HAGLROS knockdown restrained cell proliferation, migration and invasion and facilitated apoptosis in laryngeal cancer via miR-138-5p/CLN5 axis

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Abstract
Background: This work investigated the role of HAGLROS in laryngeal cancer (LC).
Methods: HAGLROS expression in the head and neck squamous cell carcinoma (HNSC), target miRNAs of HAGLROS, target mRNAs of miR-138-5p, and the binding sites of HAGLROS and miR-138-5p or CLN5 and miR-138-5p were predicted through bioinformatics. HAGLROS, miR-138-5p, CLN5, Bcl-2, and Bax levels were detected by qRT–PCR and Western blot. The biological functions of LC cells were assessed through CCK-8, colony formation assays, transwell assay, and flow cytometry assay. The targeting relationship between HAGLROS and miR-138-5p or CLN5 and miR-138-5p was confirmed by dual luciferase gene reporter analysis.
Results: HAGLROS was upregulated in LC. HAGLROS-specific small interfering RNA (Si-HAGLROS) inhibited the viability, proliferation, migration, and invasion while increased the apoptosis in LC cells. MiR-138-5p was a target of HAGLROS and the miR-138-5p inhibitor reversed the effects of si-HAGLROS on LC cells. CLN5 was a target of miR-138-5p. MiR-138-5p inhibitor raised the viability, migration and invasion, and Bcl-2 expression while declined Bax expression in LC cells, with si-CLN5 performing the opposite effects and reversing the effects of miR-138-5p inhibitor.
Conclusion: Silenced HAGLROS restrained the LC cells’ abilities to proliferate, migrate, and invade as well as facilitated apoptosis in LC via miR-138-5p/CLN5 axis.

KEYWORDS
CLN5, HAGLROS, Laryngeal cancer, MiR-138-5p

1 | INTRODUCTION

Laryngeal cancer (LC) is a malignant tumor possessing the highest morbidity and mortality in the head and neck cancer, and there will be approximately 30,832 people newly diagnosed with LC and 16,939 people dying from LC in China according to the cancer statistics in 2022.\cite{1,2} perhaps accounts for 1%-5% cancer rate in the whole world. LC usually brings about great functional and psychological inconvenience for patients, especially in the communication and eating.\cite{3} Smoking, alcohol, male gender, and old age are extensively accepted as risk factors of LC, with genetic elements also playing a vital part in LC occurrence and advancement.\cite{4,5,6} At
present, surgical resection, radiotherapy, and chemotherapy are primary therapeutic methods for LC. However, despite the high cure rate of LC in the early stage through surgery or radiotherapy, the consequences of most patients with the advanced disease remain unsatisfactory, and uncontrolled recurrence, metastasis as well as the developed resistance to radiotherapy or chemotherapy further result in a poor prognosis. What’s worse, surgical intervention probably causes a partial or complete loss of vocal function, leading to the lifelong maintenance of tracheal cannula in many LC patients because of total laryngectomy. Thus, the molecular mechanisms of LC progression should be delved into so as to provide some new insights for LC early diagnosis and treatment.

Long noncoding RNAs (lncRNAs) are defined as a class of ncRNAs ranging from 200 bp to more than a few hundred kilo-bases in size, which was widely believed to be "genomic junk" for a long time. Recently, it was reported that lncRNAs indeed participate in various cellular processes and molecular genetics through gene modulation in many aspects such as tracking between the nucleus and cytoplasm, imprinting, mRNA splicing, transcription, and epigenetic regulation. Furthermore, lncRNAs are reported to be implicated in diverse biological processes involved in physiology and pathology. Hence, lncRNAs are accepted as a novel target for cancer treatment, which has been confirmed in a number of studies. Previous researches have demonstrated that a few lncRNAs are found to be aberrantly regulated in LC tissues and several lncRNAs comprising CDKN2B-AS1, H19, NEAT1, and HOTAIR act as oncogenes to facilitate LC tumorigenesis, whereas MEG3 fulfills protective functions against LC. For example, overexpressed HAGLROS advances migratory and invasive abilities of non-small cell lung cancer and mediates autophagy to facilitate the malignant development of gastric cancer. But the role of HAGLROS in LC and the mechanism of action are still unknown.

