SEMA4D under the posttranscriptional regulation of HuR and miR-4319 boosts cancer progression in esophageal squamous cell carcinoma

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ABSTRACT

Esophageal squamous cell carcinoma (ESCC) is the major type of esophageal carcinoma, one of the main reasons of cancer-caused death. While the therapeutic effect on ESCC patients is still unsatisfactory as a result of tumor aggression, recurrence and metastasis. RNA-binding proteins, microRNAs and specific genes get involved in tumorigenesis and development of tumors in a large proportion. In several reports, SEMA4D is an oncogene and miR-4319 is a tumor suppressor. We discovered the interaction of SEMA4D with HuR and miR-4319, whereas the detailed mechanism in ESCC was yet to be researched. At first, SEMA4D was significantly overexpressed in ESCC cells, and its knockdown repressed cell proliferation and migration as well as accelerated cell apoptosis. And then HuR was proved to stabilize SEMA4D mRNA by binding to its 3′UTR. In addition, miR-4319 targeted and degraded SEMA4D. Taken together, SEMA4D was regulated competitively by HuR and miR-4319. Collectively, HuR and miR-4319 co-regulating SEMA4D affected cell proliferation, apoptosis and migration in ESCC. This research explored the regulatory mechanism on SEMA4D in ESCC and provided optional therapeutic targets for ESCC patients.

Introduction

Esophageal carcinoma is one of the main reasons for cancer-related death, among which esophageal squamous cell carcinoma (ESCC) is the major type. The treatment of ESCC which has high incidence and mortality remains difficult as a result of its extreme aggression and frequent lymph node metastasis. Hence, the research focused on the molecular mechanism in ESCC progression is worthy of further investigation.

Carcinomas generally occur with aberrant gene expression resulted from diverse aspects, among which the function of RNA-binding proteins (RBPs) was widely explored. The stability of mRNAs is controlled by numerous RBPs at transcriptional level. And dysregulation of RBPs could result in ectopic expression of cancer-associated genes, which has been largely observed in human cancers.

ELAV-like protein 1 or human antigen R (HuR) is a member of the Hu/ELAV (human/embryonic lethal abnormal vision) RBP family. Besides, HuR has also been elucidated to have oncogenic function in multiple carcinomas due to its positive regulation on RNA stability through binding to the AU-rich elements (AREs) in the 3′UTRs of target mRNAs. For instance, LncARSR enhances cell proliferation and invasion by associating with HuR and miR-200 family in epithelial ovarian cancer; PARG mRNA post-transcriptionally regulated by HuR facilitates DNA repair and is resistant to PARP inhibitors in pancreatic ductal adenocarcinomas. The LINC2707 facilitates proliferation and metastasis in gastric cancer by interplaying with HuR.

The emergence of microRNAs (miRNAs) has been exposed to be one of the crucial factors in cancer biology through the identification of alterations in microRNA processing machinery and microRNA target binding sites. And a part of miRNAs regulate biological activities including cell proliferation, apoptosis and migration and so on, which are of importance in cancer development. For example, the downregulation of microRNA-150 expression defines acute myeloid leukemia patients and the prognostic implication; miR-424 targeting AKT3 and PSAT1 has a tumor-suppressive role in colorectal cancer cells; MicroRNA-221 stimulates autophagy via suppressing HDAC6 expression and inducing cell apoptosis in pancreatic cancer.

Several studies have reported the role of miR-4319 as a tumor-suppressive gene in carcinomas with various levels of inhibition on cellular processes, which principally involves cell growth, stenness and apoptosis. Since miR-4319 was never explored in ESCC, the investigation of its possible underlying mechanism participated in ESCC progression is urgently necessary.

This paper firstly unveiled improved expression of SEMA4D in ESCC, whose silence repressed cell proliferation and migration and accelerated cell apoptosis in ESCC. Besides, HuR was proved to interact with and stabilize SEMA4D mRNA. Moreover, miR-4319 targeted and further degraded SEMA4D. Then, mechanistic assays determined that HuR and miR-4319 competitively regulated SEMA4D. Collectively, SEMA4D co-regulated by HuR and miR-4319 in opposite direction affected cell proliferation,
apoptosis and migration in ESCC. Overall, our work probed the possible regulation mechanism underlying miR-4319 and SEMA4D in ESCC process, and found an effective therapy for the cure of ESCC patients.

