miRNA-135a regulates Hut78 cell proliferation via the GATA-3/TOX signaling pathway

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Abstract. The present study investigated the role of microRNA-135a (miR-135a) in cutaneous T-cell lymphoma (CTCL) proliferation. Compared with the normal T lymphocyte control cell line, the mRNA and protein levels of GATA binding protein 3 (GATA-3) were markedly increased in the Hut78 cell line and miR-135a was markedly decreased ($P<0.05$). Based on bioinformatics, the target gene of miR-135a was identified as GATA-3. Dual luciferase and pre-miR-135a assays showed that miR-135a regulated the translation of GATA-3. In addition, the overexpression of miR-135a mimics decreased the protein levels of GATA-3 and thymocyte selection-associated high mobility group box (TOX). The substantially increased mRNA and protein levels of GATA-3 may be associated with the downregulation of miR-135a, leading to T-cell deregulation and proliferation through GATA-3/TOX regulation and subsequently causing CTCL.

Introduction

Cutaneous T-cell lymphoma (CTCL) represents serial diseases, which mainly involve malignant clonal T-lymphocytes of the CD4 phenotype due to a heterogeneous population of lymphoproliferative disorders (1). The annual incidence of primary cutaneous lymphomas is estimated to be 1:100,000, of which CTCL accounts for ~75% of cases; therefore, CTCL is the most common type of primary cutaneous lymphoma (2,3). Due to the clonal proliferation of skin-invasive mature T lymphocytes, CTCL is characterized as a type of non-Hodgkin's lymphoma (3). The pathogenesis of CTCL involves the deregulation of signaling pathways, including signal transducer and activator of transcription (STAT), Src kinases, c-Myc, cyclooxygenase-2, nuclear factor-κB, GATA binding protein 3 (GATA-3), thymocyte selection-associated high mobility group box (TOX), and embryonic stem cell regulators (4,5). GATA factors can function in undifferentiated progenitor cells and are involved in their expansion, or they can direct the coordinated maturation and cell cycle withdrawal in terminally differentiating cells. Therefore, alterations of GATA factors contribute to the development of cancer in humans. GATA3 functions in T lymphocytes, but it is also a critical regulator of mammary epithelial cells (6). The type 2 T helper cell-specific transcription factor GATA-3 is overexpressed in patients with CTCL and peripheral T-cell lymphoma (7,8). The overexpression of GATA-3 also occurs in other cancer/tumor cells, including human glioblastoma and other T-cell lymphomas (9,10). Furthermore, activated GATA-3 can promote T-cell proliferation in patients with Sézary syndrome (11), and the overexpression of GATA-3 can develop $CD4^+/CD8^+$ double-positive T-cell lymphoma (10). Therefore, the overexpression of GATA-3 promotes cancer or tumor cell proliferation and differentiation (12). However, the gene that regulates GATA-3 in CTCL and the way in which GATA-3 and its co-activators and/or co-repressors regulate the expression of disease-associated genes remain to be fully elucidated.

MicroRNAs (miRNAs), which are a class of small non-coding RNAs (18-22 nt length), are ubiquitous in eukaryotes and can regulate protein expression at the mRNA level (13-15). In CTCL, the expression levels of several miRNAs and proteins are altered (16,17). miRNA-135a (miR-135a) is involved in the regulation of several diseases, including colorectal cancer (18), blood lipid and inflammatory changes (19), senescent vascular endothelial cell calcification (20), and lung cancer metastasis and invasion (21). A low expression level of miR-135a in classic Hodgkin's lymphoma (cHL) is associated with a high likelihood of relapse and a short disease-free survival period (22). Increased expression of GATA-3 and decreased expression of miR-135a are observed in

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T-cell lymphoma. However, the association between miR-135a and GATA-3 remains to be fully elucidated.

