MiR-7 Functions as a Tumor Suppressor by Targeting the Oncogenes TAL1 in T-Cell Acute Lymphoblastic Leukemia

Hongbo Sun, MD1, Zhifu Zhang, MD1, Wei Luo, MD1, Junmin Liu, MD1, Ye Lou, MD2, and Shengmei Xia, MD3

Abstract

Background: T-cell acute lymphoblastic leukemia is a hematologic malignancy characterized by T-cell proliferation, and in many cases, the ectopic expression of the oncogenic transcription factor T-cell acute lymphocytic leukemia protein 1 (TAL1). MicroRNA-7 has been shown to play a critical role in proliferation, migration, and treatment sensitivity in a diverse array of cancers. In this study, we sought to establish a novel link between microRNA-7 and T-cell acute lymphoblastic leukemia oncogenesis. Material and Method: To do so, we characterized gene expression of microRNA-7 as well as TAL1 in both T-cell acute lymphoblastic leukemia patient-derived tissue and cell lines, as well as performing functional luciferase assays to assess microRNA-7 binding to the TAL1 3'-untranslated region. We also performed growth, apoptosis, and migration experiments using 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide, Annexin V, and transwell assays in the context of microRNA-7 overexpression. Results: We found that microRNA-7 expression is attenuated and inversely correlated with TAL1 expression in TAL1+ T-cell acute lymphoblastic leukemia cells. Additionally, microRNA-7 directly targets and suppresses TAL1 levels. Finally, microRNA-7 overexpression reduces growth, motility, and migration while inducing apoptosis in T-cell acute lymphoblastic leukemia cells, phenotypes that can be rescued by concomitant overexpression of TAL1. Conclusions: These results indicate that microRNA-7 functions as a potent tumor suppressor by inhibiting the oncogene TAL1 and suggest microRNA-7 could function as a prognostic biomarker and possible therapeutic in the clinical management of T-cell acute lymphoblastic leukemia.

Keywords
miR-7, TAL1, T-cell acute lymphoblastic leukemia

Abbreviations
ALL, acute lymphoblastic leukemia; B-ALL, B-cell acute lymphoblastic leukemia; BM, bone marrow; FBS, fetal bovine serum; mRNA, messenger RNA; miRNA, microRNA; miR-7, MicroRNA-7; MTT, 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide; NC, negative control; RT-PCR, Real-time polymerase chain reaction; T-ALL, T-cell acute lymphoblastic leukemia; 3'-UTR, 3'-untranslated region.

Introduction

Acute lymphoblastic leukemia (ALL) is the most common pediatric malignancy, comprising close to 30% of new-onset pediatric cancers. Due to significant advances in treatment regimens over the last few decades, 5-year survival is now estimated to exceed 85%.1–3 Acute lymphoblastic leukemia can be broadly subclassified into T-cell acute lymphoblastic leukemia (T-ALL) or B-cell ALL (B-ALL) depending on the cancer’s cellular origin, with T-ALL representing 12% to 15% of pediatric and 25% of adult-onset ALL cases, respectively.1,4

1 Department of Hematology, Shenzhen Longhua People’s Hospital, Shenzhen, China
2 Department of Hematology, Daqing Oilfield General Hospital, Daqing, China
3 Department of Neurology, Shenzhen Longhua People’s Hospital, Shenzhen, China

Corresponding Author:
Hongbo Sun, Department of Hematology, Shenzhen Longhua People’s Hospital, 38 Jinglong Construction Road, Shenzhen 518109, China. Email: sunhongboto@outlook.com
T-cell acute lymphoblastic leukemia has remained relatively understudied when compared with B-ALL despite the observation that it arises from biologically separate molecular pathways and has distinct disease kinetics and treatment responses. In particular, existing salvage therapy for recurrent disease is notoriously poor.\textsuperscript{5,7} Therefore, much work remains to be done to elucidate disease pathogenesis and identify novel therapeutic targets in T-ALL.

