Long non-coding RNA HAGLR inhibits growth and metastasis and reduces stemness of lung cancer cells through the microRNA-330-3p/SLC34A2 axis

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Research article

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Abstract

Background Lung cancer (LC) remains a leading cause of cancer-related mortality worldwide. Long noncoding RNAs (lncRNAs) are crucial regulatory molecules in diverse pathological processes, including cancer progression. This study was conducted to probe the influence of lncRNA HAGLR on the growth, metastasis and stemness of LC cells.

Methods Aberrantly expressed lncRNAs in LC tissues were screened out using microarray analysis. HAGLR expression in LC tissues and cells and in the paired normal ones was determined using RT-qPCR. Overexpression of HAGLR was administrated in H1299 and A549 cells to identify its function in the biological characteristics of LC cells. Sub-cellular localization of HAGLR was determined, and the downstream molecules involved in the HAGLR-mediated events were predicted on a bioinformation system and validated through dual luciferase reporter gene assay.

Results HAGLR was poorly expressed in both LC tissues and cells. Overexpression of HAGLR inhibited viability, proliferation and metastasis, and reduced the stemness of H1299 and A549 cells. HAGLR up-regulated SLC34A2 expression through sponging miR-330-3p, leading to further inactivation of the Wnt/ β -catenin signaling pathway. Artificial activation of the Wnt/ β -catenin pathway recovered the viability and promoted metastasis of LC cells inhibited by HAGLR

Conclusion HAGLR serves as a competing endogenous RNA for miR-330-3p to upregulate miR-330-3p expression and inactivate the Wnt/ β -catenin pathway, leading to inhibited growth and metastasis and reduced stemness of LC cells.

Background

Lung cancer (LC), a prevalent and highly invasive malignancy, is one of the most frequent and leading causes of cancer-related death across the globe [1]. In China, LC holds the highest incidence and mortality among all cancer types [2]. Several lifestyle and environmental factors have been suggested to be linked with the development of LC, of which cigarette smoking, as publicly accepted, is the most relevant and important, accounting for 80–90% of LC cases [3, 4]. Limited access to early diagnosis and timely treatment are main attributes that leads to poor outcomes of LC patients [5]. Since LC detected at late stages are always accompanying with high metastasis rate and the following less survival opportunity [6]. To date, despite the great progress made in conventional therapeutic approaches including surgery, chemotherapy and radiotherapy, the overall 5-year survival rate of non-small-cell lung cancer (NSCLC, the most common type of LC) is reported remaining very low [7]. Identifying novel pathophysiological mechanisms involved in LC progression is of great importance for new treatment strategy development.

Many human diseases are accompanied with mutations identified in a lot of protein-coding genes; however, most of the discovered genome is “noncoding” but transcribed [8, 9]. Besides protein-encoding RNAs, many long and short non-coding RNAs (ncRNAs) have been recognized with advances in the computational and experimental approaches of contemporary biology [10]. Among them, long non-coding

lncRNAs (lncRNAs) are a heterogeneous class that have been discovered to regulate gene expression in different levels and to play important roles in cancer progression [11]. As in human LC, lncRNA LINC00961 [12] and lncRNA CS1-IT1 [5], for example, were documented to suppress LC progression by inhibiting proliferation and metastasis but promoting apoptosis of cancer cells. Besides, another major type of ncRNAs, microRNAs (miRNAs), are crucial for post-transcriptional regulation of gene expression by binding the 3' untranslated region (3'UTR) of target mRNAs through the miRNA-response elements (MREs), leading to following mRNA degradation [13]. In addition to being identified on mRNAs, MREs can also be found on other non-coding transcripts including lncRNAs, by which the ceRNA hypothesis was proposed, which suggests that lncRNAs competes for a common pool of miRNAs and further regulates mRNA expression [14].

