RESEARCH ARTICLE

Long noncoding RNA HAGLROS regulates apoptosis and autophagy in colorectal cancer cells via sponging miR-100 to target ATG5 expression

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Abstract
The aim of this study was to explore the relationship between the expression of HOXD antisense growth-associated long noncoding RNA (HAGLROS) and prognosis of patients with colorectal cancer (CRC), as well as the roles and regulatory mechanism of HAGLROS in CRC development. The HAGLROS expression in CRC tissues and cells was detected. The correlation between HAGLROS expression and survival time of CRC patients was investigated. Moreover, HAGLROS was overexpressed and suppressed in HCT-116 cells, followed by detection of cell viability, apoptosis, and the expression of apoptosis-related proteins and autophagy markers. Furthermore, the association between HAGLROS and miR-100 and the potential targets of miR-100 were investigated. Besides, the regulatory relationship between HAGLROS and PI3K/AKT/mTOR pathway was elucidated. The results showed that HAGLROS was highly expressed in CRC tissues and cells. Highly expression of HAGLROS correlated with a shorter survival time of CRC patients. Moreover, knockdown of HAGLROS in HCT-116 cells induced apoptosis by increasing the expression of Bax/Bcl-2 ratio, cleaved-caspase-3, and cleaved-caspase-9, and inhibited autophagy by decreasing the expression of LC3II/LC3I and Beclin-1 and increasing P62 expression. Furthermore, HAGLROS negatively regulated the expression of miR-100, and HAGLROS controlled HCT-116 cell apoptosis and autophagy through negatively regulation of miR-100. Autophagy related 5 (ATG5) was verified as a functional target of miR-100 and miR-100 regulated HCT-116 cell apoptosis and autophagy through targeting ATG5. Besides, HAGLROS overexpression activated phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway. In conclusion, a highly expression of HAGLROS correlated with shorter survival time of CRC patients. Downregulation of HAGLROS may induce apoptosis and inhibit autophagy in CRC cells by regulation of miR-100/ATG5 axis and PI3K/AKT/mTOR pathway.

KEYWORDS
apoptosis, autophagy, colorectal cancer, HOXD antisense growth-associated long noncoding RNA, long noncoding RNA, microRNA-100
1 INTRODUCTION

Colorectal cancer (CRC) is the most common malignant cancers and its mortality rate ranks the third among all cancers. It is estimated that the global burden of CRC may increase by 60% by 2030. Although great progresses in diagnosis and cancer therapy have been achieved, the prognosis remains unfavorable in patients with advanced stages of CRC. Therefore, to improve clinical outcomes, it is still imperative to better elucidate the pathological mechanisms underlying CRC.

Like many other cancer types, multiple oncogenes and tumor suppressors play a key role in the progression of CRC. For instance, activation of yes-associated protein 1, an oncogene in Hippo pathway, is highly associated with poor prognosis for CRC and affects cetuximab resistance in CRC patients. DIS3 is identified as a candidate oncogene that can affect tumorigenic characteristics such as viability, migration, and invasion in CRC progression. Connexin 43 can function as a tumor suppressor in CRC development and its reduced expression is found to be associated with shorter patient survival. Moreover, accumulating evidence have disclosed the key role of microRNAs (miRNAs) in regulating CRC development. Circulating miRNAs, such as miR-34a and miR-150, have capable of distinguishing CRC patients with different disease progression.

Recently, growing incidences have highlighted the key role of long noncoding RNAs (lncRNAs) in human cancers. LncRNAs are transcribed RNAs with a length more than 200 nucleotides and with little or no protein-coding capability. In CRC pathogenesis, lncRNAs have been identified as key players. For instance, the high lncRNA-ATB expression is associated with enhanced tumor metastasis and poorer outcomes; the enhanced expression of lncRNA PANDAR promotes metastasis in CRC by regulating epithelial-mesenchymal transition; and genetic variants in lncRNA HOX transcript antisense RNA is shown to be associated with risk of CRC and rs7958904 may serve as a promising biomarker for predicting CRC risk. Given the key roles of lncRNAs in CRC pathogenesis, it is of great importance for the further identification of key lncRNAs involved in CRC progression. HOXD antisense growth-associated long noncoding RNA (HAGLROS) is a 699 base pair (bp) recently reported lncRNA that can promote the malignant progression of gastric cancer cells. Nevertheless, there is a lack of adequate knowledge of the possible roles of HAGLROS in CRC progression.