In the present study, reverse transcription-quantitative polymerase chain reaction (RT-qPCR), western blot, cell counting, dual luciferase, and pre-miR-135a assays were used to detect Hut78 cell proliferation and the gene and protein expression levels in the Hut78 cell line to investigate the role of miR-135a in regulating GATA-3 mRNA translation. The results provide evidence that the novel tumor suppressor miR-135a represses the mechanism underlying the expression of GATA-3 in CTCL.

Materials and methods

Cell culture. The cultured Hut78 human CTCL cell line (23) was purchased from American Type Culture Collection (Manassas, VA, USA). The non-malignant T-cell line (normal T lymphocytes as control cells), were established in the medical laboratory of The First Hospital of Zibo City (Zibo, China) from patients with mycosis fungoides and Sézary syndrome according to Woetmann's methods (24). Fresh blood samples were obtained from a 46-year-old male patient with mycosis fungoides and Sézary syndrome at the First Hospital of Zibo City (Zibo, China) in April 2015. The fresh blood was diluted with an equal volume of PBS and was gently dropped onto the lymphocyte separation medium (Anhui Haoyang Chemical Group Co., Ltd., Fuyang, China) which was in a 15-ml centrifuge tube. The non-malignant T-cell line was obtained from the interlayer between the blood and lymphocyte separation medium following 15-ml tube centrifugation for 15 min at 500 x g and 25˚C. Briefly, in 5% CO_2 at 37˚C, this non-malignant T-cell line was cultured in RPMI 1640 medium, containing 10% (v/v) heat-inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), L-glutamine and antibiotics (100 IU/ml of penicillin, 100 μg/ml of streptomycin). The present study was performed with the approval of the First Hospital of Zibo City. Signed written consent was obtained from the patient prior to recruitment to the study.

Reagents and instruments. The following reagents and instruments were used in the present study: miRcute miRNA isolation kit, miRcute miRNA cDNA first-strand synthesis kit, miRcute miRNA quantitative fluorescence detection kit, SuperReal PreMix (SYBR Green), and TIANScript II cDNA first-strand synthesis kit; all were purchased from Tiangen Biotech Co., Ltd. (Beijing, China). The RT-qPCR instrument (BIOER FQD-96A), GATA-3 (cat. no. ab106625), TOX (cat. no. ab155768) and β-actin (cat. no. ab8227) primary antibodies and secondary antibody (cat. no. ab7090; Abcam, Cambridge, MA, USA), TRIzol reagent (Yisheng Biology, Shanghai, China), BCA protein assay reagent kit (Zhongke Ruitai, Beijing, China), and serum RNA extraction miRNeasy serum/plasma kit (Jianlun Biology, Guangzhou, China) were also used. All plasmids/agomiR were designed and synthesized by Shanghai Biological Technology Co., Ltd. (Shanghai, China). Pre-miR-135a, miR-135a mimic, miR-135a inhibitor, and their negative controls were purchased from GeneChem Co. (Shanghai, China; Table I). Xfect transfection reagents were purchased from Takara Biotechnology Co., Ltd. (Dalian, China).

Table I. miR-135a mimics, miR-135a inhibitor and their NC sequences.

Name	Sequence (5'-3')
miR-135a mimics	UAUGGCUUUUUAUUCCUAUGUGA
Mimics NC	UUCUCCGAACGUGUCACGUTT
miR-135a inhibitor	UCACAUAGGAAUAAAAAGCCAUA
Inhibitor NC	CAGUACUUUUGUGUAGUACAA

miR, microRNA; NC, negative control.