In addition, lncRNAs can be used as recognition and regulatory elements of miRNA, and interact with miRNAs as part of the regulatory network to influence the activities of miRNAs. The interaction between lncRNAs and miRNAs can regulate the progression of many diseases, such as cancer. For example, HAGLROS accelerates the progression of lung carcinoma via sponging miR-152. In-cRNA TUG1 promotes the progression of colorectal cancer via the miR-138-5p/ZEB2 axis. miR-138-5p is a miRNA that is reported to function as a tumor suppressive role in several human cancers, such as prostate cancer, colorectal cancer, and gastric cancer, etc. In LC, miR-138-2-3p inhibited cell proliferation, induced cell apoptosis and cell cycle arrest and increase radio-sensitivity in LC stem cells. LncRNA TRPM2-AS served as a ceRNA of miR-138 to promotes cell migration and invasion in laryngeal squamous cell carcinoma. However, the biological roles of miR-138-5p in LC remain to be explored. Thus, in this study, we explored the biological role of HAGLROS and whether it can play a role in LC by regulating miR-138-5p.

2 | MATERIALS AND METHODS

2.1 | Bioinformatics

The differential expression of HAGLROS in the head and neck squamous cell carcinoma (HNSC) and normal samples were analyzed through starBase (http://starbase.sysu.edu.cn/). The Cancer Genome Atlas (TCGA) database (https://www.cancer.gov/about-ncci/organization/ccg/research/structural-genomics/tcga) was applied to predict the expression of CLN5 in HNSC and normal samples. The binding sites between HAGLROS/CLN5 and miR-138-5p were analyzed by IncSNP 2.0 (http://210.46.80.146/lnccsnp/search.php) and MiRDB (http://mirdb.org/index.html).

2.2 | Cell culture

Normal laryngeal cell line HuLa-PC and LC cell line (AMC-HN-8, SNU-46, SNU-899, and SNU-1076) bought from Fenghuishengwu (China) were incubated in RPMI-1640 medium (PM150110, Procell Life Science&Technology) enriched with 1% penicillin–streptomycin (PB180120, Procell) and 10% fetal bovine serum (FBS; 164,210, Procell) at 37°C in a humidified atmosphere containing 5% CO2.

2.3 | Cell transfection

HAGLROS specific small interfering RNA (si-HAGLROS 1/2/3; siG141231090457-1-5/siG141231090423-1-5/siG141231090406-1-5), si-CLN5 (siG000001203A-1-5), the negative control for siRNA (si-NC; siG000000002-1-10, RiboBio), miR-138-5p inhibitor and inhibitor control (I/IC, miR2N0000001-1-5/miR2N0000002-1-10) as well as miR-138-5p mimic and mimic control (M/MC, miR10000430-1-5/miR1N0000002-1-5) were ordered from RIBOBIO and transfected into SNU-46 and SNU-899 cells by transfection reagent (11668500, Thermo Fisher Scientific, USA). Cells were seeded at 5 × 104/well in 24-well plates and cultured until 50% (for siRNA) or 90% (for miR-138-5p I/IC/M/MC) confluence, subsequent to which siRNA or miR-138-5p I/IC/M/MC were added.

2.4 | Quantitative reverse transcription-polymerase chain reaction (qRT–PCR)

Total RNA kit (RK02009, Biomarker) and miRNA Isolation Kit (217004, Qiagen) were utilized for the extraction of total mRNA and miRNA from cells, respectively. Subsequently, cDNA was
suspended in 1 ml for 10 min. After removal of the supernatant, cells were re-

cultured at 37°C for 24, 48, or 72 h, following incubation with CCK-8 solution for 2 h. The optical density (OD) value at 450 nm was determined by an iMark™ Microplate Absorbance Reader (Beyotime Biotechnology).