Materials and methods

Cell lines and cell culture

ESCC cell lines containing KYSE-150, TE-10 and TE-1 were obtained from American Type Culture Collection (ATCC, Manassas, MA, USA). Normal human esophageal epithelial cell line (HEEC) and the ESCC cell line EC-109 were bought from the Cell Bank of the Chinese Academy of Sciences (Shanghai, PR China). The supported culture environment was as follows: Dulbecco’s Modified Eagle’s Medium (Wisent Bioproducts, St-Bruno, QC, Canada) with 10% FBS (Wisent Bioproducts), 100 µg/mL streptomycin and 100 U/mL penicillin in a drippy incubator (stabilized at 37°C and 5% CO₂).

Cell transfection

To knock down SEMA4D and HuR, short hairpin plasmid vectors targeting the coding area of SEMA4D (sh-SEMA4D#1 and sh-SEMA4D#2) and HuR (sh-HuR) were synthesized and achieved from GenePharma (China). Nontargeting control shRNA acted as a negative control. MiR-4319 mimics and miR-4319 inhibitor were synthesized by RiboBio (China). The plasmid vectors were transfected into KYSE-150 and TE-10 cells with Lipofectamine 2000 (Invitrogen, USA) under the manufacturer’s references.

Quantitative real-time PCR (qRT-PCR)

Following the instructions of the manufacturer, total RNA of ESCC cells was isolated with TRIzol reagent (Thermo Fisher Scientific), followed by reverse transcription into cDNA with a PrimeScript RT reagent kit (Takara, Shiga, Japan). A 7,500 Real-time PCR System (Thermo Fisher Scientific), followed by reverse transcription into cDNA with a PrimeScript RT reagent kit (Takara, Shiga, Japan). A 7,500 Real-time PCR System (Thermo Fisher Scientific) together with a SYBR Premix Ex Taq kit (Takara) was adopted to conduct the reactions. The primers for the target mRNAs and internal control were constructed as follows: SEMA4D forward: 5’-AGCTCTGCACAAAGCCATCAGC-3’ and SEMA4D reverse: 5’-CCAGCATAGAACAACCTGTGGCC-3’; HuR forward: 5’-ATGAAAGACACATGGCCGAAGCT-3’ and HuR reverse: 5’-AGTTCAAGACATGCCGAGACT-3’; GAPDH forward: 5’-CAGTCTCTGACAAAGCCATCAGC-3’ and GAPDH reverse: 5’-ACCACCCCTGTGGTGAGCAAGCT-3’ and U6 forward: 5’-CCTGCTGTGCGAGCAAGCT-3’ and U6 reverse: 5’-AACGCTTCAAGATTTGCGT-3’. The expression pattern of miR-4319 normalized to U6 and that of others to GAPDH was calculated by the \(2^{-\Delta\Delta CT}\) method. Transfected KYSE-150 and TE-10 cells (2,000 cells per well) were inoculated into 96-well dishes and grown overnight. Add 10 µL of CCK-8 solution to the serum-free medium every 24 h. After cultivating cells for 2 h, at a wavelength of 450 nm, the absorbance was tested by a microplate reader followed by plotting of proliferation curves.

Transwell assay

The migration capacity of transfected KYSE-150 and TE-10 cells was evaluated by Transwell chambers (8 µm diameter; Corning Inc., Corning, NY, USA). Cells were digested by trypsin, then collected and resuspended in RPMI-1640 medium. A total of 200 µl cell suspension was implemented into the upper chamber and RPMI-1640 medium (500 µl) with 10% FBS was added into the lower chamber. Following incubation at 37°C for 24 h, cells in the upper compartment were removed utilizing a cotton swab. At room temperature, the compartment was fixed by 4% formaldehyde for 10 min, subjected to Giemsa’s staining for 1 min and washed thrice. Migrated Cells were counted under a microscope (five fields; magnification, ×200).

Flow cytometry

Cellular apoptosis was measured by an AnnexinV/PI double staining assay, of which PI dyes the nuclei of necrotic or late apoptotic cells (red) and Annexin V-FITC dyes the membranes of early apoptotic cells (green). At room temperature, cells were washed using PBS and incubated in binding buffer (100 µl) including Annexin V-FITC and propidium iodide (PI) in the dark for 15 min. Another 400 µl binding buffer was implemented and then cells were dissected with a FACScan flow cytometer (Becton-Dickinson, San Jose, USA).