This ceRNA network hypothesis has been confirmed in several human diseases including LC. For instance, lncRNA GAS5 was documented to suppress LC cell proliferation and metastasis by serving as a ceRNA for miR-205/PTEN [15]. Likewise, a lncRNA SBF2-AS1/miR-302a/MBNL3 axis was recently identified to influence the radiosensitivity of NSCLC cells [16]. Considering the complex RNA interactions, there remains largely unknown in this field. In light of the above discussion, the study was performed to probe a novel ceRNA network in LC progression. To this end, we firstly determined the aberrantly expressed lncRNAs in LC through microarray analysis and expression detection experiments, and then had the role and the involved downstream molecules of the potential lncRNA in LC figured out.

Methods

Clinical sample collection

A total of 58 pairs of LC tumor tissue samples and the corresponding para-cancerous samples were collected from the Pathology Department of the Ninth Affiliated Hospital of Guangxi Medical University. All procedures were ratified by the Ethics Committee of the Ninth Affiliated Hospital of Guangxi Medical University and conducted as per the Declaration of Helsinki. Signed informed consent was received from each eligible participant. All tissue samples were collected after surgical resection and immediately frozen in liquid nitrogen.

Microarray analysis

Microarray analysis was performed to screen out the differentially expressed lncRNAs between tumor and normal tissues. In brief, 0.5 µg total RNA from either tumor tissues or normal tissues was reversely transcribed to cDNA using a GeneChip 3' In vitro Transcription Express Kit (Thermo Fisher Scientific, Waltham, MA, USA). Then the cDNA was hybridized with Human lncRNA Expression Array V4.0 (Arraystar, Rockville, MD, USA), and then the chip was washed and scanned on a GeneChip™ Scanner 3000 7G System (Thermo Fisher).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA from tissues and cells was extracted using a TRIzol Kit (Thermo Fisher). Next, the cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher) according to the RNA template. Then the cDNA was amplified and then diluted for RT-qPCR. The primer sequences are shown in Table 1.

Table 1
Primer sequences for RT-qPCR

Gene	Primer sequence (5'-3')
HAGLR	F: AGATCCTGGGTGCAGACTTC
	R: GTAGAAAGGGCTGGGGAAG
SLC34A2	F: CAGGCAGATCCAGCCATC
	R: TTGAGAGTCGACTGGAAAGTTCT
miR-330-3p	F: ACTTTCCAAATGAGAATCGCATGG
	R: GGTTAATTGCATGACCAGACAGTGA
U6	F: CTCGCTTCGGCAGCACA
	R: AACGCTTCACGAATTTGCGT
GAPDH	F: ACAGTCAGCCGCATCTTCTT
	R: GACAAGCTTCCCGTTCTCAG
Note: RT-qPCR, reverse transcription quantitative polymerase chain reaction; SLC34A2, solute carrier family 34 member 2; miR: microRNA, GAPDH, glyceraldehyde-3-phosphate dehydrogenase.	

Cell transfection

Human LC cell lines (H1299,95C,A549,SPC-A-1,L9981 and H1299) and human tracheal epithelial cell line (16HBE) were purchased from Cell Bank of Chinese Academy of Medical Sciences (Beijing, China). The cells were seeded into culture dishes at a density of 1 × 10⁵ cells/cm² and then filled with 10% fetal bovine serum-supplemented F12K medium (Gibco, Grand Island, NY, USA) for 48 h of incubation at 37°C with 5% CO₂. When the confluency got to 80–90%, the cells well detached in 0.025% trypsin (Gibco) and passaged.

Well growing H1299 and A549 cells were transfected with pcDNA3.1-HAGLR vector or pcDNA3.1 empty vector (all vectors were synthesized by GenePharma Co., Ltd., Shanghai, China), respectively. The transfection was performed using a Lipofectamine™ 2000 Kit (Thermo Fisher) in accordance with the manufacturer’s instructions.

Cell viability detection

Well growing H1299 and A549 cells were harvested. The viability of cells was determined using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay kit (Dojindo Laboratories, Kumamoto, Japan), while cell proliferation was determined using a 5-ethynyl-2'-deoxyuridine (EdU) labeling assay kit (iFluor 647, Abcam Co., Ltd., NY, USA). All procedures were conducted in strict accordance with the manufacturer's instructions. Transwell assays was performed to measure invasion and migration of cells as previously described [17].