In this study, the HAGLROS expression in tumor tissues isolated from CRC patients was detected, and the correlation between HAGLROS expression and survival time of CRC patients was investigated. Moreover, HAGLROS expression in CRC cells was also determined. HAGLROS was then overexpressed and suppressed in HCT-116 cells, followed by detection of cell viability, apoptosis, and the expression of apoptosis-related proteins and autophagy markers. Furthermore, HAGLROS is reported to function as a competing endogenous RNA (ceRNA) to sponge miR-100-5p in gastric cancer cells, thus, we also explored the association between HAGLROS and miR-100, as well as the functional targets of miR-100.

2 MATERIALS AND METHODS

2.1 Sample collection

Between April 2013 and October 2017, 78 CRC patients, including 51 men and 26 women, with age ranging from 32 to 86 (mean age, 56 years), were enrolled. CRC tumor tissues and matched adjacent normal tissues were collected after surgery, immediately frozen in liquid nitrogen and stored at −80°C until the extraction of total RNA. According to the TNM classification of the sixth edition AJCC, the tumor stage was classified. All recruited patients did not receive any preoperative treatments. All patients signed informed consent for research. The study was approved by the medical ethics committee of our hospital.

2.2 Cell culture and treatment

Human normal intestinal mucous cell line CCC-HIE-2 and human CRC cell lines, including CaCO-2, HCT8, HCT-116, and LoVo, were obtained from the American Type Culture Collection (ATCC, Manassas, VA). They were cultured in Dulbecco modified Eagle medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 2 mM l-glutamine (Invitrogen, Carlsbad, CA) and 10% fetal bovine serum (FBS), and maintained at 37°C incubator filling with 10% CO₂ atmosphere. In autophagy experiments, the HCT-116 cells were incubated in Earle Balanced Salt Solution (EBSS; E2888; Sigma-Aldrich) for 6 hours and the treated with chloroquine diphosphate salt (100 mM, CQ; Sigma-Aldrich) for 6 hours to block autophagy flux.
Furthermore, to confirm the association between HAGLROS and PI3K/AKT/mTOR pathway, gefitinib-resistant HT-29 cells were established. Briefly, the HT-29 cells (ATCC) were cultured in DMEM/F12 supplemented with 10% of FBS, and maintained at 37°C incubator with 5% of CO2. The cells were digested with 0.25% of trypsin (containing 0.02% ethylenediaminetetraacetic acid) solution for cell passage. After 48 hours, HT-29 cells that grew stably in the culture medium with the final drug concentration of 0.150 μM/mL were screened as gefitinib-resistant HT-29 cells.

2.3 | Transient transfection

For overexpression and knockdown of HAGLROS, pEX2-HAGLROS, and short hairpin RNA for targeting HAGLROS (sh-HAGLROS; GenePharma, China) were respectively introduced into HCT-116 cells using Lipofectamine 2000 (Invitrogen). Empty vector pEX2 and sh-NC were used as the negative controls for pEX2-HAGLROS and sh-HAGLROS, respectively. MiR-100 mimic (50 nM), mimic control (50 nM), miR-100 inhibitor (150 nM), and inhibitor NC (150 nM; GenePharma, Roche) were also introduced into HCT-116 cells to regulate expression of miR-100. For overexpression of autophagy related 5 (ATG5), the ATG5 overexpression vector (pcDNA-ATG5) was constructed by inserting the full-length ATG5 coding sequence into pcDNA3.1 and then transfected into HCT-116 cells using Lipofectamine 2000. The empty vector pcDNA3.1 was transfected as a control. After transfection for 48 hours, cells were harvested for subsequent experiments.

