Blocking Modification of Eukaryotic Initiation 5A2 Antagonizes Cervical Carcinoma via Inhibition of RhoA/ROCK Signal Transduction Pathway

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Xiaojun Liu, MS^{1,2}, Dong Chen, PhD³, Jiamei Liu, PhD²,
Zhangtao Chu, MD², and Dongli Liu, MD²

Abstract

Cervical carcinoma is one of the leading causes of cancer-related death for female worldwide. Eukaryotic initiation factor 5A2 belongs to the eukaryotic initiation factor 5A family and is proposed to be a key factor involved in the development of diverse cancers. In the current study, a series of *in vivo* and *in vitro* investigations were performed to characterize the role of eukaryotic initiation factor 5A2 in oncogenesis and metastasis of cervical carcinoma. The expression status of eukaryotic initiation factor 5A2 in 15 cervical carcinoma patients was quantified. Then, the effect of eukaryotic initiation factor 5A2 knockdown on *in vivo* tumorigenicity ability, cell proliferation, cell cycle distribution, and cell mobility of HeLa cells was measured. To uncover the mechanism driving the function of eukaryotic initiation factor 5A2 in cervical carcinoma, expression of members within RhoA/ROCK pathway was detected, and the results were further verified with an RhoA overexpression modification. The level of eukaryotic initiation factor 5A2 in cervical carcinoma samples was significantly higher than that in paired paratumor tissues ($P < .05$). And the *in vivo* tumorigenic ability of HeLa cells was reduced by inhibition of eukaryotic initiation factor 5A2. Knockdown of eukaryotic initiation factor 5A2 in HeLa cells decreased the cell viability compared with normal cells and induced G1 phase cell cycle arrest ($P < .05$). Moreover, the cell migration ability of eukaryotic initiation factor 5A2 knockdown cells was dramatically inhibited. Associated with alterations in phenotypes, RhoA, ROCK I, and ROCK II were downregulated. The above-mentioned changes in eukaryotic initiation factor 5A2 knockdown cells were alleviated by the overexpression of RhoA. The major findings outlined in the current study confirmed the potential of eukaryotic initiation factor 5A2 as a promising prognosis predictor and therapeutic target for cervical carcinoma treatment. Also, our data inferred that eukaryotic initiation factor 5A2 might function in carcinogenesis of cervical carcinoma through an RhoA/ROCK-dependent manner.

Keywords

cervical carcinoma, eukaryotic initiation 5A2, RhoA, ROCK I, ROCK II

Abbreviations

CC, cervical carcinoma; cDNA, complementary DNA; DMEM, Dulbecco modified eagle medium; eIF5A, eukaryotic initiation factor 5A; FBS, fetal bovine serum; HPV, human papilloma virus; mRNA, messenger RNA; OD, optical density; qPCR, quantitative polymerase chain reaction; ROCK, Rho-associated kinase; shRNA, short hairpin RNA; NC, negative control; TTBS, Tris-buffered saline/0.05% Tween-20; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; RhoA, Ras homolog gene family, member A.

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¹ Department of Obstetrics and Gynecology, China-Japan Union Hospital, Jilin University, Changchun, Jilin, People's Republic of China

² Department of Histology & Embryology, School of Basic Medical Sciences, Jilin University, Changchun, Jilin, People's Republic of China

³ Department of Histology & Embryology, Guangdong Medical University, Dongguan, Guangdong, People's Republic of China

Corresponding Authors:

Dong Chen, Department of Histology & Embryology, Guangdong Medical University, Songshan Lake Science & Technology Park, Dongguan, Guangdong 523808, People's Republic of China.

Email: dchen1987@sina.com

Jiamei Liu, Department of Histology & Embryology, School of Basic Medical Sciences, Jilin University, 126 Xinmin Avenue, Changchun, Jilin 130021, People's Republic of China.