TAL1 is a basic helix-loop-helix transcription factor that functions as a master regulator of hematopoiesis and cardiovascular development.\textsuperscript{8-10} Significantly, in hematopoietic stem cells, TAL1 complexes with a number of important transcription factors including GATA and RUNX1 to regulate a complex transcriptional cascade critical for differentiation, cell-fate determination, and population maintenance.\textsuperscript{11,12} Furthermore, TAL1 expression has been observed in endothelial precursors, multipotent progenitor cells, and megakaryocyte and erythrocyte lineages.\textsuperscript{12,13} Given its pivotal role in normal hematopoiesis, it is unsurprising that misregulation of TAL1 has been implicated in leukemia. Indeed, ectopic expression of TAL1 due to various chromosomal aberrations is present in 40% to 60% of T-ALL cases. In particular, translocation of the TCL promoter (t;1:14) upstream of the TAL1 gene locus as well as an intergenic deletion leading to a SIL-TAL1 fusion protein are common observations in addition to a variety of other alterations and point mutations.\textsuperscript{14-16} Thus, TAL1 has emerged as a critical target in understanding T-ALL biology and for the development of novel therapeutics.

Like in several other cancers, the role of epigenetic modifications, microRNAs (miRNAs) has been explored in T-ALL as well. Studies have elucidated the impact of transcription factors such as TAL-1 on miRNA expression profiles.\textsuperscript{17-19} However, little attention in T-ALL.

Using an in silico approach, we identified that TAL1 was a target gene for miR-7. Although miR-7 is involved in the development of multiple organs and biological function of cells, growing evidence indicates the role of miR-7 in growth, migration, and invasion of multiple cancers.\textsuperscript{19,20} Further, the expression of miR-7 in ALL has been associated with a poor prognosis. However, the role of miR-7 in the molecular subset of pediatric T-ALL has not been explored so far. We thus investigated the role of miR-7 in mediating pathogenesis of T-ALL.

**Materials and Methods**

**Patient Samples and T-ALL Cell Lines**

Primary T-ALL cells were collected from pediatric patients following acquisition of informed consent from their guardians in accordance with the Declaration of Helsinki and national ethics guidelines. This study was approved by the institutional review board of Daqing Oilfield General Hospital (approval no. DQM-yan-2019101). Age-matched participants with no manifestations of any hematological malignancy were used as control. All patients were younger than 12 years. Only cases with bone marrow (BM) samples containing 70% leukemic cells were enrolled in this study. All T cells were collected from BM prior to treatment initiation. The human T-ALL cell lines (JURKAT, PF-382, MOLT-4, LOUCY SUPT-11, ALL-SIL, SUP-1, and CCRF-CEM) were maintained in RPMI-1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. Cells were cultured at 37 °C with 5% CO\textsubscript{2}. All patients were provided the informed consent for using of their tissues in this research.

**Oligonucleotides, Cell Transfection, and Real-time Polymerase Chain Reaction**

The sequences of miRNAs used in this study were as follows: negative control (NC) miRNA; 5'-UUCUCGGAGGUUGUACUGUTT-3', miR-7; 5'-UGGAAGACUAGUGAUUUUGUUGU-3'. Control and TAL1 expression plasmids were obtained from Addgene. Negative control and miR-7 were transfected into cells at 70% to 80% confluence using Lipofectamine RNAiMAX Reagent (Thermo Fisher Scientific). Cells were cotransfected with miRNA, luciferase reporter, or TAL1 overexpression plasmids using Lipofectamine 3000 Reagent. All transfections were performed according to the manufacturer’s instructions. At 6 hours posttransfection, the medium was replaced with fresh medium containing 10% FBS. Real-time polymerase chain reaction (RT-PCR) was performed as previously described.\textsuperscript{21} MicroRNA and messenger RNA (mRNA) quantification was performed using the SYBR Green method (Applied Biosystems) on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems) in accordance with the manufacturer’s instructions. U6 snRNA was used as an internal control for miRNA quantification and GAPDH was used for mRNA quantification. Relative levels of gene expression were represented using the 2^-ΔΔCt method. The following primer sequences were used: U6 snRNA forward 5'-CTCGCTTCCGCGCACCATATACT-3' and reverse 5'-ACGCTTCACGAATTTCGCTTGTC-3'; TAL1 forward 5'-GGTCTTTGGGGAGCGGAGTG-3' and reverse 5'-ACATTCCTGCTGCCGCATCG-3'; TRAF3 forward 5'-ACAGAAGTGCAGGTCCAGACTCT-3' and reverse 5'-GCGTGGTGTAACACTGAGAGTA-3'; RAB40B forward 5'-GGGTTATGCTTTGCA-3' and reverse 5'-GAATTTCCTGGCGGCTG-3'; EPHB1 forward 5'-CTTTGACCCATCCAGATGG-3' and reverse 5'-GCTCCCTGAGTGCACACCACGC-3'; GAPDH forward 5'-GGGTTGAAACCATGGAAGTGG-3' and reverse 5'-TGATCCTCCCAGGATAACAAA-3'. The experiments were repeated in 3 times.