RT-qPCR analysis. The Hut78 cells and normal T lymphocytes were collected, and total RNAs were extracted with TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) using the phenol-chloroform extraction method. The RNA integrity was examined by gel electrophoresis, and the RNA purity was assessed according to the 260/280 ratio by spectrophotometry. Total RNA (2 μg) underwent RT to synthesize cDNA using Oligo-dT (10 μM) and Super Pure dNTPs (10 mM), according to the protocol of the TIANScript II RT kit (cat. no. KR107; Tiangen Biotech Co., Ltd.). The sequences of the GATA-3, GAPDH, miR-135a, and U6 primers used for RT-qPCR analysis are shown in Table II. GAPDH and U6 were used as internal controls for GATA-3 and miR-135a, respectively. The reaction mixture was prepared as follows, according to SuperReal PreMix Plus (SYBR Green) (cat. no. FP205; Tiangen Biotech Co., Ltd.): 10 μl SYBR Ex Taq II, 0.4 μl ROX Reference Dye, 0.8 μl each primer (final concentration, 250 nmol/l), 7 μl ddH_2O and 1.0 μl cDNA. The PCR procedure for GATA-3 was as follows: Pre-denaturation for 10 min at 95˚C, followed by 40 cycles of denaturation for 30 sec at 95˚C, annealing for 20 sec at 55˚C, and extension for 30 sec at 72˚C. The reaction conditions for miR-135a were pre-denaturation at 95˚C for 5 min, 40 cycles of denaturation at 95˚C for 20 sec, and annealing at 60˚C for 30 sec. The relative levels of GATA-3 and miR-135a were calculated using the $2^{-\Delta\Delta Cq}$ method (25).

Western blot analysis. Total protein was extracted by protein lysis, and its concentration was measured using the BCA protein assay reagent kit. Subsequently, the proteins (20 μg) were separated by 10% SDS-polyacrylamide electrophoresis and transferred onto a PVDF membrane. The membrane was blocked by 5% skim milk for 2 h. Following blocking, the primary antibodies (1:1,000) targeting GATA-3, TOX, and β-actin were added and incubated overnight at 4˚C. Subsequently, the secondary antibody (1:10,000) was added and incubated at room temperature for 1 h. The membrane was developed in ECL luminescent liquid, and the developed film was scanned using a GT 2500 scanner (Epson America, Inc., Long Beach, CA, USA) and analyzed using ImageJ 1.50i (National Institutes of Health, Bethesda, MD, USA). The relative expression level of GATA-3 with respect to β-actin was calculated based on the grey value that was obtained from Image J software.

Bioinformatics prediction of the regulatory upstream miRNA for GATA-3. Bioinformatics prediction was used to identify the upstream miRNA for GATA-3. The following

Table II. Primer sequences.

Name	Primer sequence
GATA-3	Forward: 5'-AAGAGTGCCTCAAGTATCAG-3'
	Reverse: 5'-GCGGATAGGTGGTAATGG-3'
GAPDH	Forward: 5'-CCCTCAATGACCACTTTGTG-3'
	Reverse: 5'-GGTTTGAGGGCTCTTACTCCT-3'
MicroRNA-135a	Forward: 5'-GCGCCGTATGGCTTTTTATTCCTA-3'
	Reverse: 5'-TGCAGAGATGTCCAGTCAGC-3'
U6	Forward: 5'-AACGCTTCACGAATTTGCGT-3'
	Reverse: 5'-CTCGCTTCGGCAGCACA-3'

GATA-3, GATA binding protein 3.

gene prediction software programs were used for bioinformatics prediction: miRanda (http://www.microma.org/rnicroma/home.do), TargetScan (www.targetscan.org), PiTa (http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html), RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/), and PicTar (http://pictar.mdc-berlin.de/). miR-135a was identified as the potential upstream gene of GATA-3.

Dual luciferase assay. The wild and mutant types of the miR-135a binding sequence in the 3'-untranslated region (3'-UTR) of the *GATA-3* gene were constructed by *in vitro* chemical synthesis. The cleavage sites of *Spe*1 and *Hind*III were respectively added on both ends. The two DNA fragments were cloned into pMIR-REPORT luciferase plasmids. Using the liposome method, the plasmids with the wild-type 3'-UTR and mutant-type 3'-UTR sequences were transfected into 293T cells, which were purchased from the Cell Bank of the Institute of Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Subsequently, the agomiR-135a (100 nM) was transfected into cells and incubated for 24 h. The fluorescence values were measured using the GloMax 20/20 luminometer. The Renilla fluorescent activity was used as the internal control, and all procedures were performed in strict accordance with the dual luciferase assay kit instructions.