2.5 | Cell viability assay

Cell counting kit (CCK)-8 kit (C0005, Topscience) was applied to detect cell viability. Digested and seeded in 96-well plates, cells were cultured at 37°C for 24, 48, or 72 h, following incubation with CCK-8 solution for 2 h. The optical density (OD) value at 450 nm was determined by an iMark™ Microplate Absorbance Reader (Beyotime Biotechnology).

2.6 | Colony formation assay

Trypsinized, 1 × 10^2 cells were seeded in 6-well plates and cultured with medium changed every 2 days. Two weeks later, the medium was discarded and 4% paraformaldehyde (BL-G002, SBJBIO) was mixed with cells for 20 min. After washing, cells were stained with crystal violet (BP-DL131, SBJBIO) for 10 min. The results were viewed under a CX43 microscope (AE2000, Motic, China).

2.7 | Flow cytometry assay

Apoptotic cells were determined by Annexin V-FITC/PI kit (SBJ-C0008, SBJBIO, China). Digested and collected, 1 × 10^6 cells were suspended in 1 ml Binding Buffer prior to centrifugation at 300 x g for 10 min. After removal of the supernatant, cells were re-suspended in 1 ml Binding Buffer to adjust the concentration to 1 × 10^6 cells/ml. Subsequently, 100 μl cell suspension was cultured with Annexin V-FITC and propidium iodide (PI), following which 500 μl PBS was added and gently mixed. The apoptosis was detected by a flow cytometer (AccuriC6, BD, USA).

2.8 | Transwell assay

As for detecting abilities of LC cells to migrate or invade, cells were deprived of serum and added into the upper chamber of a transwell insert coated with (invasion) or without (migration) matrigel (356,234, BD, USA). The complete medium was added to the lower chamber. After incubating for 48 h, the cells inside the inserts were removed by cotton swab, while the cells that pass through the inserts were undergo a fixation (4% paraformaldehyde, G1101, Servicebio) and staining (crystal violet, G1014, Servicebio) step. The results were observed using an optical microscope.

2.9 | Dual luciferase gene reporter (DLGR) analysis

Reporter vector (E1330, Promega Corporation) with human HAGLROS and CLN5 3′-untranslational region (UTR) sequences were constructed to acquire wild-type HAGLROS (HAGLROS-wt) and wild-type CLN5 (CLN5-wt) whereas the other reporter vector with mutative HAGLROS (HAGLROS-mut) and CLN5 (CLN5-mut) 3′-UTR sequences in the putative miR-138-5p seed region were regarded as the respective negative controls. HAGLROS-wt/HAGLROS-mut or CLN5-wt/CLN5-mut and miR-138-5p M/MC were co-transfected into SNU-46 and SNU-899 cells for 24 h. Luciferase activity of cells were analyze by DLGR system (E1910, Promega).

2.10 | Western blot

Cells centrifuged at 12,830 × g for 5 min at 4°C in RIPA lysis buffer (C500007, Sangon Biotech) to isolate total protein. Equal contents of protein (45 μg) were separated by SDS-PAGE. Then the protein was transferred into membranes blocked in 5% bovine serum albumin (BSA; BL-082, SBJBIO) at room temperature for 1 h, subsequent to which membranes were incubated with primary antibodies and secondary antibodies. The list of antibodies was shown in Table 2. Eventually, membranes were rinsed and visualization of protein expression was operated through ECL luminescence reagent (C510043, Sangon Biotech) in eZwest Lite Auto Western Blotting System (Genscript, Piscataway).

2.11 | Statistical analysis

Statistical data were analyzed by Graphpad 8.0 (GraphPad Software Inc., USA), which was presented as the means ± standard deviation. Each experiment was repeated in triplicate at least. Two groups were
compared through an independent-samples t-test while the contrast among multiple groups was made using one-way ANOVA. \( p < 0.05 \) implicated a statistically significant difference.

