min, and 85°C for 10 min. The reverse transcription products were amplified using a SYBR Premix Ex TaqII II kit (Takara) on the Bio-Rad CFX96 system at 95°C for 30 sec, followed by 40 cycles at 95°C for 5 sec, and at 60°C for 30 sec. U6 was amplified as a reference for normalization. For mRNA quantification, β-actin was selected as the internal control. Total RNA was reverse transcribed with the use of Prime Script™ RT reagent kit (Takara) following the manufacturer’s instructions. Quantitative PCR analysis were performed in real-time using SYBR Premix Ex TaqII II kit
Western blot analysis. Protein lysate was extracted from cells using RIPA lysis buffer supplemented with 1 µM PMSF (Beyotime), and protein concentration was measured with BCA Protein Assay kit (Beyotime). Western blotting was performed as previously described (28). The following primary antibodies were used in this study: rabbit anti-human cleaved-caspase-3, cleaved-caspase-8, cleaved-caspase-9, Bcl-w and Bax (immuno-noway, Newark, DE, USA); rabbit polyclonal antibody against horseradish peroxidase-conjugated goat anti-rabbit IgG (Beyotime) was used as second antibody in this study. GAPDH served as a loading control. All results were visualized with the use of BeyoECL Plus (Beyotime).

Dual-luciferase reporter assay. For dual-luciferase reporter assay, the wild-type and mutated 3'-UTR of Bcl-w mRNA was cloned into the dual luciferase reporter vectors. HEK 293T cells were seeded into 24-well plates and cotransfected with the luciferase-repoter vectors and miR-378/negative control vectors. After 48-h transfection, luciferase activity was detected using the Dual-Luciferase Reporter Assay system (Promega) according to the instructions.

Tumor growth experiments in vivo. Female 5- to 6-week-old NOD/SCID mice were obtained from Beijing HEK Bioscience. The mice were subcutaneously injected with 1x10^7 SKM-1 cells (suspended in 200 µl of RPMI-1640 medium) that infected with LV-miR-378 or LV-control. After 4 weeks, mice were sacrificed, and their tumors were carefully excised. Tomor volumes were measured and calculated using the following formula: volume (mm³) = LxW²/2 (L represents the largest diameter; W is the smallest diameter of tumor). All animal experiments were approved by the Ethics Committee of Chongqing Medical University.

Histological analysis. The xenograft tumors were fixed in 4% paraformaldehyde solution, embedded in paraffin and cut into 3-µm-thick sections. Then the paraffin sections were stained with hematoxylin and eosin (H&E). DNA fragmentation in nucleus, which is a typical features of apoptosis, can be detected by the terminal dUTP nick-end labeling (TUNEL) assay. We employed TUNEL assay to evaluate the cell apoptosis in situ using a TUNEL apoptosis assay kit (Beyotime) according to the manufacturer’s instructions. Cell counting was conducted on four random filed of each section, and cells with dark brown nuclei were identified as apoptotic cells. Final data were expressed as the percentages of apoptotic cells.

Statistical analysis. All statistical analyses were performed with SPSS software 20.0. Results were presented as mean ± standard deviation (SD). Two-tailed Student’s t-test was carried out to determine significance when only two groups were compared. Variations between groups were analyzed with one-way ANOVA, followed by Tukey multiple comparison test. P<0.05 was considered statistically significant.

Results

Expression level of miR-378 in MDS patients. BM-MNCs are widely used samples in the miRNA profiling studies of MDS. They are unsorted cells containing not only stem cells but also maturing white cells, which may fully reflect aberrant expression of miRNAs and maximize total RNA yield from the precious patient samples. With the utilization of qRT-PCR, we demonstrated that the expression of miR-378 is obviously decreased in BM-MNCs from MDS patients in contrast with healthy controls (Fig. 1).

