Long non-coding RNA HAGLROS promotes the development of diffuse large B-cell lymphoma via suppressing miR-100

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Abstract

Background: Long non-coding RNAs (lncRNAs), a vital component of functional regulators, are involved in various human cancers development, including diffuse large B-cell lymphoma (DLBCL). In particular, lncRNA HAGLROS has been reported to be associated with several types of cancer in humans. Nevertheless, the role of HAGLROS in DLBCL has yet to be described.

Methods: The HAGLROS expression patterns and its relationship with clinicopathological features and survival were investigated in DLBCL patients. CCK-8 and transwell assays were used to analyze the cell proliferation, migration, and invasion capacities. AGO2-RIP, dual-luciferase assay, RT-qPCR, and rescue experiments were fulfilled to measure the physical interaction between HAGLROS and miR-100. Xenograft assay was conducted to test tumor growth ability.

Results: HAGLROS was upregulated in DLBCL tissues and cells, and closely associated with advanced clinicopathological features. Upregulation of HAGLROS resulted in poor survival outcomes in DLBCL patients. In addition, HAGLROS knockdown inhibited the proliferation, migration, and invasion of DLBCL cells in vitro. Further experiments revealed that HAGLROS negatively regulated the expression of miR-100 in DLBCL, and the expression of miR-100 and HAGLROS showed an inverse correlation in DLBCL tissues. HAGLROS functioned as a competing endogenous RNA for miR-100 in DLBCL cells, and miR-100 overexpression abolished the oncogenic effects of HAGLROS upregulation on DLBCL progression. Besides, in-vivo assays revealed that HAGLROS knockdown suppressed tumor growth in nude mice.

Conclusion: HAGLROS overexpression contributes to DLBCL development and poor prognosis via targeting miR-100, which could be a potential prognostic biomarker and therapeutic target for DLBCL patients.

Keywords: biomarker, DLBCL, HAGLROS, miR-100, prognosis
INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most prevalent subtype of B-cell-derived non-Hodgkin’s lymphomas (NHLs), accounting for 30% to 40% of all NHL cases in China.1 DLBCL is an invasive lymphoma with high heterogeneity in terms of genetic findings, treatment response, and clinical prognosis.2 Chemotherapy is the current first-line treatment modality for patients with DLBCL, which has achieved significant results in the therapeutic response rate.3 However, there are still about 1/3 of patients insensitive to the first-line treatment, often resulting in refractory and relapse, and ultimately increasing the mortality of DLBCL.4 It has been shown that some genomic alterations play a vital role in the initiation and development of DLBCL, although the pathogenesis of DLBCL has yet to be fully elucidated.5 Therefore, there is a clear need to identify more specific biomarkers for the early surveillance and effective treatment for DLBCL patients as early as possible.

Long non-coding RNAs (lncRNAs) are a kind of non-coding RNA transcripts with a length of more than 200 nucleotides.6 LncRNAs have no protein-coding capacity, while they are actively involved in an assortment of physiological and pathological processes, modulating gene expression, cancer initiation and metastasis, and disease prognosis.7–9 Recently, growing evidence has demonstrated that there exists a wealth of lncRNAs, including MALAT1, SNHG14, and NEAT1, were differentially expressed in DLBCL cells, and are closely correlated with the malignant processes of DLBCL.10–12 LncRNA HAGLROS, 699 nucleotides in length, is recently reported to participate in the malignant processes of some cancers, including gastric cancer,13 hepatocellular carcinoma,14 intrahepatic cholangiocarcinoma,15 osteosarcoma,16,17 lung cancer,18,19 and ovarian cancer.20,21 However, the specific role of HAGLROS in DLBCL has yet to be described.

In this study, we detected the expression profile of HAGLROS in DLBCL tissues and cells, and then investigated the association between HAGLROS expression level and clinicopathological features and survival outcomes in patients with DLBCL. We also investigated the biological performances of HAGLROS with regards to the proliferation, migration, and invasion of DLBCL cells. Furthermore, HAGLROS is reported to play a crucial role in gastric cancer by functioning as a competing endogenous RNA (ceRNA) to sponge miR-100.22 We explored whether HAGLROS modulated the progression of DLBCL cells by competitively sponging miR-100. This study for the first time provided a novel insight into the oncogenic role of HAGLROS in DLBCL, which could result in better understanding of DLBCL pathogenesis and treatment.