### 3 | RESULTS

#### 3.1 | HAGLROS knockdown restrained abilities of LC cells to proliferate, migrate, and invade yet facilitated apoptosis of LC cells

HAGLROS was predicted to be upregulated in HNSC by starBase based on 502 cancer and 44 normal samples (Figure 1A, \( p = 1.3e-29 \)). HAGLROS expression in LC cell lines (AMC-HN-8, SNU-46, SNU-899, SNU-1076) was evidently higher than normal laryngeal cell line HuLa-PC, of which SNU-46 and SNU-899 cells possessed relatively high level of HAGLROS (Figure 1B, \( p < 0.001 \)). Therefore, the two cell lines were selected for the later experiments. In addition, HAGLROS expression of SNU-46 or SNU-899 cells was appreciably down-regulated after transfection of si-HAGLROS 1, si-HAGLROS 2, and si-HAGLROS 3 relative to transfection of si-NC, among which the effect of si-HAGLROS 3 was the most obvious (Figure 1C,D, \( p < 0.01 \)). Thus, we chose si-HAGLROS 3 for the subsequent researches (hereafter represented as si-HAGLROS).

During cell functional assay, both SNU-46 and SNU-899 cells in si-HAGLROS group decreased OD value (Figure 1E,F, \( p < 0.05 \)), colony formation rate (Figure 1G,H, \( p < 0.001 \)), migration rate and invasion rate (Figure 1I,J, \( p < 0.001 \)) when contrasted with si-NC group. On the contrary, a higher apoptosis rate was viewed in the two cells transfected with si-HAGLROS in comparison with those transfected with si-NC (Figure 2A,B, \( p < 0.001 \)).

#### 3.2 | HAGLROS regulated proliferation, migration, invasion and apoptosis of LC cells by sponging miR-138-5p

The binding sites between the miR-138-5p and HAGLROS were predicted by IncSNP 2.0 (Figure 3A). The DLGR analysis was then carried out to verify the predication, which discovered that miR-138-5p M dramatically reduced the luciferase activity of HAGLROS-wt in SNU-46 and SNU-899 cells, but not of HAGLROS-mut (Figure 3B,C, \( p < 0.01 \)). To further investigate whether HAGLROS regulated LC cell proliferation and apoptosis via miR-138-5p, miR-138-5p I was transfected into LC cells. The transfection efficacy was determined by qRT-PCR, which showed that miR-138-5p expression of SNU-46 and SNU-899 cells was prominently declined after transfection of \( \text{I} \) when compared with transfection of IC (Figure 3D,E, \( p < 0.001 \)). Moreover, it was found that the knockdown of HAGLROS raised miR-138-5p level in SNU-46 and SNU-899 cells, which could also reversed miR-138-5p down-regulation induced by the miR-138-5p inhibitor in the two cells (Figure 3F,G, \( p < 0.01 \)). In contrast with si-NC+IC group, the colony formation rate (Figure 3H,I), migration rate (Figure 4A,C) and invasion rate (Figure 4B,D) of SNU-46 and SNU-899 cells in si-HAGLROS+IC group was decreased while that in si-NC+I group was elevated, with the colony formation rate, migration rate, and invasion rate of SNU-46 and SNU-899 cells in si-HAGLROS+I group higher than si-HAGLROS+IC group and lower than si-NC+I group (Figures 3H,I and 4A-D, \( p < 0.01 \)). And the results of flow cytometry assay revealed that miR-138-5p I reversed si-HAGLROS-induced apoptosis in SNU-46 and SNU-899 cells (Figure 3J,K, \( p < 0.01 \)).