Overexpression of miR-378 by lentivirus in SKM-1 cells. To clarify the role of miR-378 in the human MDS, SKM-1 cells were transfected with LV-miR-378 for upregulating the expression of miR-378. After infection of LV-miR-378, SKM-1 cells were observed under fluorescence microscope to generally evaluate the infection efficiency (Fig. 2A and B). By using flow cytometry, we found the percentage of green fluorescent protein (GFP)-positive cells was >70% after transfection with LV-miR-378 (Fig. 2C), suggesting that the lentivirus successfully transduced into SKM-1 cells. Next, expression of miR-378 was detected using qRT-PCR. Lentivirus expressing miR-378 significantly increased the miR-378 level in SKM-1 cells (Fig. 2D).

Overexpression of miR-378 inhibited SKM-1 cell growth in vitro. To investigate the effect of miR-378 on cell proliferation, we conducted CCK-8 assay. After 3 days, the OD value was significantly decreased in SKM-1 cells transfected with LV-miR-378 compared with cells transfected with LV-control and untransfected SKM-1 cells (Fig. 3). The results proved that overexpression of miR-378 inhibited cell growth ability in SKM-1 cells.

Effects of miR-378 on apoptosis and cell cycle of SKM-1 cells. The effect of miR-378 on SKM-1 cell apoptosis was assessed
KUANG et al: miR-378 INHIBITS GROWTH IN MDS CELLS

1924

using Annexin V/7-AAD staining assay. It showed that overexpression of miR-378 induced apoptosis in SKM-1 cells, and the percentage of apoptotic cells in miR-378 overexpressing group was significantly higher than that of other two groups (Fig. 4A and D). We also observed apoptotic state in each group by Hoechst 33258 staining. Fluorescence microscope imaging displayed that more cells in miR-378 overexpressing group showed apoptotic morphological characteristics (Fig. 4B). In addition, cell cycle distribution was evaluated using flow cytometry. Results showed that miR-378 significantly increased the proportion of cells in G0/G1 phase and decreased the percentage of cells in S phase (Fig. 4C and E).

miR-378 induces apoptosis in SKM-1 cells by activating both intrinsic and extrinsic apoptosis pathway. To further investigate the specific mechanisms by which miR-378 induces apoptosis in MDS cells, we employ western blot assay to detect the expression of caspase-3, -8, -9 and Bax. As shown in Fig. 6, upregulation of miR-378 obviously increased the expression of...
cleaved-caspase-3, -8 and -9. Also, overexpression of miR-378 led to upregulated expression of proapoptotic protein Bax. Taken together, these results confirmed that overexpression of miR-378 activated caspase-dependent apoptosis in MDS cells.

Figure 4. miR-378 suppresses SKM-1 cell growth by inducing cell apoptosis and G1-phase cell cycle arrest. (A) Cell apoptosis was assessed by Annexin V/7-AAD staining after infection with LV-miR-378 and LV-control. (B) Apoptotic cells were also detected by nuclear staining with Hoechst 33258. (C) Representative images depicting cell cycle distribution of SKM-1 cells overexpressing miR-378, SKM-1 cells transfected with LV-control and normal SKM-1 cells, respectively. (D) Apoptosis rate of SKM-1 cells transfected with LV-miR-378 significantly increased, when compared with LV-control transfected SKM-1 cells and normal SKM-1 cells. *P<0.01 compared with other two groups. (E) Proportion of cells in various phases of the cell cycle. The proportion of LV-miR-378 transfected cells increased in G0/G1 phase, but it decreased in S phase. *P<0.05 compared with the other two groups.
Expression of target genes are suppressed by miR-378. To explore the apoptosis-related target genes of miR-378 in SKM-1 cells, we performed bioinformatics algorithms Targetscan (http://www.targetscan.org/) and PicTar (http://pictar.mdc-berlin.de/). Both bioinformatic analyses indicated that anti-apoptotic protein gene Bcl-w is a putative target of miR-378. Then we conducted dual-luciferase reporter assay to determine whether Bcl-w is a direct target of miR-378. As shown in Fig. 7B, overexpression of miR-378 resulted in a remarkable diminution of luciferase activity in 293T cells transfected with Bcl-w wild-type 3'UTR vector. By contrast, there was no obvious change of luciferase activity in SKM-1 cells cotransfected with miR-378 vectors and Bcl-w mutant-type 3'UTR vectors. We further conducted qRT-PCR and western blotting to determine whether overexpression of miR-378 could result in a downregulation of Bcl-w expression. As a result, overexpression of miR-378 in SKM-1 cells resulted in a marked decrease of Bcl-w protein expression level, whereas the expression of Bcl-w mRNA was not affected obviously (Fig. 7C). We also examined the expression of CDC40, which is a proven target of miR-378 (29), by using a western blot assay. It showed that miR-378 significantly diminished the expression of CDC40 in SKM-1 cells (Fig. 7D).
Discussion