Self-renewal assay

Well growing H1299 and A549 cells (1 cell/ μ L) were sorted into 96-well plates (100 μ L/well) and cultivated for 7 d. Then the number of formed cell spheres was observed under a microscope (Olympus Optical Co., Ltd., Tokyo, Japan).

Flow cytometry

Well growing H1299 and A549 cells were collected. The apoptosis of each group of cells was determined using Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double staining method on a flow cytometer (AccuriC6, BD Company, USA). Thereafter, cells were labeled with CD44 (ab216647, Abcam) and CD133 (ab19898, Abcam) and detected on the flow cytometer. The ratio of cancer stem cells (CD44⁺ and CD133⁺) were evaluated.

Immunofluorescence staining

Expression of Sex-determining region Y-box 9 (SOX9) and Octamer 4 (OCT4) in H1299 and A549 cells was detected using Immunofluorescence staining. In brief, cells growing on slides were washed 3 times with phosphate buffer saline (PBS), fixed in 4% paraformaldehyde at 4°C for 15 min, and then treated with 0.5% Triton-100 X for 20 min. Next, the cell slides were incubated with primary antibodies SOX9 (1:200, ab185230, Abcam) and OCT4 (1:100, ab19857, Abcam) at 4°C overnight. Next, the cell slides were washed with PBS and incubated with the secondary antibody (1:5000, ab150088, Abcam) at 37°C for 1 h. Thereafter, the cells were further stained with 4', 6-diamidino-2-phenylindole and then observed under a fluorescence microscope (Leica DM 3000).

Sub-cellular localization of HAGLR

Sub-cellular localization of HAGLR was at first predicted on LncAtlas (LncAtlas.org). Next, the sub-cellular localization of HAGLR in H1299 and A549 cells was further measured using a nucleoplasmic RNA separation kit (PARIS kit, Cat. No. AM1921, Invitrogen, Inc., Carlsbad, CA, USA) as per the kit's protocol.

Dual luciferase reporter gene assay

The binding relationships between miR-330-3p and HAGLR and between miR-330-3p and solute carrier family 34 member 2 (SLC34A2) were validated by dual luciferase reporter gene assay. The cDNA sequence of HAGLR and SLC34A2-3'UTR sequence containing the binding site with miR-134 were designed by GenePharma Co., Ltd. (Shanghai, China). The sequences were inserted into pMIR-REPORT™ vector (Thermo Fisher) [18] to construct pMIR-HAGLR-wide type (WT) and pMIR-SLC34A2-WT vectors, and

the corresponding pMIR-HAGLR-mutant type (MUT) and pMIR-SLC34A2-MUT vectors were constructed as well based on the mutated binding sequences. The transfection was performed using a Lipofectamin™ 2000 Kit (Invitrogen). The relative luciferase activity was determined on aDual Luciferase Reporter Assay System (Promega, Madison, WI, USA) [19].

Western blot analysis

The expression of marker proteins of the Wnt/ β -catenin signaling pathway in H1299 and A549 cells was determined using western blot analysis as previously described [20]. The primary antibodies used were Wnt (1:2000, ab15251, Abcam), β -catenin (1:500, ab6302) and horseradish peroxidase-labeled secondary antibody immunoglobulin G (1:50000, ab7090).

Statistical analysis

SPSS 21.0 (IBM Corp. Armonk, NY, USA) was applied for data analysis. Kolmogorov-Smirnov checked that data were in normal distribution. Measurement data were exhibited as mean $\pm$ standard deviation (SD). Differences between every two groups were compared utilizing the t test, while those among multiple groups were compared using one-way or two-way analysis of variance (ANOVA) with Tukey's multiple comparison test used for post hoc test. The survival curve for patients was recorded using the Kaplan-Meier method and analyzed using log rank test. Correlations between molecular expression were compared using Pearson's correlation analysis. The p value was acquired from two-tailed tests, and $p < 0.05$ was considered to present significant difference.