Cell transfection. The plasmids pre-miR-135a, miR-135a mimic, miR-135a inhibitor, and their negative controls were transfected into cells using Xfect transfection reagents, according to the manufacturer's protocol.

Statistical analysis. Processed by SPSS18.0 (SPSS, Inc., Chicago, IL, USA), all data are presented as the mean ± standard deviation and a normality test performed. Multiple groups of measurement data were analyzed using one-way analysis of variance (26). $P<0.05$ was considered to indicate a statistically significant difference.

Results

mRNA and protein expression of GATA-3. To detect the mRNA and protein expression levels of GATA-3 in the Hut78 CTCL cells and normal T lymphocytes, RT-qPCR and western blot analyses were performed, respectively. As shown in Fig. 1, the mRNA (Fig. 1A) and protein (Fig. 1B) levels of GATA-3 in the CTCL Hut78 cells were significantly increased compared with those in the normal T lymphocytes ($P<0.01$). Therefore, the expression level was GATA-3 is increased in the CTCL Hut78 cells.

Bioinformatics and dual luciferase assay. To identify the upstream regulatory miRNA of GATA-3, bioinformatics prediction was performed. miR-135a was found to be the potential upstream gene of GATA-3. The wild and mutation types of the binding sequence are shown in Fig. 2A. To determine whether miRNA-135 directly targets GATA-3, a dual luciferase assay was performed. The fluorescence values were significantly decreased following co-transfection with agomiR-135a and the pMIR-REPORT plasmid ($P<0.01$; Fig. 2B). No statistically significant differences in fluorescence values were observed compared with the mutation group ($P>0.05$); however, miR-135a was shown to bind directly with GATA-3 at the 3'-UTR to regulate its expression.

Expression of miR-135a in Hut78 cells. To detect changes in the expression of miR-135a in the CTCL Hut78 cells, RT-qPCR analysis was performed. The expression level of miR-135a in the CTCL Hut78 cells was significantly decreased compared with that in the normal T lymphocytes ($P<0.01$; Fig. 3). Therefore, miR-135a may have a regulatory role in the pathological process of CTCL through GATA-3.

Overexpression of miR-135a in Hut78 cells. The proliferation curves of Hut78 CTCL cells were drawn according to the instructions of the Cell Counting Kit-8. The proliferation of the Hut78 CTCL cells transfected with the pre-miR-135a plasmid was increasingly inhibited on the third day (Fig. 4). On the fourth and fifth days, the proliferation of cells was further increased.

miR-135a downregulates the levels of GATA-3 and TOX. According to the bioinformatics prediction, one of the target genes of miR-135a was identified as GATA-3. The potential binding sequence for miR-135a was identified in the 3'-UTR of GATA-3 (Fig. 2A). As predicted, the results of the western blot analysis showed that the overexpression of miR-135a mimics decreased the expression of GATA-3, whereas miR-135a increased its expression upon transfection (Fig. 5A). Simultaneously, the expression level of *TOX*, which is a downstream gene of GATA-3, was also decreased in the miR-135a-overexpressing cells (Fig. 5A). Additionally the results of the RT-qPCR assay showed that the mRNA level of GATA-3 in the miR-135a mimics group was marginally decreased compared with its level in the other