#### 3.3 | CLN5 acted as a target of miR-138-5p

Through TCGA search, it was found that CLN5 was up-regulated in HNSC (Figure 5A, \( p = 4.48 \times 10^{-5} \)). MiRDB also predicted the complementary binding sites between CLN5 and miR-138-5p (Figure 5B), which was confirmed by DLGR analysis owing to the fact that co-transfection of CLN5-wt and miR-138-5p M appreciably reduced luciferase activity of SNU-46 and SNU-899 cells relative to co-transfection of CLN5-wt and MC, while the luciferase activity did not differ markedly between cells co-transfected with miR-138-5p M and CLN5-mut and those co-transfected with MC and CLN5-mut (Figure 5C,D, \( p < 0.01 \)). During Western blot, both in SNU-46 and SNU-899 cells, CLN5 protein expression was declined in si-HAGLROS+IC group whereas CLN5 protein expression was increased in si-NC+I group, and CLN5 protein expression in si-HAGLROS+I group was higher than si-HAGLROS+IC group and lower than si-NC+I group (Figure 5E,F, \( p < 0.05 \)).

### TABLE 2: Antibodies used in this study

| Name            | Catalog | Molecular weight | Dilution | Manufacturer   |
|-----------------|---------|------------------|----------|----------------|
| CLN5            | ab170899| 41kDa            | 1/1000   | abcam, UK      |
| Bcl-2           | ab182858| 26kDa            | 1/2000   | abcam, UK      |
| Bax             | ab32503 | 21kDa            | 1/1000   | abcam, UK      |
| GAPDH           | ab8245  | 36kDa            | 1/10000  | abcam, UK      |
| Goat anti rabbit| ab205718| –                | 1/2000   | abcam, UK      |
| Goat anti mouse | ab205719| –                | 1/2000   | abcam, UK      |
FU et al. FIGURE 1 HAGLROS knockdown restrained viability and proliferation of LC cells. (A) The differentially expressed HAGLROS between HNSC and normal samples was predicted by starBase. (B) HAGLROS expression among normal laryngeal cell line HuLa-PC and LC cell lines (AMC-HN-8, SNU-46, SNU-899, and SNU-1076) was detected through qRT-PCR. GAPDH was the loading control. (C–D) HAGLROS expression of SNU-46 (C) and SNU-899 (D) cells was detected through qRT-PCR after transfection of si-HAGLROS 1, si-HAGLROS 2, and si-HAGLROS 3. GAPDH was the loading control. (E, F) OD value at 24, 48 or 72 h of SNU-46 (E) and SNU-899 (F) cells was tested by CCK-8 assay after transfection of si-HAGLROS. (G, H) Colony formation rate of SNU-46 (G) and SNU-899 (H) cells was assessed through colony formation assay after transfection of si-HAGLROS. $p < 0.01$, $p < 0.001$ vs. HuLa-PC cells; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. si-NC group. All experiments were repeated independently at least three times. Data was performed as the means ± standard deviation. CCK-8, cell counting kit-8; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HNSC, head and neck squamous cell carcinoma; LC, laryngeal cancer; OD, optical density; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; si-HAGLROS, HAGLROS specific small interfering RNA; si-NC, negative control for siRNA.
3.4 | MiR-138-5p regulated abilities of LC cells to migrate and invade as well as expressions of apoptosis-related proteins in LC cells by targeting CLN5