Email: jiameiliu2541@outlook.com

Introduction

Carcinoma of the uterine cervix is the major cause of cancer-related death among women in developing countries.¹ Overwhelming evidence now shows that most cases (80%-90%) of cervical carcinoma (CC) are infected by high-risk human papilloma virus (HPV), and persistent infection of a certain type of HPV genotypes has been taken as an indispensable step for the progression of CC.^{2,3} However, except for HPV infection, the development of CC also depends on extensive genetic alternations, which will interact with external pathogenic factors including HPV in complicated ways. Over the past 20 years, circa 200 candidate genes with more than 500 molecular variants have been comprehensively investigated for their association with the oncogenesis and development of CC.⁴ Although the function of most targeted biomarkers in CC was only verified in certain populations, some of these genes might have true impact on the carcinogenesis of CC.

Translational control of gene expressions is critical in the proliferation of cells; unfortunately, the process is often dysregulated in cancers.⁵ As a key member participating in the modulation of the proliferation of eukaryotic cells, eukaryotic initiation factor 5A (eIF5A) initiates the synthesis of methionyl-puromycin, a model assay for the first ribosomal peptidyl transfer reaction.⁶ Homo sapiens have 2 *eIF5A* isoforms: *eIF5A1*, which is expressed in many normal tissues, and *eIF5A2*, which has relatively limited expression and distribution. Although the exact physiological activity of this family has yet to be revealed, a strong correlation between *eIF5A* overexpression and development of cancers has been established. As reported by Memin *et al*, blocking modification of *eIF5A1* reduced the proliferation of CC cells and suppressed the expression of cancer-related genes.⁷ The study of Chen *et al* has concluded that the overexpression of *eIF5A2* is an independent predictor for the prognosis of urothelial carcinoma of the bladder,⁸ and this result is further explained by the study of Luo and his colleagues.⁹ Additionally, the elevated level of *eIF5A2* was proved to promote the cytoskeleton rearrangement in hepatocellular carcinoma through an RhoA/Rho-associated kinase (ROCK) I-dependent manner.¹⁰ This RhoA/ROCK-dependent regulation on cancers by *eIF5A* was validated with pancreatic cancer as well, which implicated that *eIF5A* was not only a cytoskeletal rheostat controlling RhoA/ROCK protein expression but also an RhoA/Rac-dependent modulator of cell migration and invasion.¹¹ But even with extensive investigations on the function of *eIF5A* in multiple cancer types, little progression of the function of *eIF5A2* in CC attack has been made. Based on this information, it is valuable to assess the function of *eIF5A2* in CC to promote the understanding of the role of this factor in CC and the development of *eIF5A*-dependent anti-CC therapies.

Thus, in the present study, the expression status of *eIF5A2* was first detected with clinical CC samples to determine the possible involvement of *eIF5A2* in the carcinogenesis of CC. To reveal the function of *eIF5A2* in CC, the effect of *eIF5A2* inhibition on the cell proliferation, cell cycle distribution, and

cell mobility was measured. As previously reported, *eIF5A2* exerted its function in tumors through an RhoA/ROCK-dependent manner; therefore, the regulation of *eIF5A2* interfering on the expression of RhoA and ROCKs was quantified as well. With the above-mentioned work, we expected to verify the function of *eIF5A2* in CC and to preliminarily reveal the pathways through which *eIF5A2* contributes to the formation and progression of this malignancy.

Materials and Methods

Chemicals, Animals, and Cell Cultures

Antibodies against *eIF5A2*, RhoA, ROCK I, and ROCK II were purchased from Abcam (Cambridge, Massachusetts). Human CC cell line HeLa preserved in our laboratory was cultured in Dulbecco modified eagle medium (DMEM) medium supplemented with 20% fetal bovine serum (FBS) and 1% (vol/vol) antibiotics mixture in an atmosphere of 95% air and 5% CO₂ at 37°C. Cells from 3 to 6 passages were used for further studies. BALB/c-nu mice housed in our laboratory were used for *in vivo* tumor induction experiments and maintained in cages at room temperature (20°C-25°C) with a constant humidity (55% ± 5%) with food and water available ad libitum. All the assays with animals were performed following the Institutional Animal Ethics Committee and Animal Care Guidelines for the Care and Use of Laboratory Animals of The Guangdong Medical University.