Luciferase reporter assay

The potential binding site between SEMA4D and miR-4319 was predicted using TargetScan7 (http://www.targetscan.org/vert_71/) tool. The wild-type (WT) SEMA4D sequences containing the predicted miR-4319 binding site were amplified and embedded into a pmirGLO dual-luciferase vector (Promega Corp., Madison, WI, USA) in order to establish the reporter vector pmirGLO-SEMA4D-WT. The putative binding site of miR-4319 in the 3’-UTR of SEMA4D was mutated employing a GeneArt™ Site-Directed Mutagenesis PLUS System (Cat. no. A14604; Thermo Fisher Scientific, Inc.), then embedded into a pmirGLO vector to construct the reporter vector pmirGLO-SEMA4D-Mut. MiR-4319 mimics or miR-NC and the respective reporter vector were co-transfected into 2 × 10⁴ ESCC cells, followed by 48 h of incubation. Thereafter, a Dual-Luciferase Reporter Assay System (Promega Corp.) was adopted to examine luciferase activities normalized to Renilla using the Dual-Luciferase Reporter Gene Assay kit (Promega Corp.) following the manufacturer’s brochure.
RNA immunoprecipitation (RIP) assay

A Magna RIP kit (EMD Millipore, Billerica, MA, USA) was utilized for RIP assay following the guidelines of the manufacturer. Whole-cell lysate was grown with RIP buffer added with magnetic beads that aimed to conjugate with human antibody anti-HuR, anti-Ago2 or normal mouse IgG as endogenous control. These antibodies were all from Millipore. The immunoprecipitated RNAs were harvested, refined, and analyzed by qRT-PCR to determine the binding of target RNAs.

RNA pull-down

In RNA pull-down experiments, a Pierce Magnetic RNA-Protein Pull-Down Kit (Thermo, Waltham, MA, USA) was employed. Briefly, the sequence of SEMA4D binding with HuR (or miR-4319 with SEMA4D) was amplified, which was purified utilizing the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), biotinylated by a Pierce RNA 3’ End Desthiobiotinylation Kit (Thermo, Waltham, MA, USA) and purified again utilizing TRIzol. Next, ESCC cells were lysed in Pierce IP Lysis Buffer (Thermo, Waltham, MA, USA). Complexes were subsequently eluted to test the expression of target genes by western blot and qRT-PCR.

Western blot

Following washing in ice-cold PBS for three times, cells were lysed in a 300 mL of cell lysis buffer with a mixture of protease inhibitors and 1 mM phenylmethylsulfonyl fluoride (PMSF). After 30 min of incubation, centrifuge the cell lysate, then harvest total protein from the supernatant, and quantify the concentration with a bicinchoninic acid (BCA) protein assay kit (Beyotime, Hangzhou, China). Lysates were divided using an SDS-PAGE gel, transferred onto PVDF membranes and then inflicted western blot evaluation. The PVDF membranes were cultivated with primary antibodies anti-HuR (Millipore) and anti-GAPDH (Abcam) at 4°C overnight, followed by 2 h of incubation with the appropriate peroxidase-conjugated secondary antibody at room temperature. The membranes were washed thrice with PBS and blotted with an enhanced chemiluminescent (ECL) detection system.

Statistical analysis

Data are universally expressed as the mean ± SD. All statistical results were obtained using SPSS 16.0 (SPSS, Chicago, IL, USA) and also GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Student’s t-test was utilized to estimate comparisons between groups. Each sample was assessed in triplicate and \( P < .05 \) was considered to present significant differences.

Results

SEMA4D is significantly overexpressed and its knockdown represses cancer progression in ESCC

To investigate whether SEMA4D was implicated in ESCC, we examined its expression level in a panel of ESCC cell lines (KYSE-150, TE-10, EC-109 and TE-1) and normal human esophageal epithelial cell line (HEEC). SEMA4D expression level was markedly overexpressed in ESCC cell lines compared with the control group (Figure 1(a)), suggesting the possible participation of SEMA4D in ESCC progression. Then, we performed loss-of-function assays to explore its function role in ESCC. The expression level of SEMA4D was dramatically knocked down...
under the treatment of sh-SEMA4D#1 or sh-SEMA4D#2 in KYSE-150 and TE-10 cells (Figure 1(b)) compared with Mock and sh-NC group. CCK-8 assay showed that cell proliferation of KYSE-150 and TE-10 cells was inhibited by the silencing of SEMA4D (Figure 1(c)). Flow cytometry analysis of cell apoptosis exhibited that cell apoptosis rate of KYSE-150 and TE-10 cells was increased when SEMA4D was downregulated (Figure 1(d)). And transwell migration assay indicated the repression effects of SEMA4D knockdown on cell migration in KYSE-150 and TE-10 cells (Figure 1(e)). All these data demonstrate that SEMA4D is up-regulated in ESCC and its knockdown suppresses cell proliferation and migration as well as induces cell apoptosis in ESCC.