**Luciferase Assays**

The wild-type full-length TAL1 3’-untranslated region (3’-UTR) was amplified and ligated into the psi-CHECKTM luciferase reporter vector (Promega). The mutated TAL1 3’-UTR was generated utilizing the TAL1 3’-UTR plasmid as a template and mutating the miR-7 seed binding site using the
QuikChange Multi Site-Directed Mutagenesis kit (Stratagene). JURKAT and CCRF-CEM cells were cultured for 24 hours and transfected with a mixture of 2 μL Lipofectamine 2000 (Life Technologies), 200 μL of OPTIMEM medium (Thermo Fisher Scientific), 100 ng of the reporter vector, and 100 nM of miR-7 or NC. After 48 hours, cells were lysed and processed according to the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase and Renilla luciferase activity were measured and calculated as described previously. The experiments were repeated in 3 times.

**Western Blot**

Western blot was performed as previously described. Anti-bodies including anti-TAL1, anticleaved caspase-3, anti-Bcl-2, and anti-β-actin were obtained from Santa Cruz Biotechnology. The experiments were repeated in 3 times.

**3-(4,5-dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide Assays**

3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide assays were performed to examine cell growth. 5 × 10^5 JURKAT or CCRF-CEM cells per well were seeded into a 96-well plate and maintained as described above. The MTT reagent (20 μL/well) was added and the plate was incubated for 3 to 4 hours at 37 °C. The MTT reagent was then removed and 150 μL of dimethyl sulfoxide was added to each well to solubilize the formazan. Absorbance was measured at 590 nm using a microplate reader. The experiments were repeated in 3 times.

**Colony Formation**

Colony formation was performed as previously described. The experiments were repeated in 3 times.

**Annexin-V FITC Apoptosis Assay**

The Annexin-V Fluorescein isothiocyanate (FITC) apoptosis assay was performed using the Annexin-V: FITC apoptosis detection kit 1 (BD Biosciences). Cells (5 × 10^5) were centrifuged at 1100 rpm for 5 minutes and resuspended in 0.5 mL binding buffer. Annexin-V FITC (5 μL) and propidium iodide (50 μg/mL, 5 μL) were then added into the cell suspension. Cells were incubated at room temperature for 5 minutes in the dark and analyzed by flow cytometry within 30 minutes. The experiments were repeated in 3 times.

**TUNEL Assay**

Apoptotic cells on coverslips were stained with DeadEnd Fluorometric Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) System (Promega) according to the manufacturer’s protocol. Coverslips were mounted on glass slides with VECTRASHIELD (Vector Laboratories). Detection of free 3'-OH ends was performed through the addition of digoxigenin-conjugated nucleotides or fluorescein-12-deoxyuridine triphosphate (dUTP) by terminal deoxynucleotidyl transferase and visualized by fluorescent microscopy. In each sample, at least 100 transfected cells were counted. The experiments were repeated in 3 times.