Results

HAGLR is abnormally lowly expressed in LC tissues and cells

lncRNAs with differential expression in LC were determined using microarray analysis based on 5 pairs of LC tissues and para-cancerous tissues and then validated by RT-qPCR. The results suggested that HAGLR was significantly lowly expressed in LC tissues (Fig. 1A-B). Next, we further assessed HAGLR expression in LC cells and human tracheal epithelial cells, which found that HAGLR was expressed at low levels in LC cells as well, particularly in H1299 and A549 cell lines (Fig. 1C). Moreover, data on the KM-plotter website (<http://kmplot.com/analysis/>) suggested that LC patients with lower HAGLR expression presented lower survival rate and less median survival time (Fig. 1D). To further identify the function of HAGLR in LC, artificial overexpression of HAGLR was administrated in H1299 and A549 cell lines, and RT-qPCR found the transfection was successfully performed since HAGLR expression was increased in both cell lines (Fig. 1E).

Overexpression of HAGLR inhibits viability of LC cells

The EdU labeling and MTT assay results suggested that overexpression of HAGLR inhibited viability and proliferation while promoted apoptosis of H1299 and A549 cells (Fig. 2A-C). In addition, the Transwell assay results suggested that the invasion and migration abilities of LC cells was notably decreased as well following HAGLR overexpression (Fig. 2D-E).

Overexpression of HAGLR reduces the stemness of H1299 and A549 cells

Following the findings above, we further explored the role of HAGLR in stemness of H1299 and A549 cells. After 7 d of incubation, the formation of tumor spheres by non-adherent H1299 and A549 cells was evaluated. It was found that overexpression of HAGLR inhibited the size and number of formed tumor spheres by H1299 and A549 cells (Fig. 3A). Next, flow cytometry was performed to measure the number of cancer stem cells (CD44⁺/CD133⁺) in H1299 and A549 cells, which suggested that overexpression of HAGLR reduces the stemness of H1299 and A549 cells (Fig. 3B). In addition, the immunofluorescence staining suggested that the expression of SOX9 and OCT4 in both cells was decreased following HAGLR overexpression (Fig. 3C).

HAGLR up-regulates SLC34A2 expression through sponging miR-330-3p

To explore the potential molecular mechanisms involved in the HAGLR mediation, we firstly determined the sub-cellular localization of HAGLR in cells. The data on LncAtlas (<http://lncatlas.crg.eu/>) suggested that HAGLR is mainly localized in cytoplasm (Fig. 4A), this was further identified by the RNA nuclear-cytoplasm separation assay, where HAGLR was mainly expressed in cytoplasm (Fig. 4B).

This finding indicated that miR-330-3p might regulate growth and stemness of LC through the ceRNA network. Thereafter, the online prediction on StarBase (<http://starbase.sysu.edu.cn/>) and dual luciferase reporter gene assay identified that HAGLR could directly bind to miR-330-3p (Fig. 5A). Additionally, miR-330-3p was found to be highly expressed in LC patients according to RT-qPCR (Fig. 5B) and was negatively correlated with HAGLR expression (Fig. 5C).

Moreover, prediction on StarBase and dual luciferase reporter gene assay also validated that miR-330-3p could directly bind to the 3'UTR of SLC34A2 (Fig. 5D). SCL34A2 was lowly expressed in LC patients (Fig. 5E), presenting a positive correlation with HAGLR expression (Fig. 5F). Moreover, data in the TCGA DataBase (<http://www.tcg.org/>) also suggested that SLC34A2 is lowly expressed in LC (Fig. 5G). In addition, we further found that overexpression of HAGLR led to considerably decreased miR-330-3p expression while increased SLC34A2 expression (Fig. 5H).

Overexpression of HAGLR up-regulates SLC34A2 expression and inhibits the Wnt/ β -catenin signaling pathway.

Next, we assessed the levels of Wnt/ β -catenin pathway-related proteins Wnt1 and β -catenin in cells. It was found that over-expression of HAGLR inhibited the Wnt1 and β -catenin expression in H1299 and A549 cells (Fig. 6A). In addition, IQ-1 (1 mg, ab142079), a Wnt/ β -catenin signaling pathway agonist, was introduced into H1299 cells and A549 cells following HAGLR overexpression, after which the viability of H1299 and A549 cells was partly recovered, and the migration and invasion abilities of cells were promoted (Fig. 6B-E).