2.4 | Quantitative reverse-transcription polymerase chain reaction

TRIzol reagent (Invitrogen) was applied for extraction of total RNA. Reverse-transcription (RT) reactions for complementary DNA synthesis were then conducted using an M-MLV Reverse Transcriptase Kit (Invitrogen). For detection of gene expression, real-time RT quantitative polymerase chain reaction (qRT-PCR) was then carried out using a standard SYBR Green PCR Kit (Toyobo, Osaka, Japan) by means of a Rotor-Gene RG-3000A (Corbett Life Science, Sidney, NSW, Australia). Glyceraldehyde 3-phosphate dehydrogenase was used as the endogenous control of HAGLROS; and U6 and β-actin were used as references for miRNAs and messenger RNAs (mRNAs), respectively. Relative quantification of gene expression levels was then determined using the 2−ΔΔCt method.

2.5 | Dual-luciferase reporter assay

The fragments of HAGLROS containing the predicted miR-100 binding site and flanking sequence were cloned into a pmirGLO Dual-luciferase miRNA Target Expression Vector (Promega, Madison, WI) to form the reporter vectors HAGLROS wild-type (wt) (HAGLROS-wt, 5′-GGACGCCUU GUGCCGCG-3′) and HAGLROS mutated-type (HAGLROS-mut, 5′-GGACGGAAGCACCACCG-3′), respectively. Furthermore, to verify the target relationship between miR-100 and ATG5, the fragments of ATG5 containing the predicted miR-100 binding site and flanking sequence were also cloned into the pmirGLO vector to establish the reporter vectors ATG5-wt (5′-AAAUUGUAAGCUUA-3′) and ATG5-mut (5′-AAAUUGUAAGCCG-3′), respectively. The constructed reporter vectors were then transfected into HCT-116 cells, along with miR-100 mimic or mimic control. Luciferase activity then was measured using the Dual-Luciferase Reporter Assay System (E1910; Promega) at 48 hours of posttransfection.

2.6 | RNA binding protein immunoprecipitation

RNA binding protein immunoprecipitation (RIP) assay was carried out using the RNA-Binding Protein Immunoprecipitation Kit (Millipore) according to the manufacturer’s instruction. Followed by lysis preparation, magnetic beads were prepared by being conjugated with human anti-Ago2 (Millipore), which was used to enrich HAGLROS and miR-100. Normal mouse anti-IgG (Millipore) was used as negative control. Immunoprecipitation and RNA purification were performed, followed by qRT-PCR analysis.

2.7 | RNA pull-down assay

To explore whether HAGLROS and miR-100 were in the same RNA-induced silencing complex (RISC) complex, RNA pull-down assay was performed using synthesized HAGLROS as a probe to detect Ago2 from the pellet using Western blot analysis and miR-100 using qRT-PCR. In brief, the DNA fragment containing the whole HAGLROS sequence and IncRNA loc285194 (positive control) was amplified and then cloned into pCR8 (Invitrogen). After restriction enzyme digestion, the resultant plasmid DNA was linearized. Biotin-labeled RNAs were reversely transcribed using Biotin RNA Labeling Mix (Roche Diagnostics) and T7 RNA polymerase (Roche, Switzerland). Followed by purification of the products using RNase-free DNase I (Roche Diagnostics, Mannheim, Germany) and the RNeasy Mini Kit (Qiagen), RNA was extracted for subsequent qRT-PCR or for Western blot analysis.
All experiments were repeated three times. The measurements were then performed using the primary antibodies to Bcl-2, Bax, cleaved-caspase-3, procaspase-3, cleaved-caspase-9, procaspase-9, LC3II, LC3I, Beclin-1, P62, ATG5, PI3K, p-PI3K, AKT, p-AKT, p-mTOR, mTOR, PTEN, and β-actin (1:1000, Abcam, Cambridge, UK) overnight at 4°C. β-Actin was used as the control. The signals were revealed after incubation with the recommended secondary antibodies conjugated with horseradish peroxidase (1:2000; Santa Cruz Biotechnology) using an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE).