**Cell Migration and Invasion Assays**

Cell migration and invasion assays were performed using 8 μM-pore membrane Transwell inserts (BD Biosciences). For the migration assay, JURKAT or CCRF-CEM cells (1 × 10^5 cells per chamber) were seeded into the upper chambers in serum-free RPMI-1640 medium supplemented with 0.1% bovine serum albumin (BSA). Complete RPMI-1640 medium with 10% FBS was placed into the lower chambers. After 24 hours, cells on the upper surface of the chamber were removed with a cotton swab, and the inserts were fixed with 4% paraformaldehyde and stained with crystal violet. Cells attached to the lower surface of the filter were counted in 6 randomly selected areas using a microscope. For invasion assays, the Transwell inserts were coated with a layer of Matrigel Growth Factor Reduced Matrix (BD Biosciences). For time-lapse video assessment of cell movement, phase-contrast images were obtained every 60 seconds for 30 minutes at 37 °C with 5% CO₂. The velocities and accumulated distances of 20 randomly selected T-ALL cells were determined by manually tracking individual cells using ImageJ (NIH) software and a manual tracking plugin. The experiments were repeated in 3 times.

**Statistics**

Data are expressed as mean ± standard deviation of 3 independent experiments. Differences among groups were analyzed using 1-way analysis of variances or paired t tests. A P value <.05 was considered statistically significant. All statistical analyses were conducted using GraphPad Prism 6 (GraphPad Software).

**Results**

**MicroRNA-7 Expression Is Suppressed in T-ALL**

To investigate the role of miR-7 in T-ALL, we first analyzed the expression levels of miR-7 in 2 publicly available data sets. We found that T-ALL primary cells and cell lines express lower levels of miR-7 than thymocytes, BM precursors, and CD34⁺ hematopoietic progenitor cells (Figure 1A). MicroRNA-7 (miR-7) levels were also significantly lower in T-ALL primary cells and cell lines compared with normal hematopoietic T-cells (Figure 1B). These results indicate that miR-7 is downregulated in human T-ALL, suggesting a functional role in disease pathogenesis.

**MicroRNA-7 Directly Targets TAL1**

Next, we assessed the expression of miR-7 in various genetic subtypes of human T-ALL. We found miR-7 expression negatively correlated with TAL1 expression in 40 primary T-ALL patient samples (Figure 2A and B). Consistent with our
findings in clinical samples, miR-7 was highly expressed in TAL1-negative cell lines as compared to the TAL1-positive cell lines (Figure 2C). To identify potential miRNAs, targeting TAL1, we undertook a bioinformatic analysis using TargetScan. Through our analysis, we found that miR7 targets TAL1 at sites depicted in Figure 2D. To experimentally confirm, whether TAL1 is a target gene of miR-7, we fused firefly luciferase to the 3'-UTR of wild-type or mutant TAL1 to form reporter constructs. These were then cotransfected into JURKAT or CCRF-CEM cells along with either a miRNA NC or miR-7. Using a luciferase assay, we then demonstrated that the reporter harboring the wild-type TAL1-3'-UTR was significantly reduced to 30% of baseline ($P < .01$) by miR-7 as compared to the NC. However, mutant TAL1-3'-UTR was unaffected by miR-7 (Figure 2E). Furthermore, RT-PCR and Western blot analysis revealed that the expression of TAL1 was significantly downregulated in miR-7-transfected JURKAT and CCRF-CEM cells (Figure 2F and G). MicroRNA-7 overexpression suppressed TAL1 downstream genes including TRAF3, RAB40B, and EPHB1 (Figure 2H). Finally, we examined miR-7 expression levels in LOUCY and SUPT-1 cells overexpressing TAL1 and identified significantly decreased expression of miR-7 (Figure 2I). Taken together, these data suggest that TAL1 is a direct target of miR-7 and that TAL1 also inhibits miR-7 expression through negative feedback.

**MicroRNA-7 Impairs Growth and Induces Apoptosis of T-ALL Cells by Regulating TAL1**

We therefore investigated the functional impact of miR-7 on the growth of T-ALL cells. Both JURKAT and CCRF-CEM cells transfected with miR-7 demonstrated significant reductions in growth compared with those transfected with NC (Figure 3A and B). Anchorage-independent growth, as assessed by soft agar assays, was significantly reduced in miR-7-transfected cells to about 60% of that of NC-transfected cells (Figure 3C and D). These effects were corroborated by investigations of cell apoptosis, in which we found that miR-7 overexpression led to an increased number of Annexin V positive cells, increased caspase-3 levels, and decreased Bcl-2 expression (Figure 3E and F). Additionally, the number of TUNEL-positive cells was significantly higher in T-ALL cells transfected with miR-7 as compared to control cells (Figure 3G and H). We then tested whether the decrease in TAL1 expression observed after miR-7 transfection was responsible, at least in part, for growth inhibition and apoptosis. We therefore cotransfected TAL1 and miR-7 or NC into T-ALL cells. As expected, anchorage-independent cell growth and apoptosis were rescued in the context of TAL1 overexpression (Figure 3A-H).