Discussion

Though it has become a common sense that cigarette smoking is the major cause of LC, LC continues to be one of the most prevalent malignancies and the most common causes of cancer deaths globally, leaving development of novel therapeutic options of great significance to the modern society [3]. HAGLR, also known as HOXD-AS1, whose aberrant expression has been documented to link with cancer progression [21]. Here in this study, we reported that HAGLR could serve as a competing endogenous RNA for miR-330-3p to upregulate miR-330-3p expression and inactivate the Wnt/ β -catenin pathway, leading to inhibited growth and metastasis and reduced stemness of LC cells.

Initially, our study found that HAGLR was aberrantly lowly expressed in LC tissues and cells according to microarray analysis and RT-qPCR. Artificial up-regulation of HAGLR inhibited viability and metastasis of H1299 and A549 cells. As aforementioned, uncontrolled metastasis is a major reason leading to death of late-stage LC patients [6]. HAGLR is localized between the HOXD1 and HOXD3 genes in the HOXD cluster and are usually serves as an oncogene in several cancer types [21]. However, its anti-tumor has been documented before, where HAGLR has been demonstrated to inhibit growth and metastasis of colorectal carcinoma by suppressing HOXD3-induced Integrin β 3 transcription and the following MAPK/AKT activation [22]. Similarly, low expression of HAGLR was found to be associated with increased cell growth and decreased survival time of patients with lungadenocarcinoma, a major subtype of NSCLC [23]. Moreover, overexpression of HAGLR was found to reduce the stemness of LC cells, presenting as decreased cell number with CD44⁺ and CD133⁺, as well as decreased expression of SOX9 [24] and OCT4 [25] in cells. It is well-known that cancer stem cells are closely correlated with carcinogenesis, tumor progression, relapse and metastasis, leaving reducing stemness of stem cells a great challenge as well as a promising therapeutic approach in cancer treatment [26]. These findings further validated the potential inhibiting role of HAGLR in LC progression.

The findings above triggered us to figure out the potential underlying molecular mechanisms. HAGLR was suggested to be sub-localized in cytoplasm, indicating it might exert functions through the ceRNA networks. Then, online prediction and the dual luciferase reporter gene assay identified that HAGLR could directly bind to miR-330-3p, which was further found to be highly expressed in LC patients. miR-330-3p has been documented to serve as an oncogene in pancreatic cancer [27]. Importantly, previous studies have suggested that miR-330-3p could promote NSCLC LC progression by inhibiting malignant behaviors of LC cells including proliferation, invasion, migration and metastasis [28, 29]. Moreover, we further found that miR-330-3p could directly bind to SLC34A2. SLC34A2 was found to be down-regulated in surgical

samples of NSCLC as compared to the adjacent normal lung tissues [30]. Quite in line with our study, SLC34A2 was found to be lowly-expressed in LC cell lines, and elevated SLC34A2 was shown to inhibit viability and invasion of LC cells [31]. In addition, SLC34A2 was found as a favorable prognostic marker in LC patients, with higher SCL34A2 levels leading to higher overall survival rates [32]. Furthermore, our study found that the expression of Wnt1 and β -catenin in H1299 and A549 cells was decreased following HAGLR over-expression. Administration of IQ-1, a Wnt/ β -catenin agonist, recovered viability and invasiveness of H1299 and A549 cells. Since HAGLR could promote SCL24A2 expression, and SCL24A2 has been found to negatively regulate the Wnt/ β -catenin pathway in NSCLC cells [33]. The Wnt/ β -catenin signaling is implicated in multiple physiological processes including cell migration, polarization, as well as in maintenance and proliferation of cancer stem cells in the tumor bulk [34]. These findings suggested that activation of Wnt/ β -catenin might be responsible for the growth and metastasis of LC cells.