Detection of cell apoptosis by flow cytometry
Following different treatments, HCT-116 cells were harvested, washed twice with prechilled PBS and resuspended in 100 μL binding buffer at a concentration of 1 × 10^6 cells/mL. According to the manufacturer’s protocol of the Annexin V-FITC Apoptosis Detection Kit (BD Bioscience, San Jose, CA), HCT-116 cells were double-stained with Annexin V and propidium iodide (PI). The apoptotic cells were then detected within 1 hour using a BD LSRII flow cytometer (BD Biosciences). The obtained data were then analyzed with FACSDiva Software (BD Biosciences).

Western blot analysis
Total protein was extracted from HCT-116 cells in different groups by lysing with cell lysis buffer (Beyotime, Haimen, China). After determining the protein concentrations using a bicinchoninic acid assay (Biotech), equal amounts of proteins (30 μg/lane) were separated on 12% SDS-polyacrylamide gels and transferred onto polyvinylidene fluoride membranes (Millipore) by means of a semidyry electroblot apparatus (Bio-Rad). Immunoblotting was then performed using the primary antibodies to Bcl-2, Bax, cleaved-caspase-3, procaspase-3, cleaved-caspase-9, procaspase-9, LC3II, LC3I, Beclin-1, P62, ATG5, PI3K, p-PI3K, AKT, p-AKT, p-mTOR, mTOR, PTEN, and β-actin (1:1000, Abcam, Cambridge, UK) overnight at 4°C. β-Actin was used as the control. The signals were revealed after incubation with the recommended secondary antibodies conjugated with horseradish peroxidase (1:2000; Santa Cruz Biotechnology) using an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE).

Statistical analysis
All experiments were repeated three times. The measurement data are presented as the mean ± SD. The differences between groups were evaluated with two-tailed Student t tests or one-way analysis of variance. The gene expression levels of HAGLROS between CRC tissues and normal adjacent normal tissues were compared with the Wilcoxon test, and the associations between HAGLROS expression and clinical characteristics were analyzed by the χ^2 test. Survival curves were estimated by the Kaplan-Meier method and the statistical differences between survival curves were assessed using the log-rank test. To further evaluate the association between HAGLROS expression and survival, Cox proportional hazards analysis was conducted to calculate the hazard ratio (HR) and the 95% confidence interval (CI). In addition, the correlation analysis of HAGLROS expression and miR-100 expression in CRC tissues was evaluated using the Pearson correlation coefficient. All statistical analyses were carried out using SPSS Statistics 20.0 Software (IBM, Armonk, NY) and a value of P < 0.05 indicated statistically significant.

RESULTS

HAGLROS was highly expressed in CRC tissues and its high expression correlated with a shorter survival time in CRC patients
To investigate whether HAGLROS played a key role in CRC development, we firstly detected the expression of HAGLROS in CRC tissues and matched adjacent nontumor tissues. As shown in Figure 1A, HAGLROS expression was higher expressed in CRC tissues compared with that in matched adjacent nontumor tissues (P < 0.001). Moreover, we investigated the correlation between HAGLROS expression and clinical characteristics of CRC patients. Among these 78 CRC patients, 36 patients were classified as high-HAGLROS expression group (above the mean) and the rest of the 42 patients were classified as low-HAGLROS expression group (below the mean) based on the mean value of HAGLROS expression levels. Survival analysis showed that the higher expression of HAGLROS correlated with a shorter survival time of CRC patients (P = 0.0010; Figure 1B). Furthermore, as presented in Table 1, HAGLROS expression correlated with tumor stage and tumor differentiation but had no significant correlation with other clinical characteristics, such as age, sex, tumor location, and tumor size.