**MicroRNA-7 Suppressed Migration, Invasion, and Cell Motility of T-ALL Cells by Regulating TAL1**

Using the transwell migration assay, we found that overexpression of miR-7 resulted in decreased directional cell migration and invasion in control JURKAT and CCRF-CEM cells, but not in cells overexpressing TAL1 (Figure 4A and B). This suggests that miR-7 negatively regulates cell migration and invasion via inhibition of TAL1. Using time-lapse microscopy, we found that miR-7 overexpression resulted in slower migration velocity and shorter migration distance over a span of 30 minutes (Figure 4C-E). By contrast, the inhibition of cell motility by miR-7 was not detected in TAL1-overexpressed T-ALL cells, which indicates that miR-7 reduces random movement of T-ALL cells via TAL1 as well. Together, these data indicate that miR-7 negatively regulates the migration, invasion, and motility of T-ALL cells. This phenotype is likely to be underpinned mechanistically by a reduction of TAL1 via miRNA-mediated decay.

**Discussion**

In this study, we have identified a critical and novel role for miR-7 in the pathogenesis of T-ALL. We found that the miR-7
expression levels are decreased in T-ALL cells compared with healthy controls and that the expression of miR-7 controls important cellular processes including cell growth, apoptosis, motility, and migration. Furthermore, we identified the T-ALL-associated transcription factor TAL1 as a target of miR-7, showing an inverse correlation between miR-7 and TAL1 expression, and showing evidence for direct binding of miR-7 to the TAL1 3′-untranslated region (3′-UTR). Furthermore, concomitant TAL1 overexpression attenuates the proapoptotic and migration-inhibitory effects of miR-7 expression, providing functional insight into the tumor-suppressive ability of miR-7 as well as mechanistic understanding concerning the role of TAL1 in T-ALL oncogenesis.

T-cell acute lymphoblastic leukemia is an important yet understudied subtype of ALL. Because of this, treatment regimens are typically like those used in B-ALL, despite abundant evidence indicating that T-ALL is governed by distinct biological processes. Induction usually entails use of an anthracycline (doxorubicin or daunorubicin), vincristine, a steroid such as dexamethasone, and intrathecal methotrexate. It has also been shown that asparaginase improves efficacy in conjunction with methotrexate, especially for pediatric patients. Despite great success with these treatment modalities, nearing 85% for new-onset disease, much work remains. For instance, ideal consolidation therapy in T-ALL is an area of active research. More saliently, outcomes in recurrent disease are notoriously poor. Although the purine nucleoside analog Nelarabine has had promising results in recent clinical trials, new therapeutics are needed. Targeting specific molecular pathways has been...
successful for other hematologic cancer subtypes and may therefore be effective here.

Although transcription factor TAL1 plays an important role in normal hematopoiesis, its expression is strictly regulated by several tumor-suppressive E-box binding proteins including E2A and HEB. With ectopic expression of TAL1 due to translocation events, it cooperates with several other complex factors including GATA3 and RUNX1 to both increase their own expression in an autoregulatory feedback loop as well as inhibit expression of tumor suppressor E-box proteins. This leads to a differentiation failure, allowing for mutations in NOTCH1 to accumulate that ultimately result in a proliferative phenotype and oncogenesis. Similar to other cancers, several miRNA genes have been identified in the context of