Conclusion

To sum up, the current study provided evidence that HAGLR could inactivate the Wnt/ β -catenin pathway through the miR-330-3p/SCL24A2 ceRNA network, leading to suppressed growth and metastasis and reduced stemness of LC cells. Thus, HAGLR might serve as a novel therapeutic approach for LC treatment. However, though we identified a novel ceRNA network in this study, the specific role of miR-330-3p or SCL24A2 alone in LC cell behaviors was not included. Besides, more specific mechanisms by which SCL24A2 inactivates the Wnt/ β -catenin pathway remains unelucidated. We hope more studies will be carried out in the near future to validate our findings, and we will focus on more specific roles of miR-330-3p and SCL24A2 in LC progression and in Wnt/ β -catenin mediation.

Abbreviations

ANOVA, analysis of variance; ceRNA, competing endogenous RNA; EdU, 5-ethynyl-2'-deoxyuridine; FITC, fluorescein isothiocyanate; LC, lung cancer; LncRNAs, long non-coding RNAs; mean $\pm$ SD, mean $\pm$ standard derivation; miRNAs, microRNAs; MREs, miRNA-response elements; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; MUT, mutant type; NC, negative control; ncRNAs, non-coding RNAs; NSCLC, non-small-cell lung cancer; OCT4, Octamer 4; PBS, phosphate buffer saline; PI, propidium iodide; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SLC34A2, solute carrier family 34 member 2; SOX9, sex-determining region Y-box 9; WT, wide type; 3'UTR, 3'untranslated region.

Declarations

Ethics approval and consent to participate

All procedures were ratified by the Ethics Committee of the Ninth Affiliated Hospital of Guangxi Medical University and conducted as per the *Declaration of Helsinki*. Signed informed consent was received from each eligible participant.

Consent for publication

Not applicable.

Availability of data and materials

All the data generated or analyzed during this study are included in this published article.

Competing interests

All authors declare that there is no conflict of interest in this study.

Funding

Not applicable.

Authors' contributions

CXY contributed to the conception of the study. CXY and DKC designed the study. XLZ provided advice on statistical methods and the analyses of the data. CXY and DKC prepared the draft of the manuscript, tables and figures. FDY provided comments on the final draft of the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1 Primer sequences for RT-qPCR

Gene	Primer sequence (5'-3')
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	R: GTAGAAAGGGCTGGGAAG
SLC34A2	F: CAGGCAGATCCAGCCATC
	R: TTGAGAGTCGACTGGAAAGTTCT
miR-330-3p	F: ACTTTCCAAATGAGAATCGCATGG
	R: GGTTAATTGCATGACCAGACAGTGA
U6	F: CTCGCTTCGGCAGCACA
	R: AACGCTTCACGAATTTGCGT
GAPDH	F: ACAGTCAGCCGCATCTTCTT
	R: GACAAGCTTCCCGTTCTCAG

Note: RT-qPCR, reverse transcription quantitative polymerase chain reaction; SLC34A2, solute carrier family 34 member 2; miR: microRNA, GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Figures

Figure 1

HAGLR is abnormally lowly expressed in LC tissues and cells. A, Heatmap for the top 30 differentially expressed lncRNAs in 5 pairs of LC tissues and the paired adjacent tissues. B, HAGLR expression in 58 pairs of LC tissues and normal tissues was determined using RT-qPCR. C, HAGLR expression in LC cell lines (H1299,95C,A549,SPC-A-1,L9981 and H1299) and human tracheal epithelial cell line (16HBE) was

determined using RT-qPCR. D, prognostic value of HAGLR expression in LC patients was analyzed on the KN-plotter website (<http://kmplot.com/analysis/>). Then, HAGLR overexpressing vector or the empty vector was transfected into H1299 and A549 cell lines. E, HAGLR expression in cells was determined using RT-qPCR. Data were exhibited as mean $\pm$ SD. Data in panel B were compared using unpaired t test, and data in panels C and E were analyzed by one-way ANOVA and Tukey's multiple comparison test, while in panel D, the survival curve was produced using the Kalpan-Meier method and analyzed using log rank test. Repetition = 3. *, $p < 0.05$.