CLN5 expression of SNU-46 and SNU-899 cells was evidently down-regulated after transfection of si-CLN5 when contrasted with transfection of si-NC, implying that the transfection succeeded (Figure 6A,B, p < 0.001). Compared with si-NC+IC group, si-CLN5+IC group decreased CLN5 mRNA and protein levels while si-NC+I group increased CLN5 mRNA and protein levels, with CLN5 mRNA and protein levels in si-CLN5+I group higher than si-CLN5+IC group and lower than si-NC+I group (Figure 6C-F, p < 0.05). In addition, CLN5 knockdown reduced OD value (Figure 6G,H), migration rate (Figure 7A,C) and invasion rate (Figure 7B,D) of SNU-46 and SNU-899 cells and reversed the elevated OD value, migration and invasion rate induced by miR-138-5p I (Figures 6G,H and 7A–D, p < 0.05). In contrast with co-transfection of si-NC and IC, co-transfection of si-CLN5 and IC declined Bcl-2 protein level while increased Bax protein level, with Bcl-2 protein level of the two cells in si-CLN5+I group higher than si-CLN5+IC group and lower than si-NC+I group whereas the converse results obtained in Bax protein level (Figure 6I,J, p < 0.05).
Figure 3  HAGLROS regulated LC cell proliferation and apoptosis by sponging miR-138-5p. (A) The binding sites between HAGLROS and miR-138-5p were predicted through LncSNP 2.0. (B, C) Luciferase activity of SNU-46 (B) and SNU-899 (C) cells was determined by dual luciferase gene reporter assay. (D, E) The transfection efficiency of miR-138-5p I in SNU-46 (D) and SNU-899 (E) cells was detected through qRT-PCR. U6 was the loading control. (F, G) miR-138-5p expression of SNU-46 (F) and SNU-899 (G) cells was detected through qRT–PCR after co-transfection of si-HAGLROS/siNC and miR-138-5p I/IC. U6 was the loading control. (H, I) Colony formation rate of SNU-46 (H) and SNU-899 (I) cells was assessed through colony formation assay after co-transfection of si-HAGLROS/siNC and miR-138-5p I/IC. (J, K) Apoptosis rate of SNU-46 (J) and SNU-899 (K) cells was evaluated by flow cytometry assay after co-transfection of si-HAGLROS/siNC and miR-138-5p I/IC.

**p < 0.01, ***p < 0.001 vs. MC group; &&&p < 0.001 vs. IC group; &p < 0.01, &&p < 0.001 vs. si-NC + IC group; ***p < 0.001 vs. si-HAGLROS + IC group. All the experiments were repeated independently at least three times. Data was performed as the means ± standard deviation. I, inhibitor; IC, inhibitor control; LC, laryngeal cancer; M, mimic; MC, mimic control; qRT–PCR, quantitative reverse transcription–polymerase chain reaction; si-HAGLROS, HAGLROS specific small interfering RNA; si-NC, negative control for siRNA.
FIGURE 4  HAGLROS regulated LC cell migration and invasion by sponging miR-138-5p. (A, B) Migration rate (A) and invasion rate (B) of SNU-46 cells was evaluated by transwell assay after co-transfection of si-HAGLROS/siNC and miR-138-5p I/IC (magnification, 250×, scale bar = 50 μm). (C, D) Migration rate (C) and invasion rate (D) of SNU-899 cells was evaluated by transwell assay after co-transfection of si-HAGLROS/siNC and miR-138-5p I/IC (magnification, 250×, scale bar = 50 μm). ***p < 0.001 vs. si-NC + IC group; ^^^p < 0.001 vs. si-HAGLROS + IC group; +++p < 0.001 vs. si-HAGLROS + I group. All the experiments were repeated independently at least three times. Data were performed as the means ± standard deviation. I, inhibitor; IC, inhibitor control; LC, laryngeal cancer; si-HAGLROS, HAGLROS specific small interfering RNA; si-NC, negative control for siRNA.
DISCUSSION

HAGLROS has been identified as an oncogenic role in a number of malignant tumors. However, few researches expound its effects on LC. In line with the analysis of crucial lncRNAs with diagnostic and prognostic value for HNSC based on TCGA database, our study exhibited the abnormal overexpression of HAGLROS in HNSC through the prediction of starBase, verifying the feasibility of HAGLROS as a diagnostic biomarker for HNSC. With LC a common type of head and neck cancer, it was presumed that HAGLROS might also have a certain relationship with LC. Former works have determined the upregulation of HAGLROS in gastric cancer, colorectal cancer, osteosarcoma, etc. Similarly, we found that HAGLROS was upregulated in LC as well. Those discoveries implied that HAGLROS might serve as an innovative biomarker for the advancement of LC.

To explore the functions of HAGLROS in LC, loss-of-function experiments were performed through construction of the gene silence model. Accumulating evidence has revealed that HAGLROS is characterized as an oncogene in multiple kinds of malignancies, the overexpression of which can facilitate proliferative, migratory and invasive abilities of cancer cells while inhibit apoptosis. Similarly, the consequences of our study presented that the knockdown of HAGLROS restrained the LC cells abilities to proliferate, migrate and invade as well as facilitated cell apoptosis in LC, which suggested the positive role of HAGLROS in LC development.