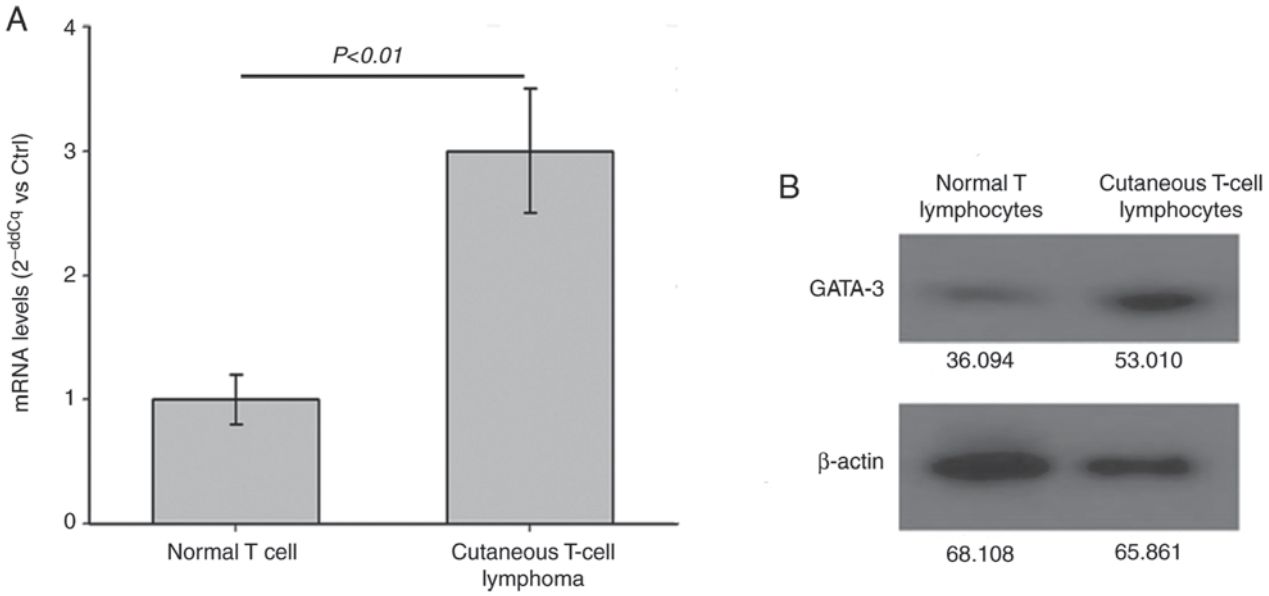

Figure 1. Analysis of the expression of GATA-3. (A) mRNA expression levels of GATA-3 in the Hut78 CTCL cells and normal T lymphocytes. (B) Protein expression levels of GATA-3 in the Hut78 CTCL cells and normal T lymphocytes. Gene expression levels are shown relative to the control (normal T lymphocytes). Normalization of expression was achieved against the endogenous genes GAPDH and β-actin. Each bar of the histogram corresponds to the mean ± standard deviation of three independent experiments. CTCL, cutaneous T-cell lymphoma; GATA-3, GATA binding protein 3; Ctrl, control.