MATERIALS AND METHODS

2.2 | Cell culture

We purchased human normal B-cell-immortalized cell line (HMy2. CIR) and DLBCL cell lines (Farage and SU-DHL-4) from Shanghai Chinese Academy of Sciences Cell Bank (China). HMy2.CIR was cultured in Iscove’s modified Dulbecco’s medium (IMDM) (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Life Technologies) and 1% penicillin-streptomycin. Farage and SU-DHL-4 were cultured in RPMI 1640 medium (Gibco) containing 10% FBS and 1% penicillin-streptomycin. All cells were cultured under a humidified incubator at 37°C with 5% CO2.

2.3 | RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using TRizol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Complementary DNA (cDNA) was then synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher, USA) in accordance with the manufacturer’s protocol. RT-qPCR reactions were then conducted using the SYBR® Premix Ex Taq (Takara Bio, Inc.) on an ABI prism 7500 sequence detection system (Applied Biosystems). The relative expression levels of HAGLROS and miR-100 were normalized to GAPDH and U6 using the 2−ΔΔCt method, respectively.22 All sequences of the oligonucleotides (GenePharma Co., Ltd, Shanghai, China) used in this study are shown in Table S1.

2.4 | Cell transfection

DLBCL cells were transfected with siRNAs for HAGLROS (si-HAGLROS#1 or #2) and its negative control (si-NC), the overexpressed previous radiotherapy and chemotherapy and their diagnosis was pathologically confirmed by biopsy. The control group consisted of 51 patients with reactive hyperplasia of lymph nodes, and the lymphmatic tissue samples were obtained via biopsy from them. All of the clinical samples were immediately frozen in liquid nitrogen after biopsy and then frozen at −80°C to await analysis. The DLBCL cohort included 45 males (53.0%) and 55 females (47.0%), aged 19–74 years, with a median age of 51.2 years (Table 1). The control group included 24 females and 27 males, aged 16–64 years, with a median age of 44 years. All DLBCL patients had received follow-up after initiate treatment by the same team between January 2012 and January 2021. A range of clinical data, including age, gender, B symptoms, extrathyroidal extension, tumor-node-metastasis (TNM) stage, extra-nodal invasion, and International Prognostic Index (IPI) were recorded. All patients signed an informed consent form. This study was approved by the ethics committee of The Affiliated Hospital of Nantong University and was conducted in accordance with the Declaration of Helsinki.

2.1 | Clinical samples

A total of 100 patients diagnosed with DLBCL were recruited from The Affiliated Hospital of Nantong University between January 2012 and December 2014. The recruited DLBCL patients did not undergo
plasmids for HAGLROS (pc-HAGLROS) and its negative control (pcDNA3.1), miR-100 mimics and NC-mimics (GenePharma Co., Ltd, Shanghai, China). In brief, DLBCL cells were seeded in 6-well plates at a density of $1 \times 10^5$ cells per well for 24 h. Cell transfection was then conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) based on the manufacturer's instructions. Transfected cells were then cultured for an additional 48 h at 37°C before being used in downstream experiments.

### 2.5 | Cell counting kit-8 (CCK-8) proliferation assays

We used CCK-8 solution (Dojindo, Kumamoto, Japan) to detect cell proliferation. 100 µL of DLBCL cell suspension ($2 \times 10^4$ cells ml$^{-1}$) were seeded into a 96-well plate. Then, 10 µg of CCK-8 solution was added to each well after cultured for 1, 2, 3, and 4 days. Optical density (OD) values were then measured using an automatic microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at an absorbance of 450 nm.