It has been elucidated that lncRNAs is able to act as competing endogenous RNAs (ceRNAs) to upregulate mRNAs by sponging miRNAs, thereby forming a regulatory network LncRNA-miRNA-mRNA at post-transcription level to achieve various biological effects in a number of cancers including LC. For the investigation on molecular mechanisms of HAGLROS in LC, we first probed into the interaction between HAGLROS and possible miRNAs. Several papers have illuminated that HAGLROS mediates tumor progression by sponging many miRNAs, such as miR-100, miR-5095, and miR-152. In this research, miR-138-5p was a target miRNA of HAGLROS, and the following DLGR analysis validated that HAGLROS could directly bind to miR-138-5p specifically in LC cells. MiR-138-5p acts as a cancer suppressor in gastric cancer, prostate cancer, and colorectal cancer, but its role in LC has not been reported. Consistent with previous studies, we found that miR-138-5p also acts as a tumor suppressor in LC. In addition, miR-138-5p reversed the effects of siHAGLROS, indicating that the oncogenic
role of HAGLROS in LC partially attributed to its interaction with miR-138-5p.

We then investigated the downstream mRNAs of miR-138-5p I to delve into the functions of HAGLROS in LC. CLN5 is a lysosome protein. Jiexia Xing et al.\textsuperscript{51} reported that knocking out CLN5 inhibited the malignant biological behavior of glioma. Li-rong Renz et al.\textsuperscript{52} pointed out that inhibition of CLN5 expression could inhibit the development of ovarian cancer by targeting lysosomal signals. In this study, CLN5 depletion offset the functions of miR-138-5p I on LC, indicating that miR-138-5p exerts a tumor suppressor in LC by targeting CLN5. And the results of western blot on apoptosis-associated factors revealed that miR-138-5p I promoted Bcl-2 (an anti-apoptotic molecule\textsuperscript{53}) whereas repressed Bax (a pro-apoptotic factor\textsuperscript{53}) in LC, while CLN5 knockdown had the contrary effects and reversed the effects of miR-138-5p inhibition on the two factors associated with apoptosis. Those findings implicated that the miR-138-5p I fulfilled its functions on LC via activating CLN5.

**FIGURE 6** miR-138-5p regulated viability of LC cells and apoptosis-related proteins in LC cells by targeting CLN5. (A, B) The transfection efficiency of si-CLN5 in SNU-46 (A) and SNU-899 (B) was detected through qRT-PCR. GAPDH was the loading control. For (C, J), LC cells were co-transfected with siCLN5/siNC and miR-138-5p I/IC. (C, D) CLN5 mRNA expression of SNU-46 (C) and SNU-899 (D) cells was detected through qRT-PCR. GAPDH was the loading control. (E, F) CLN5 protein expression of SNU-46 (E) and SNU-899 (F) cells was detected through western blot. GAPDH was the loading control. (G, H) OD value at 24, 48 or 72h of SNU-46 (G) and SNU-899 (H) cells was tested by CCK-8 assay. (I, J) Bcl-2 and Bax protein levels of SNU-46 (I) and SNU-899 (J) cells was detected through Western blot. GAPDH was the loading control. All the experiments were repeated independently at least three times. Data was performed as the means ± standard deviation. CCK-8, cell counting kit-8; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; I, inhibitor; IC, inhibitor control; LC, laryngeal cancer; OD, optical density; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; si-CLN5, CLN5 specific small interfering RNA; si-NC, negative control for siRNA.
In conclusion, this study determined the oncogenic role of HAGLROS in LC, and further demonstrated that HAGLROS, miR-138-5p, and CLN5 formed a ceRNA regulatory network in LC progression. The identification of the role and mechanism of HAGLROS in LC probably brought a promising biomarker and therapeutic target for LC.
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