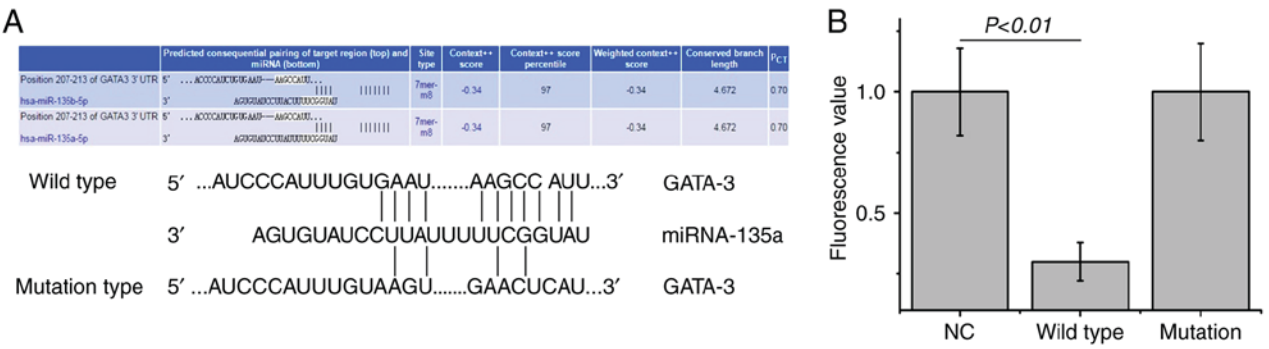

Figure 2. miR-135a-targeting GATA-3 experiments. (A) Wild-type and mutation binding sequences of miR-135a with GATA-3. (B) Dual luciferase assay. Each bar of the histogram corresponds to the mean ± standard deviation of three independent experiments. miR/miRNA, microRNA; GATA-3, GATA binding protein 3; 3'-UTR, 3'-untranslated region; NC, negative control.

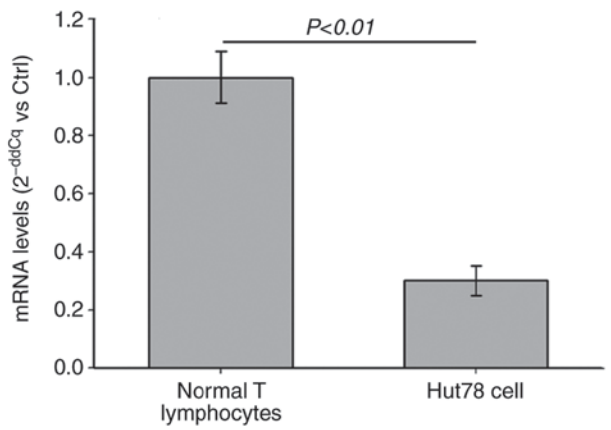

Figure 3. Expression of miR-135a in the Hut78 cutaneous T-cell lymphoma cell and normal T lymphocytes. Gene expression levels are shown relative to the control (normal T lymphocytes). Normalization of expression was achieved against the endogenous U6 gene. Each bar of the histogram corresponds to the mean ± standard deviation of three independent experiments. miR, microRNA; Ctrl, control.

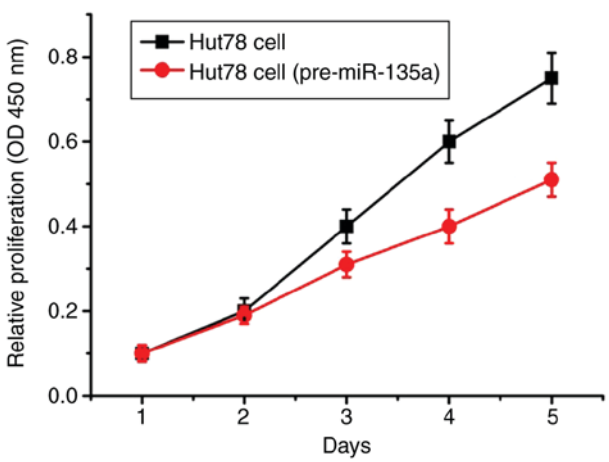

Figure 4. Effect of the overexpression of miR-135a on Hut78 cell proliferation. The Hut78 cell number was the same at the beginning. Following miR-135a transfection, Hut78 cell proliferation curves were drawn according to the instructions of the Cell Counting Kit-8. Data are reported as the mean ± standard deviation of three experiments. miR, microRNA.