### 2.6 | Transwell assays

Polyethylene membranes (24-well inserts; 8.0 µm; Corning, Inc.) were used to detect the migration and invasion abilities of DLBCL cells. Chambers were precoated with 50 µl of Matrigel (BD Biosciences) at 37°C for 1 h and used for invasion assays, while uncoated chambers were used for migration assays. Cell suspensions containing $1 \times 10^5$ cells in 100 µL of FBS-free RPMI 1640 were seeded into the upper chamber. Meanwhile, the lower chamber was covered with 500 µL of RPMI 1640 supplemented with 10% FBS. After cultured at 5% CO$_2$ and 37°C for 48 hours, the migration and invasive cells were fixed with 4% polyoxymethylene, and then stained with 0.1% crystal violet. Cells were counted in five randomly selected fields using a light microscope (Leica Microsystems GmbH, Wetzlar, Germany).

### 2.7 | RNA immunoprecipitation (RIP) assay

The RIP assay was conducted using the RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA). Human anti-Ago2 antibodies (Millipore, USA) were utilized to capture the RNAs used for RT-qPCR analysis. HAGLROS or miR-100 expression were measured by RT-qPCR.

### 2.8 | Luciferase reporter assay

Cells were placed in a 24-well plate and co-transfected with mutant (MUT) or wild-type (WT) HAGLROS, together with miR-100 mimics and NC-mimics (GenePharma Co., Ltd, Shanghai, China). In brief, DLBCL cells were seeded in 6-well plates at a density of $1 \times 10^5$ cells per well for 24 h. Cell transfection was then conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) based on the manufacturer's instructions. Transfected cells were then cultured for an additional 48 h at 37°C before being used in downstream experiments.

### TABLE 1 | HAGLROS expression and clinicopathological features in 100 cases of DLBCL

| Variables          | Total (n = 100) | HAGLROS, n (%) | P-value |
|--------------------|----------------|----------------|---------|
|                    |                | High expression (n = 50) | Low expression (n = 50) |
| Age, year          |                |                |         |
| <60                | 49             | 23 (46.0)      | 26 (52.0) | 0.548 |
| ≥60                | 51             | 27 (54.0)      | 24 (48.0) |         |
| Gender             |                |                |         |
| Male               | 45             | 21 (42.0)      | 24 (48.0) | 0.546 |
| Female             | 55             | 29 (58.0)      | 26 (52.0) |         |
| B symptoms         |                |                |         |
| Absent             | 61             | 28 (56.0)      | 33 (66.0) | 0.305 |
| Present            | 39             | 22 (44.0)      | 17 (34.0) |         |
| TNM Stages         |                |                |         |
| I-I                | 54             | 20 (40.0)      | 34 (68.0) | 0.005 |
| III-IV             | 46             | 30 (60.0)      | 16 (32.0) |         |
| Extra-nodal invasion |            |                |         |
| Yes                | 57             | 35 (70.0)      | 22 (44.0) | 0.009 |
| No                 | 43             | 15 (30.0)      | 28 (56.0) |         |
| IPI score          |                |                |         |
| 0-2                | 43             | 16 (32.0)      | 27 (54.0) | 0.026 |
| 3-5                | 57             | 34 (68.0)      | 23 (46.0) |         |

Note: Abbreviations: DLBCL, diffuse large B-cell lymphoma; IPI, International Prognostic Index. $p < 0.05$ is showed in bold.
or NC-mimics using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 48 h of co-transfection, cells were evaluated for luciferase with a dual-luciferase reporter assay system (E1910, Promega, WI, USA).

### 2.9 | Xenograft assay

Male BALB/c nude mice (aged 4–5 weeks, \(n = 8\), weighing 18–20 g) were purchased from SLAC Laboratory Animal Company (Shanghai, China). Farage cells were transfected with si-HAGLROS#1 or si-NC. Subsequently, Farage cells were suspended in phosphate-buffered saline at a density of \(1 \times 10^7\) cells ml\(^{-1}\), following that 100 µl of the cell suspension were injected into the right flanks of nude mice to form tumor (\(n = 4\) for each group). A Vernier caliper was used to measure the length and width of tumors every week, and the tumor size was calculated using the equation: length \(\times\) width\(^2\) \(\times 0.5\). All mice were euthanized by isoflurane after 4 weeks, and the tumors were excised and weighed. All animal experimental procedures were approved by the Ethics Committee of The Affiliated Hospital of Nantong University.