Effects of HAGLROS expression on cell viability, cell apoptosis, and autophagy
To further explore the role of HAGLROS in CRC, qRT-PCR was also performed to detect the HAGLROS expression in CRC cells including CaCO-2, HCT8, HCT-116, and LoVo cells. The results showed that the
HAGLROS expression was significantly increased in these CRC cells in comparison with that in normal intestinal mucus CCC-HIE-2 cells \((P < 0.01; \text{Figure 1C})\). Because HAGLROS expression in HCT-116 cells was highest, HCT-116 cells were used for subsequent experiments. By transfection with pEX2-HAGLROS and sh-HAGLROS, HAGLROS was successfully overexpressed and suppressed in HCT-116 cells \((P < 0.01; \text{Figure 1D})\). Also, the inhibitory effects of sh-HAGLROS#2 was stronger than sh-HAGLROS#1, thus sh-HAGLROS#2 was selected for subsequent knockdown experiments. Furthermore, the results of MTT assay showed that the viability of HCT-116 cells in the pEX2-HAGLROS group was significantly increased compared with that of the pEX2 group \((P < 0.05)\), and HCT-116 cell viability of sh-HAGLROS#2 group was markedly decreased relative to that of sh-NC group \((P < 0.05; \text{Figure 1E})\). The results of flow cytometry showed that the percentage of apoptosis cells in the sh-HAGLROS#2 group was markedly increased in comparison with that in the sh-NC group \((P < 0.001; \text{Figure 1F})\). Further Western blot analysis showed that knockdown of HAGLROS markedly inhibited the expression of Bcl-2 and promoted the expression of Bax, cleaved-caspase-3, and cleaved-caspase-9 in HCT-116 cells (Figure 1F). However, overexpression of HAGLROS had no significant effects on HCT-116 cell apoptosis (Figure 1F). To explore the role of autophagy in CRC, after transfection with the pEX2-HAGLROS, HCT-116 cells were further treated with chloroquine diphosphate salt (CQ; 100 mM) for 6 hours to inhibit autophagy. The results showed that overexpression of HAGLROS significantly increased the expression of LC3II/LC3I and Beclin-1 and decreased the expression of P62, whereas knockdown of HAGLROS had opposite effects on the expression levels of these autophagy markers (all \(P < 0.05; \text{Figure 1G}\)). Moreover, CQ treatment markedly reversed the effects of overexpression of HAGLROS on the expression levels of these autophagy markers (all \(P < 0.05; \text{Figure 1G}\)).
the expression of miR
clinicopathological characteristics of colorectal cancer
markedly decreased in pEX2
that in pEX2 group and obviously increased in sh‐
HAGLROS#2 group compared with that in sh‐NC group
(all P < 0.05; Figure 2A), implying the negative correlation
between HAGLROS and miR‐100. To verify it, the HCT‐116
cells were transfected with pEX2‐HAGLROS‐MS2 or pEX2‐
HAGLROS‐mut‐MS2 with miR‐100 mimic or mimic control,
and dual‐luciferase reporter assay showed that HAGLROS
could target miR‐100 (P < 0.05; Figure 2B). To further
confirm that that HAGLROS and miR‐100 were in the same
RISC, we performed a RIP assay using anti‐Ago2. The
results showed that anti‐Ago2 precipitated the Ago2 protein
from the cell lysates (Figure 2C, up panel), and higher
HAGLROS and miR‐100 were detected in the Ago2 pellet
than those in the input control (Figure 2C, down panel).
Furthermore, RNA pull‐down assay was performed, and the
results of Western blot analysis showed that Ago2 was
detected after pull‐down experiment with streptavidin beads,
suggesting HAGLROS directly interacted with Ago2 (Figure
2D, up panel). Moreover, a significant amount of miR‐100 in
the HAGLROS pulled down pellet was revealed compared
with control, while the amount of miR‐100 in the loc285194
pulled down pellet was only slightly increased (Figure 2D,
down panel). These data confirm the negative relationship
between HAGLROS and miR‐100.