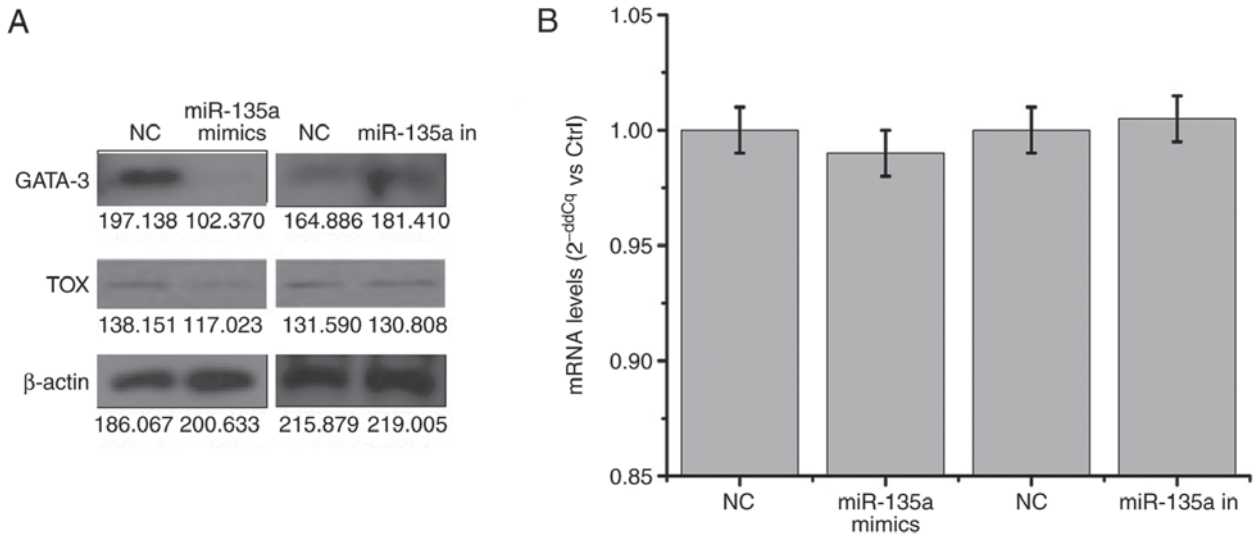

Figure 5. Effect of miRNA-135a on mRNA and protein expression in the Hut78 cells. (A) Western blot analysis showed decreased expression levels of GATA-3 and TOX in the miR-135a mimics group of Hut78 CTCL cells compared with those in the NC and miR-135a inhibition groups of CTCL cells. For each sample, cell lysate was extracted and probed with antibodies specific for GATA-3, TOX, and β-actin proteins. (B) Reverse transcription-quantitative polymerase chain reaction analysis showed a marginal decrease in the expression of GATA-3 in the miR-135a mimics group of Hut78 CTCL cells compared with levels in the NC and miR-135a inhibition groups of CTCL cells. Normalization of expression was achieved against the endogenous GAPDH gene. Each bar of the histogram corresponds to the mean ± standard deviation of three independent experiments. miR/miRNA, microRNA; GATA-3, GATA binding protein 3; TOX, thymocyte selection-associated high mobility group box; NC, negative control; miR-135a in, miR-135a inhibition; Ctrl, control.

groups (Fig. 5B), and the protein expression of GATA-3 was substantially downregulated in the Hut78 cells. The primers for RT-qPCR analysis were located at the middle of GATA-3 mRNA, and the PCR product belonged to one of several exons in the GATA-3 pre-mRNA. The existence of GATA-3 pre-mRNA may have resulted in the marginal decrease in the mRNA expression of GATA-3 in the RT-qPCR assay. Furthermore, GATA-3 mRNA was destroyed by miR-135a following pre-mRNA maturation. Therefore, miR-135a led to the destruction of GATA-3 mRNA and inhibited the translation of GATA-3 mRNA.

Discussion

GATA-3, which can be upregulated in lymphoma or CTCL, has binding sites in the TOX promoter (11,27,28). In CTCL, the expression level of GATA-3 in Hut78 cells was higher than that in normal T lymphocytes. To understand the mechanism underlying the changes in the expression of GATA-3, the present study performed a search for the miRNA that targets GATA-3 mRNA via bioinformatics analysis; this was found to be miR-135a.