### 2.10 | Statistical analyses

All data are expressed as mean ± standard deviation of at least three experiments. Statistical evaluations were performed using SPSS 20.0 (IBM SPSS Inc., Chicago, IL, USA). Differences between two groups were analyzed using the unpaired Student’s t-test. Comparisons of multiple groups were analyzed by analysis of variance (ANOVA) followed by Dunnett’s test. Categorical data were compared using the chi-squared test. Overall survival (OS) of patients with DLBCL were then evaluated using Kaplan-Meier curves and compared using the log-rank test. Prognostic factors were analyzed by Cox regression proportional hazards analysis. Correlation analysis was performed using Pearson’s method. Differences were considered to be significant when \(p < 0.05\).

## 3 | RESULTS

### 3.1 | HAGLROS is upregulated in human DLBCL tissues and correlates with poor prognosis

First, we investigated the expression profiles of HAGLROS in DLBCL tissues. Then, a total of 100 DLBCL tissues and 51 lymphatic tissues were collected for RT-qPCR analysis. Analysis showed that HAGLROS expression levels were significantly upregulated in DLBCL tissues when compared to controls (\(p < 0.001\), Figure 1A). Subsequently, the relative expression levels of HAGLROS were evaluated in human normal B-cell-immortalized cell line (HMy2.CIR) and DLBCL cell lines (Farage and SU-DHL-4). RT-qPCR data showed that HAGLROS expression was significantly higher in DLBCL cells than in HMy2.CIR cells (\(p < 0.001\), Figure 1B).

Next, we analyzed the relationship between HAGLROS expression levels in DLBCL tissues and the clinicopathological features of these DLBCL patients. First, we separated the 100 DLBCL patients into a HAGLROS low expression group (\(n = 50\)) and a HAGLROS high expression group (\(n = 50\)) based on the median values of HAGLROS expression in DLBCL tissues. The expression levels of HAGLROS in tumor tissues were significantly correlated with TNM stage, extranodal invasion, and IPI score (Table 1). Next, the Kaplan-Meier curves indicated that higher expression level of HAGLROS was associated with shorter OS (Figure 1C; \(p = 0.001\)). By using univariate and multivariate COX regression analysis (Table 2), we found that TNM stage (HR: 1.854, 95% CI: 1.512–2.615, \(p < 0.001\)), extra-nodal invasion (HR: 1.677, 95% CI: 1.321–1.826, \(p = 0.012\)), IPI score (HR: 1.548, 95% CI: 1.312–1.846, \(p = 0.001\)), and high HAGLROS expression (HR: 1.330, 95% CI: 1.218–1.624, \(p = 0.005\)) were independent and significant indicators of a poor OS in DLBCL patients. Collectively,
our data indicated that HAGLROS is an independent unfavorable prognostic factor for DLBCL patients.

3.2 | HAGLROS knockdown suppresses the proliferation, migration, and invasion ability of DLBCL cells

Next, we investigated the proliferation, migration, and invasion abilities of HAGLROS in DLBCL tumor progression using CCK-8 and Transwell assays. First, we knocked down the expression levels of HAGLROS both in Farage and SU-DHL-4 cells using two HAGLROS siRNAs (p < 0.001, Figure 2A-B). CCK-8 assay results showed that the silencing HAGLROS expression significantly reduced the cell proliferation abilities both in Farage and SU-DHL-4 (p < 0.01, Figure 2C-D). Transwell assays demonstrated that downregulation of HAGLROS led to a significant reduction in the migratory and invasive capability in the above two cells (p < 0.001, Figure 2E-F).