HuR binds to SEMA4D and stabilizes SEMA4D mRNA level

RNA-binding proteins have been reported to exert their functions through interacting with miRNAs in cancers. As shown in Figure 2(a), we gained the predicted interaction between HuR and SEMA4D from starBase v2.0. We transfected sh-HuR into KYSE-150 and TE-10 cells, with sh-NC as a negative control. The mRNA and protein levels of HuR were strikingly reduced as measured by qRT-PCR and western blot experiments (Figure 2(b,c)). In RNA pull-down assay, HuR was only abundant in the complex pulled down by bio-SEMA4D in KYSE-150 and TE-10 cells (Figure 2(d)). In RIP assay, SEMA4D was enriched in the mixture immunoprecipitated by anti-HuR (Figure 2(e)). These two experiments proved the interaction between HuR and SEMA4D bilaterally. qRT-PCR assay found that SEMA4D level was relatively decreased when HuR was silenced in KYSE-150 and TE-10 cells (Figure 2(f)). After the addition of Actinomycin D into KYSE-150 and TE-10 cells, the mRNA half-life of SEMA4D was declined by HuR silence, supporting that the mRNA stability of SEMA4D was regulated by HuR (Figure 2(g)). All of these results elucidate that HuR binds to SEMA4D and stabilizes the mRNA level of SEMA4D.

MiR-4319 targets and degrades SEMA4D

MicroRNAs have also been exposed to play pivotal parts in human tumors via targeting mRNAs at a large proportion.
From TargetScan7 prediction, we obtained the putative binding sequences between SEMA4D and miR-4319 (Figure 3)(a). Firstly, miR-4319 mimics obviously lifted miR-4319 expression in KYSE-150 and TE-10 cells as tested through qRT-PCR assay (Figure 3(b)). Luciferase reporter experiment demonstrated the repression of miR-4319 overexpression on the luciferase activity of WT-SEMA4D (Figure 3(c)) and RIP experiment demonstrated the enrichment of miR-4319 and SEMA4D in the compound precipitated by anti-Ago2 (Figure 3(d)), both of which suggested the combination between miR-4319 and SEMA4D. Furthermore, qRT-PCR assay detected that miR-4319 overexpression overtly lowered SEMA4D expression in KYSE-150 and TE-10 cells (Figure 3(e)). Briefly, SEMA4D is a target gene of miR-4319 and its expression was negatively regulated by miR-4319 in ESCC.

**SEMA4D is regulated competitively by HuR and miR-4319**

Previous researches have studied the competition between RBPs and miRNAs on the regulation of mRNAs. Considering the respective modulation of HuR and miR-4319 on SEMA4D in ESCC, we supposed that HuR and miR-4319 competitively bound to SEMA4D so as to mediate cellular activities of ESCC. In pull-down assay, SEMA4D mRNA was gathered in the complex pulled down by biotinilated-miR-4319, which was strengthened when HuR was downregulated (Figure 4(a)). In RIP assay, SEMA4D mRNA was concentrated in the mixture precipitated by anti-HuR, and this phenomenon was suppressed by the overexpression of miR-4319 (Figure 4(b)). ESCC cells were separately co-transfected with sh-NC and miR-4319 mimics or sh-HuR and miR-4319 mimics, compared with NC group. As estimated through qRT-PCR assay, SEMA4D expression was inhibited by miR-4319 mimics, also restrained by HuR down-regulation and further repressed by miR-4319 up-regulation (Figure 4(c)). Besides, qRT-PCR assay revealed that HuR expression had no variation under miR-4319 overexpression in KYSE-150 and TE-10 cells (Figure 4(d)). Taken together, HuR and miR-4319 regulate SEMA4D at posttranscriptional level competitively.