3.4 | HAGLROS‐regulated HCT‐116 cell
apoptosis and autophagy through miR‐100
To verify the role of miR‐100 in CRC development, the
expression of miR‐100 in CRC tissues and cells were
detected. As shown in Figure 2E, the miR‐100 expression in
CRC tissues was significantly lower than that in matched
adjacent nontumor tissues (P < 0.01). Moreover, the corre‐
lation analysis of HAGLROS expression and miR‐100 expression in CRC tissues was performed. The results showed that there was stronger negative correlation between HAGLROS expression and miR‐100 expression in CRC patients (R² = 0.6346, P < 0.001; Figure 2F). In addition, in comparison with normal intestinal mucous
CCC‐HIE‐2 cells, the miR‐100 expression in CRC cells
including CaCO‐2, HCT8, HCT‐116, and LoVo cells were all
markedly decreased (P < 0.05; Figure 2G). To further detect
whether the role of HAGLROS in CRC cells was achieved
by miR‐100, the miR‐100 expression was overexpressed and
inhibited in HCT‐116 cells by transfection with miR‐100 mimic and miR‐100 inhibitor, respectively (P < 0.001;
Figure 2H). HCT‐116 cells were then cotransfected with
sh‐HAGLROS#2 and miR‐100 inhibitor for further detect‐
ing the synergistic effects of HAGLROS knockdown and
miR‐100 inhibition on cell apoptosis and autophagy. The
results showed that the effects of HAGLROS knockdown on
HCT‐116 cell apoptosis and the expression levels of
apoptosis‐related proteins (Figure 2I) as well as the expression
levels of autophagy markers (Figure 2J) were
significantly reversed by inhibition of miR‐100 at the same
time, indicating that effects of HAGLROS downregulation
on HCT‐116 cell apoptosis and autophagy were achieved by
negative regulation of miR‐100.

### Table 1. Correlation between HAGLROS expression and
clinicopathological characteristics of colorectal cancer

| Characteristics | Cases | Low | High | P value |
|----------------|-------|-----|------|---------|
| Sex            |       |     |      |         |
| Male           | 51    | 29  | 22   | 0.663   |
| Female         | 27    | 13  | 14   |         |
| Age            |       |     |      |         |
| <60            | 48    | 28  | 20   | 0.275   |
| ≥60            | 30    | 14  | 16   |         |
| Tumor location |       |     |      | 0.584   |
| Colon          | 46    | 24  | 22   |         |
| Rectum         | 32    | 18  | 14   |         |
| T stage        |       |     |      |         |
| T1‐2           | 13    | 12  | 1    | 0.023   |
| T3‐4           | 65    | 30  | 35   |         |
| N stage        |       |     |      |         |
| N0             | 41    | 32  | 9    | 0.024   |
| N1‐2           | 37    | 10  | 27   |         |
| M stage        |       |     |      |         |
| M0             | 58    | 36  | 22   | 0.032   |
| M1             | 20    | 6   | 14   |         |
| Differentiation|       |     |      |         |
| Low            | 18    | 3   | 15   | 0.033   |
| Medium         | 53    | 34  | 19   |         |
| High           | 7     | 5   | 2    |         |
| Size, cm       |       |     |      |         |
| <4.5           | 36    | 20  | 16   | 0.788   |
| ≥4.5           | 42    | 22  | 20   |         |

Abbreviation: HAGLROS, HOXD antisense growth‐associated long
noncoding RNA.

markers in pEX2‐HAGLROS‐CQ group (all P < 0.05; Figure 1G).

3.3 | HAGLROS negatively regulated
the expression of miR‐100

Accumulating evidence has reported that HAGLROS serves
as a sponge for miR‐100‐5p in gastric cancer cells, the
association between HAGLROS and miR‐100 was investig‐
ated. The results showed that miR‐100 expression was
markedly decreased in pEX2‐HAGLROS group relative to
that in pEX2 group and obviously increased in sh‐
HAGLROS#2 group compared with that in sh‐NC group
(all P < 0.01; Figure 2A), implying the negative correlation
between HAGLROS and miR‐100. To verify it, the HCT‐116
cells were transfected with pEX2‐HAGLROS‐MS2 or pEX2‐
3.5 | ATG5 was verified as a functional target of miR-100 and miR-100 regulated HCT-116 cell apoptosis and autophagy through targeting ATG5

To further explore the regulatory mechanism of miR-100, the potential targets of miR-100 were predicted by HumanTargetScan and ATG5 was identified (http://www.targetscan.org/cgi-bin/targetscan/vert_71/view_gene.cgi?rs=ENST00000360666.4&taxid=9606&members=miR-100-3p&showcnc=0&shownc=0&showncf1=1&showncf2=1&subset=1). Luciferase reporter assay further confirmed that only the relative luciferase activity of ATG5 3′-untranslated region (3′-UTR)-wt were significantly inhibited after
cotransfection with miR-100 mimic ($P<0.05$; Figure 3B), indicating that miR-100 could target ATG5. Moreover, the expression of ATG5 at both mRNA and protein levels were significantly downregulated in miR-100 mimic group compared with that in mimic NC group, while markedly upregulated in miR-100 inhibitor group relative to that in inhibitor NC group ($P<0.05$; Figure 3C and 3D). These data indicated that ATG5 was target of miR-100.