miR-378 has been proven to participate in the initiation and development of various cancers (16,19,29,30). However, the role of miR-378 in cancer cells seems controversial because it plays a tumor-promoting role in non-small cell lung cancer and nasopharyngeal carcinoma (19,30), but exerts tumor-suppressive effect on gastric cancer and colorectal cancer (16,29). Recently, a study identified markedly downregulated expression of miR-378 in BM-MNCs from MDS patients (25), suggesting that miR-378 may participate in the molecular pathogenesis of MDS. However, the knowledge of its pathogenic role in MDS is still lacking.

In this study, we identified a lower expression level of miR-378 in MDS patients compared with healthy controls. SKM-1 is a malignant cell line established in the leukemic phase during MDS progress towards AML (26), and it is an eligible model to investigate the mechanisms underlying the pathogenesis and transformation of MDS (31). In functional studies, we observed that overexpression of miR-378 in SKM-1 cells attenuated cell proliferation in vitro by promoting cell apoptosis and blocking cell cycle progression. These results indicated that miR-378 played a tumor-suppressive role in the MDS/AML cell line, and it may contribute to the transformation of MDS to AML.

However, the effect of miR-378 is heterogeneous because it exerts a totally reverse role in different types of cancers, which may due to miR-378 targeting different genes in these cancers and further regulates diverse signaling pathways. In agreement with our results, Wang et al (29) found that miR-378 inhibited colorectal cancer cell growth via inducing cell cycle arrest and apoptosis. They further proved that CDC40, a critical cell-cycle regulator promoting G1/S-phase transition (32), was a direct target of miR-378 and responsible for cell cycle arrest in colorectal cancer cells. We also found the enhanced expression of miR-378 increased CDC40 protein expression, presenting a potential mechanism by which miR-378 caused G1-phase cell cycle arrest in SKM-1 cells.

Our in vitro study showed that overexpression of miR-378 could inhibit proliferation and promote apoptosis of SKM-1 cells, suggesting that miR-378 played an inhibitory role in the pathogenesis and progression of MDS. However, the initiation and development of cancer is not only related to the characteristics of the cancer cells, but also closely related to the complex interaction between the cancer and the host or the microenvironment in vivo (33). The immunodeficient mouse models, especially those of SCID mice and NOD/SCID mice, have been used to study the growth and tumorigenesis of hematological malignant tumors in vivo (34,35). In order to determine whether miR-378 overexpression could affect the growth and apoptosis of MDS cells in vivo, we conducted
NOD/SCID mice lack both functional T- and B-cells (36), which may result in easier formation of MDS cell-derived solid tumors in these mice.