**HuR and miR-4319 co-regulating SEMA4D affect cell proliferation, apoptosis and migration in ESCC**

In order to confirm the regulation mechanism on SEMA4D in ESCC, we performed rescue experiments. MiR-4319 was clearly restrained by miR-4319 inhibitor in KYSE-150 and TE-10 cells as assessed by qRT-PCR assay (Figure 5(a)). CCK-8 experiment displayed that miR-4319 downregulation and SEMA4D inhibition, respectively, encouraged and repressed cell proliferation of KYSE-150 and TE-10 cells, and the prohibitive role of SEMA4D silencing could be restored by the restraint of miR-4319 (Figure 5(b)). Flow cytometry presented that cell apoptosis rate of KYSE-150 and TE-10 cells were inhibited by miR-4319 knockdown but enhanced by SEMA4D silencing. Moreover, the effect of silenced SEMA4D on apoptosis was abolished by miR-4399 suppression (Figure 5(c)). At last, transwell assay illustrated that miR-4319 inhibitor promoted cell migration, and the restraint of SEMA4D down-regulation on cell migration was abrogated by the silencing of miR-4319 in KYSE-150 and TE-10 cells (Figure 5(d)). To sum up, HuR and miR-4319 regulate SEMA4D expression in ESCC...
cell proliferation, apoptosis and migration in ESCC by regulating SEMA4D in a competitive way.

**Discussion**

Semaphorin 4D (SEMA4D), located in chromosome 9q22.2 and also known as Cluster of Differentiation 100 (CD100), is a member of the semaphorin family. The oncogenic role of SEMA4D in stimulating tumor invasiveness and progression has been demonstrated in a big amount of carcinomas. For instance, semaphorin 4D enhances skeletal metastasis of breast cancer cells; Semaphorin 4D and hypoxia-inducible factor-1α upregulation is associated with prognosis of colorectal carcinoma patients; Semaphorin 4D plays a biomarker role in tumor development and angiogenesis of human breast cancer; The role and underlying mechanisms of SEMA4D were exposed in vasculogenic mimicry formation in NSCLC.

Consistently, the present study found that SEMA4D was expressed at high levels and its knockdown repressed cell proliferation, migration and EMT process in ESCC. It was the first time that SEMA4D was probed in ESCC, further indicating its expression profile and functional role.

HuR, a well-established RNA-binding protein, has already been recognized as a cancerogenic gene and reported to exert its function via targeting mRNAs at posttranscriptional level in numerous carcinomas. For example, circAGO2 impels cancer progression by facilitating HuR-repressed functions on AGO2-miRNA complexes; LncRNA B4GALT1-AS1 attracts HuR to boost osteosarcoma cells migration and stemness by reinforcing YAP transcriptional activity; BAG3 regulates IL-8 mRNA stability through the interplay between HuR and miR-4312 in PDACs. Our study represented that HuR interacted with SEMA4D mRNA and further stabilized its mRNA level in ESCC.

Moreover, miR-4319 has been exposed to its anti-tumor effects in triple-negative breast cancer, prostate cancer and acute myeloid leukemia, affecting cell stemness, growth and apoptosis. It was mentionable that this was the first exploration of miR-4319 in ESCC. In the current research, miR-4319 targeted SEMA4D so as to degrade the expression level of SEMA4D in ESCC.

A diverse range of reports has indicated the competitive regulation of RBPs and miRNAs on particular mRNAs. Take some examples, RNA-binding protein Dnd1 enhances cell apoptosis via stabilizing the Bim mRNA and provides a shelter from miR-221 targeting in breast cancer; the inflammatory marker C-Reactive Protein is under the posttranscriptional regulation of HuR and miR-637; Transformer 2β and miR-204 modulate apoptosis by competitively binding to BCL2.
mRNA 3’UTR. Hence, we studied the influences of HuR and miR-4319 on the regulation of SEMA4D in combination. In accordance with our assumptions, mechanism experiments unveiled the co-regulation of HuR and miR-4319 on SEMA4D in inverse directions.

In the end, rescue experiments affirmed that HuR and miR-4319 competitively regulate SEMA4D to affect cell proliferation, apoptosis and migration in ESCC. This paper uncovered the expression profile and promising regulatory mechanism on SEMA4D in ESCC, preparing for the targeted molecular therapy for patients with ESCC.

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