To further confirm whether ATG5 was a functional target of miR-100, ATG5 expression was overexpressed by transfection with pcDNA-ATG5. The results showed that mRNA and protein expression levels of ATG5 were markedly increased after transfection with pcDNA-ATG5 compared with pcDNA3.1 ($P<0.001$; Figure 4A), suggesting that the transfection was successful. HCT-116 cells were then cotransfected with miR-100 mimic and pcDNA-ATG5 for further investigating the synergistic effects of miR-100 overexpression and ATG5 overexpression on cell apoptosis and autophagy. The results showed that miR-100 overexpression significantly induced apoptosis in HCT-116 cells ($P<0.01$) by decreasing Bcl-2 expression and increasing the expression of Bax, cleaved-caspase-3 and cleaved-caspase-9 (Figure 4B). Moreover, miR-100 overexpression markedly inhibited autophagy in HCT-116 cells by decreasing the expression of LC3II/LC3I and Beclin-1 and increasing the expression of P62 (all $P<0.05$; Figure 4C). Furthermore, the effects of miR-100 overexpression on HCT-116 cell apoptosis and autophagy were significantly reversed after miR-100 overexpression and ATG5 overexpression at the same time (all $P<0.05$; Figure 4B and 4C). These data confirmed that ATG5 was a functional target of miR-100 and miR-100 regulated HCT-116 cell apoptosis and autophagy through targeting ATG5. Notably, the results showed that ATG5 expression was markedly decreased sh-HAGLROS#2 group compared with that in sh-NC group ($P<0.01$; Figure 4D), implying that ATG5 might be a downstream target of HAGLROS/miR-100 axis.

3.6 | The effects of HAGLROS in CRC cells were achieved possibly by regulating the activation of PI3K/AKT/mTOR pathway

The PI3K/AKT/mTOR signaling are crucial to many aspects of cell growth and survival in pathological conditions (eg, cancer). We thus explored the association between HAGLROS and PI3K/AKT/mTOR pathway. As shown in Figure 5A, HAGLROS overexpression alone resulted in a significant decrease in the expression levels of PTEN and obvious increases in the expression of p-PI3K, p-AKT, and p-mTOR in HCT-116 cells. The expression changes of these proteins were significantly counteracted by overexpression of HAGLROS and miR-100 synchronously, but further enhanced after overexpression of HAGLROS, miR-100, and ATG5 concurrently (Figure 5A). Notably, gefitinib-resistant HT-29 cells were established to confirm the association between HAGLROS and PI3K/AKT/mTOR pathway. The results showed that overexpression of HAGLROS alone significantly activated the PI3K/AKT/mTOR pathway (Figure 5B). Consistent results were also obtained after overexpression of HAGLROS, miR-100, and ATG5 concurrently (Figure 5B). These data indicated that the effects of HAGLROS in CRC cells were achieved possible by regulating the activation of PI3K/AKT/mTOR pathway.

4 | DISCUSSION

As the rapid development of biological technologies, especially high throughput sequencing, a large amount of important noncoding RNAs such as miRNAs and lncRNAs, have been discovered to be involved in many diseases, including cancer. LncRNA was first discovered by Okazaki et al in 2002, and has become a research focus in disease field following miRNA. The key lncRNAs involved in disease development is still a tip of the iceberg and needs further exploration.

In this study, we found that HAGLROS was highly expressed in CRC tissues and cells. Highly expression of HAGLROS correlated with shorter survival time of CRC patients. Moreover, knockdown of HAGLROS in HCT-116 cells induced apoptosis and inhibited autophagy.
FIGURE 4  Continued.
Furthermore, HAGLROS negatively regulated the expression of miR-100, and HAGLROS controlled HCT-116 cell apoptosis and autophagy through negatively regulation of miR-100. ATG5 was verified as a functional target of miR-100 and miR-100 regulated HCT-116 cell apoptosis and autophagy through targeting ATG5. Besides, HAGLROS overexpression activated PI3K/AKT/mTOR pathway. Taken together, the potential regulatory mechanism graph of HAGLROS in CRC was shown in Figure 6.