As described before, Wang et al (29) reported similar suppressive effect of miR-378 on colorectal cancer cells. However, the study of Wang and colleagues only clarified the cell cycle-related mechanisms involved in the action of miR-378, but did not further address the mechanisms underlying the miR-378-induced apoptosis. To explore the mechanisms underlying apoptosis caused by miR-378 in SKM-1 cells, we employed two bioinformatics algorithms to predict potential target genes of miR-378. Both bioinformatic analyses implied that anti-apoptotic protein gene Bcl-w is a putative target for miR-378. As a member of B cell lymphoma 2 (Bcl-2) family, Bcl-w plays an anti-apoptotic function in both healthy cells and cancer cells (37,38). Bcl-2 family of proteins, which are a group of important regulators in the mitochondria-related apoptosis pathway, contain anti-apoptotic proteins (Bcl-2, Bcl-xl, Bcl-w) and pro-apoptotic proteins (Bax, Bad, Bid, Bak) (39). Bcl-w could inhibit mitochondria-mediated apoptosis by directly interacting with pro-apoptotic proteins Bax and Bad (38,40). In addition to blocking apoptosis of mitochondrial pathway, it also protects cells from apoptosis by suppressing the activation of stress-activated protein kinase (41). Moreover, this anti-apoptotic protein has been found commonly expressed in various cancers (41-43), promoted tumor cell growth and invasion (44,45). In this study, we proved that Bcl-w was a direct target of miR-378 using a dual-luciferase reporter assay. In agreement with this, western blotting showed that miR-378 reduced the expression of Bcl-w protein. By contrast, the Bcl-w mRNA level was not affected by ectopic expression of miR-378. All these findings indicated that miR-378 negatively regulated the expression of Bcl-w protein by directly binding the 3'UTR of Bcl-w mRNA.

In addition, we evaluated the expression of cleaved-caspase-3, cleaved-caspase-8 and cleaved-caspase-9, which are critical regulators of apoptosis. There are two major pathways involved in apoptosis, i.e., the extrinsic death receptor pathway and the intrinsic mitochondrial pathway (46). The extrinsic pathway is triggered by the tumor necrosis factors and further leads to the cleavage and activation of caspase-8 (47). In the intrinsic mitochondrial pathway, caspase-9 is activated by apoptosis, which is a complex that formed of cytochrome c and apoptotic protease activating factor-1 (48). Both extrinsic pathway and intrinsic pathway lead to the cleavage and activation of caspase-3 and finally cause apoptosis (49). In this study, we observed that enforced expression of miR-378 led to increased levels of cleaved caspase-3, -8 and -9, indicating that miR-378 induced apoptosis in SKM-1 cells via both extrinsic pathway and intrinsic pathway. Besides, we found that expression of pro-apoptotic protein Bax was notably upregulated in miR-378-overexpressing SKM-1 cells. Several studies have reported high ratio of Bcl-2 family pro-apoptotic proteins versus anti-apoptotic proteins correlates with increased apoptosis in MDS cells (50-52). A recent study proved that increased Bax/Bcl-w ratio contributed to the activation of caspase-dependent apoptosis (45). Based on these findings, we propose a potential mechanism behind apoptosis induced by overexpression of miR-378 in SKM-1 cells. The upregulation of Bax and downregulation of Bcl-w caused by miR-378 result in a remarkable increase in the Bax/Bcl-w ratio. Increased Bax/Bcl-w ratio directly or indirectly leads to activation of caspase-3, -8 and -9 that further induces caspase-dependent apoptosis. However, how miR-378 upregulates the expression level of Bax protein is still unknown.

In conclusion, this study pointed to a pronounced downregulation of miR-378 in MDS patients. Overexpression of miR-378 can suppress MDS/AML cell growth by promoting apoptosis and blocking G1/S-phase transition, at least, partly via negatively regulating Bcl-w and CDC40 expression. However, because each miRNA can regulate multiple target genes and the miRNA-regulated signaling pathways are complex, the specific mechanisms of miR-378 in the development of MDS are still needed to be elucidated in further studies. Recently, a study found that treatment of miRNA inhibitor could reverse the phenotypes of mouse model resembling MDS (13), suggesting that miRNA-based treatment may be a new strategy for therapy of MDS.

Acknowledgements

We are grateful to Chongqing Key Laboratory of Ophthalmology for the technical assistance. We also thank Mr. Nian Zhou and Miss Xinxin Li for their great help in this study. This study was supported by the National Natural Science Foundation of China (nos. 30971277 and 81250034), the Natural Science Foundation of Chongqing (CSTC, 2009BB5070), the bureau of Chongqing (2013-2-023), the Foundation of Chongqing Graduate Student Innovative Research (CYS14123) and the Project Foundation of Chongqing Municipal Education Committee (2013).

References