3.3 | HAGLROS serves as a sponge for miR-100

It is well known that IncRNAs could influence the expression of miRNAs on the identify of ceRNA. HAGLROS could targeted miR-100 to modulate various cancers based on StarBase v2.0 (http://starbase.sysu.edu.cn/mirLncRNA.php) software and previous studies. Based on these predictions, we speculated the association between HAGLROS and miR-100 in DLBCL. The clinical tissue analysis showed that miR-100 expression levels were significantly downregulated in DLBCL tissues when compared to controls (p < 0.001, Figure 3A). Moreover, as confirmed by the Pearson correlation analysis, the expression of miR-100 and HAGLROS showed an inverse correlation in DLBCL samples (R = 0.73, p < 0.001, Figure 3B). The reduced expression of miR-100 was also discovered in DLBCL cell lines (Farage and SU-DHL-4) when compared to human normal B-cell-immortalized cell line (HM2.CIR) (p < 0.001, Figure 3C). In the DLBCL cells, enhanced overexpression of HAGLROS by cDNA caused miR-100 downregulation, while HAGLROS knockdown lead to miR-100 upregulation (p < 0.001, Figure 3D). These results show that HAGLROS and miR-100 are competitively expressed. To further determine the interaction of miR-100 and HAGLROS, we conducted the anti-AGO2-RIP and dual-luciferase reporter assays. HAGLROS has been reported to be mainly cytoplasmic located; thus, we supposed that HAGLROS may form an RNA-induced silencing complex with miRNAs in DLBCL. The data of anti-AGO2-RIP revealed a significant enrichment of HAGLROS and miR-100 in DLBCL cells (p < 0.001, Figure 3E). Moreover, the dual-luciferase reporter assay showed that significant decreased luciferase activity was observed in DLBCL cells transfected with wild-type HAGLROS and miR-100 mimics, but not the mutant HAGLROS (p < 0.001, Figure 3F).

3.4 | HAGLROS promotes the proliferation, migration, and invasion of DLBCL cells via sponging miR-100

To determine whether miR-100 is involved in HAGLROS oncogenic role in the progression of DLBCL, we transfected miR-100 mimics or NC-mimics in HAGLROS overexpressing Farage cells. As demonstrated in Figure 4A-C, HAGLROS overexpression alone distinctly increased the proliferation, migration, and invasion abilities of Farage cells, while these impacts were obviously weakened after overexpression of miR-100. Moreover, the inhibitory function of miR-100 in relation to cell proliferation, migration, and invasion was determined in Farage cells. All of these data showed that HAGLROS accelerates the proliferation, migration and invasion of DLBCL cells by interacting with miR-100.

3.5 | HAGLROS knockdown suppresses tumor growth in vivo

The above findings of in-vitro assays prompted us to analyze the function of HAGLROS on tumor growth in vivo. Hence, the xenograft assay was performed to validate the findings. The representative photo of excised tumors is shown in Figure 5A. The tumor growth

| TABLE 2 | Univariate and multivariate analysis for overall survival in 100 cases of DLBCL |
|---|---|---|---|
| Variables | Univariate | P-value | Univariate | P-value |
| | HR (95% CI) | | HR (95% CI) | |
| Age, ≥60 vs. <60 | 1.121(0.794–1.961) | 0.542 | 1.854(1.512–2.615) | <0.001 |
| Gender, male vs. female | 1.052(0.885–1.366) | 0.354 | 1.677(1.321–1.826) | 0.012 |
| B symptoms, present vs. absent | 1.254(0.854–1.985) | 0.154 | 1.564(1.398–1.814) | 0.005 |
| Stages, III-IV vs. I-II | 2.057(1.657–2.954) | <0.001 | 1.548(1.312–1.846) | 0.001 |
| Extra-nodal invasion, yes vs. no | 1.845(1.542–2.154) | 0.002 | 1.330(1.218–1.624) | 0.001 |
| IPI score, 3–5 vs. 0–2 | 1.745(1.451–2.054) | <0.001 | |
| HAGLROS expression, high vs. low | 1.564(1.398–1.814) | 0.001 | |

Note: Abbreviations: DLBCL, diffuse large B-cell lymphoma; IPI, International Prognostic Index. p < 0.05 is showed in bold.
curve demonstrated that HAGLROS knockdown notably hindered the tumor growth of Farage cells in vivo (Figure 5B). Moreover, the tumor weight was markedly reduced after HAGLROS knockdown (Figure 5C).