Figure 2

Overexpression of HAGLR inhibits viability of H1299 and A549 cells. A, proliferating cells were observed by EdU labeling. B, viability of H1299 and A549 cells was determined using MTT assay. C, apoptosis of H1299 and A549 cells was determined using Annexin V-FITC/PI staining on a flow cytometer. D-E, migration and invasion abilities of H1299 and A549 cells were detected by Transwell assays. Data were

exhibited as mean $\pm$ SD. In panels A, C, D and E, data were analyzed using one-way ANOVA, while data in panel B were analyzed using two-way ANOVA, and Tukey's multiple comparison test was applied for post hoc test. Repetition = 3. *, $p < 0.05$.

Figure 3

Overexpression of HAGLR reduces the stemness of H1299 and A549 cells. A, self-renewal ability of H1299 and A549 cells was determined using tumor sphere formation assay. B, number of cancer stem cells (CD44+/CD133+) was screened out by flow cytometry. C, expression of cancer stem cells markers SOX9 and OCT4 in H1299 and A549 cells was assessed by immunofluorescence staining. Data were exhibited as mean $\pm$ SD. In panels A, C, D and E, data were analyzed using one-way ANOVA, while data in panel B were analyzed using two-way ANOVA, and Tukey's multiple comparison

Figure 4

HAGLR is sub-localized in cytoplasm. A, HAGLR is sub-localized in cytoplasm, as predicted by LncAtlas. B, nuclear and cytoplasmic expression of HAGLR in H1299 cells and A549 cells was determined by RT-qPCR. Data were exhibited as mean $\pm$ SD. Data were analyzed using two-way ANOVA, and Tukey's multiple comparison test was applied for post hoc test. Repetition = 3. *, $p < 0.05$.

Figure 5

HAGLR up-regulates SLC34A2 expression through sponging miR-330-3p. A, binding relationship between HAGLR and miR-330-3p was predicted on StarBase (<http://starbase.sysu.edu.cn/>) and validated through dual luciferase reporter gene assay. B, miR-330-3p expression in 58 pairs of LC tissues and normal tissues was detected using RT-qPCR. C, miR-330-3p expression was negatively correlated with HAGLR expression in LC patients. D, binding relationship between miR-330-3p and SLC34A2 was predicted on StarBase and validated through dual luciferase reporter gene assay. E, SLC34A2 expression in 58 pairs of LC tissues and normal tissues was detected using RT-qPCR. F, miR-330-3p expression was positively correlated with HAGLR expression in LC patients. G, SLC34A2 expression was examined on TCGA DataBase (<http://www.tcg.org/>). H, expression of miR-330-3p and SLC34A2 in H1299 and A549 cells following HAGLR expression was measured by RT-qPCR. Data were exhibited as mean $\pm$ SD. Data were analyzed using two-way ANOVA and Tukey's multiple comparison test. Correlations between HAGLR and miR-330-3p expression, and between miR-330-3p and SLC34A2 expression was analyzed using Pearson's correlation analysis. Repetition = 3. *, $p < 0.05$.

Figure 6

Overexpression of HAGLR up-regulates SLC34A2 expression and inhibits the Wnt/ β -catenin signaling pathway. A, levels of Wnt/ β -catenin pathway-related proteins Wnt1 and β -catenin in H1299 and A549 cells were determined by western blot analysis. Then, a Wnt/ β -catenin signaling pathway agonist IQ-1 was introduced into H1299 cells and A549 cells following HAGLR overexpression. B, proliferating cells were observed by EdU labeling. C, apoptosis of H1299 and A549 cells was determined using Annexin V-FITC/PI staining on a flow cytometer. D-E, migration and invasion abilities of H1299 and A549 cells were detected by Transwell assays. Data were exhibited as mean $\pm$ SD. Data in panel A were analyzed using

two-way ANOVA, while data in panels B-E were analyzed using one-way ANOVA, and Tukey's multiple comparison test was applied for the post hoc test. Repetition = 3. *, $p < 0.05$.

Supplementary Files

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