Increasing evidence have revealed that lncRNAs can function as ceRNAs to regulate gene expression by sponging miRNAs, thus play a key role in many diseases.28,29 In a previous study, HAGLROS could competitively sponge miR-100-5p to increase mTOR expression in gastric cancer cells.23 Consistent with this finding, we also found HAGLROS negatively regulated the expression of miR-100. Growing studies have reported that downregulation of miR-100 is associated with tumor metastasis and poor prognosis in CRC.30,31 Moreover, miR-100 is confirmed to play a suppressor role in regulating the proliferation and invasion of SW620 CRC cells.32 Besides, Yang et al33 demonstrated that miR-100 upregulation increased the radiosensitivity of CRC cells, suggesting that miR-100 might act as a promising clinical target for CRC radiotherapy. Thomas revealed that miR-100 could induce cetuximab resistance in CRC,34 hinting the role of miR-100 in chemotherapy. These data support the key role of miR-100 in CRC progression and treatment. In our study, highly expression of HAGLROS correlated with shorter survival time.
of CRC patients. Moreover, HAGLROS controlled HCT-116 cell apoptosis and autophagy through negatively regulation of miR-100. Therefore, we speculate that HAGLROS may contribute to CRC development via sponging miR-100.

Furthermore, ATG5 was verified as a functional target of miR-100. ATG5 is considered as a key player in the autophagy process. Increasing evidence has confirmed that alterations of autophagy processes result in the development of cancers, including CRC. The increased expression of ATG5 is also found to be associated with lymphovascular invasion in CRC tissues. Additionally, Won et al reported that Justicidin A-induced autophagy flux enhances apoptosis of CRC cells through regulating class III PI3K and ATG5 pathway. In the current study, miR-100 regulated HCT-116 cell apoptosis and autophagy through targeting ATG5. Given the key role of ATG5 in autophagy process, our results prompt us to speculate that HAGLROS may regulate ATG5 expression by functioning as a ceRNA of miR-100, thus regulating the apoptosis and autophagy process in CRC cells.

Remarkably, the association between HAGLROS and PI3K/AKT/mTOR pathway was explored in this study. The PI3K/AKT/mTOR signaling are found to play crucial roles in many aspects of cell growth and survival under pathological conditions (eg, cancer). Activation of the PI3K/AKT/mTOR pathway is a key mechanism to mediate the role of S100A4 in promoting viability and migration of CRC cells. Importantly, salidroside is found to trigger apoptosis and autophagy in CRC cells via suppressing the PI3K/AKT/mTOR pathway and celastrol orbiculatus extract can induce apoptosis and autophagy in CRC cells via inhibiting the activation of PI3K/AKT/mTOR pathway, implying that targeting PI3K/AKT/mTOR pathway may be a promising therapeutic strategy for CRC. In this study, our results showed that HAGLROS overexpression alone activated PI3K/AKT/mTOR pathway both in HCT-116 cells and gefitinib-resistant HT-29 cells, which was counteracted by overexpression of HAGLROS and miR-100 synchronously, but further enhanced after overexpression of HAGLROS, miR-100 and ATG5 concurrently. Considering the role of PI3K/AKT/mTOR pathway in CRC development, we speculated that the effects of HAGLROS/miR-100/ATG5 axis on the apoptosis and autophagy of CRC cells may be achieved possible by regulating the activation of PI3K/AKT/mTOR pathway.

In conclusion, our findings indicated that highly expression of HAGLROS correlated with shorter survival time of CRC patients. Downregualtion of HAGLROS may induce apoptosis and inhibit autophagy in CRC cells by negative regulation of miR-100/ATG5 axis. The roles of HAGLROS/miR-100/ATG5 axis in regulating the apoptosis and autophagy of CRC cells may be achieved possible by activation of PI3K/AKT/mTOR pathway. Our findings may provide an experimental basis for the development of targeted therapy for CRC.

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