4 | DISCUSSION

Recently, growing attention has been focused on a range of IncRNAs that play fundamental biological roles in the progression of DLBCL. For instance, Zhao et al. demonstrated that SMAD5-AS1 overexpression could inhibit DLBCL proliferation in vitro and in vivo by sponging miR-135b-5p to upregulate APC expression.²⁴ Shi et al. reported that IncRNA FIRRE functioned as an oncogene by promoting cell proliferation and reducing cell apoptosis in DLBCL.²⁵ Various studies have associated HAGLROS with tumor progression. For instance, Chen et al. reported that HAGLROS was upregulated and correlated with poor outcomes in patients with gastric cancer and silencing HAGLROS expression could suppressed the cell proliferation, invasion and migration of gastric cancer.¹³ Wu et al. demonstrated that HAGLROS was frequently upregulated in osteosarcoma tissue and cells, and high HAGLROS expression was related to poor survival outcomes.¹⁷ Moreover, Chen et al. revealed that HAGLROS was significantly overexpressed in non-small cell lung cancer and associated with poor survival.¹⁸ These results highlighted the tumor-promotive roles of HAGLROS in above cancers.

In the current study, we analyzed clinical samples and found that HAGLROS was upregulated in DLBCL tissues and cells. Our study also revealed the close relationship between HAGLROS expression and a range of important clinical features. High expression levels of HAGLROS were closely correlated with advanced clinicopathological characteristics and poor survival outcomes. The functional assays showed that downregulation of HAGLROS could inhibit
the proliferation, migration, and invasion of DLBCL cells in vitro. Moreover, the in vivo experiment showed that HAGLROS knockdown suppressed the tumor growth of Farage cells in nude mice.

Increasing evidence have revealed that lncRNAs play a key role in many diseases via functioning as ceRNAs to regulate gene expression by sponging miRNAs. In gastric cancer cells, HAGLROS could increase mTOR expression by competitively sponging miR-100-5p to in gastric cancer cells. In line with this finding, we also found the negative correlation between HAGLROS and miR-100. The dual-luciferase reporter assays, AGO2-RIP, RT-qPCR, and rescue experiments confirmed that miR-100 was a direct target of HAGLROS. In previous studies, downregulation of miR-100 is observed in several
cancers, including lung cancer,\textsuperscript{26} hepatocellular carcinoma,\textsuperscript{27} and bladder cancer,\textsuperscript{28} and miR-100 is generally reported to be a tumor suppressor. In this study, miR-100 was found to be downregulated in human DLBCL tissues and cells, and miR-100 overexpression could inhibit the proliferation, migration, and invasion of DLBCL cells. Taken together, our results demonstrate that HAGLROS was upregulated in DLBCL and correlated with poor prognosis. Moreover, HAGLROS exerted a tumor-promoting role during the progression of DLBCL via targeting miR-100. This study may provide an original insight into the biology of DLBCL. However, this study had a clear limitation in that the deep underlying mechanisms of HAGLROS biological function remain unclear and the in vivo assays are not available.

**FIGURE 4** HAGLROS promotes proliferation, migration, and invasion of Farage cells through targeting miR-100. (A) Farage cell proliferation was measured by CCK-8 assay after co-transfection of HAGLROS plasmid and miR-100 mimics. (B) Farage cell migration and invasion were measured by transwell assay after co-transfection of HAGLROS plasmid and miR-100 mimics. (C) Calculation of Farage cells that migrated or invaded through the filter following eosin staining by transwell assay. ***p < 0.001

**FIGURE 5** HAGLROS knockdown suppressed tumor growth in vivo. (A) Representative images of the excised tumors in si-HAGLROS-1 and si-NC groups. (B) The tumor size curves from two different groups. (C) The tumor weight from two different groups. **p < 0.01, ***p < 0.001

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Not applicable.

**CONFLICT OF INTEREST**
No conflict of interest was declared in the study.

**DATA AVAILABILITY STATEMENT**
All data generated or analyzed during this study are available from the corresponding author on reasonable request.

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Supporting Information

Additional supporting information may be found in the online version of the article at the publisher’s website.