Figure 3. MicroRNA-7 (miR-7) impairs growth and induces apoptosis of T-cell acute lymphoblastic leukemia (T-ALL) cells, which is reversed by TAL1 overexpression. 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay of JURKAT (A) or CCRF-CEM (B) cells transfected with negative control (NC), miR-7, or miR-7 + TAL1 plasmid at day 0, 1, 2, 3, 4, and 5. **P < .001 compared to NC-transfected cells. Soft agar assay of JURKAT (C) or CCRF-CEM (D) Cells transfected with NC, miR-7, or miR-7 + TAL1 plasmid. Representative images are shown (left panel) as well as colony numbers (right panel). **P < .001 compared to NC-transfected cells. Cell apoptosis characterized by Annexin V positive cells (E), cleaved caspase-3, and Bcl-2 level (F) and TUNEL staining (G, H) in JURKAT or CCRF-CEM cells transfected with NC, miR-7, or miR-7 + TAL1 plasmid. **P < .001 compared to NC-transfected cells.
T-ALL. Some of the genes found to be significantly regulated upon overexpression or silencing specifically of TAL1 were miR-135a, miR-223, miR-330-3p, miR-146b-5p, miR-545. Although most of the studies were identifying regulation of miRNAs by TAL, we evaluated the regulation of TAL1 through epigenetic regulation mediated by miRNA. Through our bioinformatic analysis, we confirmed miR-7 as the miRNA with target sites on TAL1. Consistent with other reports, our in vitro data suggest that miR-7 negatively regulates TAL1, by directly targeting TAL1 for degradation, likely functions as a tumor suppressor by inhibiting this tumorigenic molecular cascade.

Figure 4. MicroRNA-7 (miR-7) suppresses migration, invasion, and cell motility in T-cell acute lymphoblastic leukemia (T-ALL) cells, which can be rescued by TAL1. JURKAT or CCRF-CEM cells transfected with NC, miR-7, or miR-7 + TAL1 plasmid. (A) Transwell migration analysis of JURKAT or CCRF-CEM cells transfected with NC, miR-7, or miR-7 + TAL1. (B) Transwell invasion analysis of JURKAT or CCRF-CEM cells transfected with NC, miR-7, or miR-7 + TAL1. (C) Representative migration images of individual JURKAT (top panel) or CCRF-CEM (bottom panel) cells recorded for 30 minutes by time-lapse video microscopy (n = 20). Mean velocity (D), accumulated, and traveled distance (E) of 20 individual cells transfected with NC, miR-7, and miR-7 + TAL1 assessed in (C). **P < .001 compared to miR-7 transfected cells.
MicroRNA-7 is well known as a tumor regulator in various malignant tumors besides T-ALL. For instance, Jiang and colleagues provided evidence that miR-7 regulates proliferation in chronic myeloid leukemia. The expression of miR-7 has been related to cellular migration in several cancer subtypes. It has also been shown to have prognostic value in breast cancer and to regulate migration and metastasis. Furthermore, miR-7 regulates sensitivity and modulates resistance to a number of chemotherapeutic agents in a variety of tumor types. Our findings concerning the role of miR-7 in T-ALL proliferation and migration are consistent with these studies, which in turn suggest that miR-7 may play an important role in drug sensitivity and resistance in T-ALL. This should be an avenue of further research.

Conclusions
Although we have uncovered evidence that miR-7 functions as a tumor suppressor by acting through TAL1, much further work remains. For instance, it would be important to elucidate the mechanism whereby TAL1 inhibits miR-7 expression and whether this process can be modified. Additionally, it is currently unclear what role, if any, that miR-7 plays in TAL1-negative T-ALL. Although the in vitro findings need an in vivo validation, it would be of interest to explore potential therapeutic avenues for miR-7 in the treatment of T-ALL and to identify further miRNAs that regulate other members of the TAL1 signaling cascade.

Authors’ Note
The data used to support the findings of this study are available from the corresponding author upon request. H.S. and Z.Z. contributed equally to this work. H.S. designed the manuscript; H.S., Z.Z., and W.L. performed experiments; H.S. and S.X. collected the data; H.S., Z.Z., and J.L. analyzed the results; Z.Z. and Y.L. wrote the manuscript.

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Declaration of Conflicting Interests
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ORCID iD
Hongbo Sun https://orcid.org/0000-0002-8404-4996

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