Increasing levels of mature miR-135a can cause cHL cell apoptosis and growth reduction through miR-135a-regulating Janus kinase 2 (JAK2) (22). As confirmed by a dual luciferase assay, an increased level of miR-135a inhibited Hut78 cell proliferation via the role of miR-135a in targeting GATA-3 mRNA. However, the level of miR-135a was lower in CTCL Hut78 cells than in normal T lymphocytes, suggesting that miR-135a may be involved in the pathogenesis of CTCL.

The transcription factor of *TOX* is GATA-3, which is involved in the signal cascades governing T-cell development (11,29). In addition, the transcript levels of TOX are markedly increased in CTCL, compared with those in normal skin or benign inflammatory dermatoses (4). Therefore, the enhanced transcription of TOX may be induced by the overexpression of GATA-3 due to the decreased expression of regulatory miRNA-135a in CTCL.

In the present study, the miRNA functioning as the upstream regulator of GATA-3 was investigated via bioinformatics analysis. miRNAs are small, endogenous, non-coding RNAs, which can dissect and inhibit target mRNA translation for its deregulation (30,31). miRNAs are vital in regulating disease development, physiology and pathogenesis (32). According to the bioinformatics prediction, miR-135a was identified as one of the potential upstream genes that may regulate the expression GATA-3. The dual luciferase assay further confirmed that miR-135a can directly bind with the 3'UTR of GATA-3, suggesting that miR-135a can directly regulate the translation of GATA-3. Activated GATA-3 can promote T-cell proliferation in patients with Sézary syndrome (11), and the overexpression of GATA-3 promotes cancer/tumor cell proliferation and differentiation (10,12). In the present study, the expression of GATA-3 was upregulated due to the downregulated expression of miR-135a in the Hut78 CTCL cell line and exhibited enhanced cell proliferation.

The assays involving miR-135a mimics showed that the mRNA level of GATA-3 in the miR-135a mimics group was marginally decreased compared with those in other groups, and the protein level of GATA-3 was substantially downregulated in the Hut78 cells. Unfortunately, whether the miR-135a mimics altered the level of miR-135a in the Hut78 cells was not examined. Previous reports have confirmed that miRNA mimics can induce the upregulation of target gene mRNA and decrease the protein level of the target gene (33,34). The results of the present study suggest that the enhanced gene transcription of GATA-3 was activated in the miR-135a mimics assay owing to the decreased

protein expression of GATA-3, which is a key molecule in the signaling pathway.

Cell apoptosis is a complex, multistage process that involves numerous genes. Apoptosis can be induced by endoplasmic reticulum stress, the mitochondrial pathway, and the death receptor pathway. The mitochondrial pathway has been relatively well researched and is controlled predominantly by members of caspase-3, cleaved caspase-3, and B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein. Certain natural products, including Euphorbia factor L2 and bruceine D, can induce cell apoptosis through the mitochondrial pathway (35,36). Cell apoptosis can be induced by miR-133b and miR-135a *in vitro* via a signaling cascade, involving JAK2, STAT3 and Bcl-2 (37). In the present study, miR-135a also induced cell apoptosis by targeting GATA-3 and regulating GATA-3/TOX signaling.

In conclusion, the mRNA and protein expression levels of GATA-3 were markedly increased in CTCL. This finding may be associated with the downregulated expression of miR-135a, leading to T-cell deregulation and proliferation through GATA-3/TOX regulation and subsequently causing CTCL. However, immunohistochemical analysis is required to further examine the expression of GATA-3 in more tissue samples of CTCL.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HW and JW designed the study. Western blot analysis was conducted by HW and RL. RT-qPCR analysis was performed by HW, XG and YZ. Other experiments were conducted by HW, BS and JW. HW and JW analyzed and interpreted the data, and drafted the manuscript. All authors critically revised the manuscript, and read and approved the final version of the manuscript.

Ethics approval and consent to participate

Ethical approval was provided by the Medical Ethics Committee of The First Hospital of Zibo City (reference no. 201503045).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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