Patients and Tissue Specimen Collection

In the current study, for the detection of *eIF5A2* expression levels in clinical samples, 15 paired CC and paratumor samples were collected from CC patients from March 2011 to April 2015 in Biobank of China-Japan Union Hospital, Jilin University and preserved in liquid nitrogen for further detection. All the patients enrolled in the present study should meet the following criteria: (1) tumor samples from the resection must be confirmed to be primary CC, and paratumor tissues sampled from the patients must contain no CC cells. (2) All the cases involved in the analysis should have detailed information of clinicopathological and prognostic characteristics. Those who had undergone any therapy against CC before surgical operation were excluded. The study was approved by ethics committee of the China-Japan Union Hospital of Jilin University (Changchun, China). The ethics committee approved the relating screening, inspection, and data collection of the patients, and all participants signed a written informed consent form. All works were undertaken following the provisions of the Declaration of Helsinki.

Knockdown of *eIF5A2* Gene in HeLa Cells by Short Hairpin RNA

Eukaryotic initiation factor 5A-specific (5'-GGAUCUAAAA-CUGCCAGAATT-3') and nontargeting (5'-UUCUCCGAAC-GUGUCACGUTT-3') short hairpin RNAs (shRNAs) were

synthesized by Wanlei Life Science (Shenyang, China) according to the previous study.¹⁰ Sequences of different shRNAs were ligated into pGCsi-H1 plasmid to form transfection vectors (pGCsi-H1-NC and pGCsi-H1-eIF5A2). Transfection was conducted using transfection agents of Applygen Technologies Inc (No. c1507, Beijing, China), according to the manufacturer's instruction. To detect the effect of *eIF5A2* knockdown on biological processes in HeLa cells, 3 experimental groups were set up: (1) control group, HeLa cells; (2) NC group, HeLa cells transfected with pGCsi-H1-NC plasmid; and (3) shIF5A2 group, HeLa cells transfected with pGCsi-H1-eIF5A2 plasmid. Each treatment was represented by at least 3 replicates. The knockdown efficiency of *eIF5A2* by shRNA was assessed using quantitative polymerase chain reaction (qPCR) and Western blotting assay as described subsequently and illustrated in Supplemental File 1. Stable transfected cells for further experiments were screened in medium with the presence of G418 (0.5 µg/µL).

Tumorigenicity Assay

Eighteen healthy BALB/c-nu mice were selected and randomly divided into 3 groups for tumor induction: (1) control group, mice were subcutaneously injected with 0.2 mL (5×10^7 /mL) HeLa cells at oter; (2) NC group, mice were subcutaneously injected with 0.2 mL (5×10^7 /mL) pGCsi-H1-NC plasmid-transfected HeLa cells; and (3) shIF5A2 group, mice were subcutaneously injected with 0.2 mL (5×10^7 /mL) pGCsi-H1-eIF5A2 plasmid-transfected HeLa cells. Then, the mice were raised under the same conditions for 27 days. The volumes, major axis, and minor axis of the solid tumors were measured every 3 days since the day tumor could be observed with naked eyes. Upon completion of the assay, all the mice were killed using air embolism method, and tumor tissues were harvested and preserved at -80°C for qPCR validation of the expression of *eIF5A2*.

Real-Time qPCR

Whole RNA in samples was extracted using RNA simple Total RNA Kit according to the manufacturer's instruction (No. DP419; TIANGEN, Beijing, China). β -Actin was selected as the reference gene. Then, the RNA was reverse transcribed to complementary DNA (cDNA) templates using Super M-MLV reverse transcriptase (No. RP6502; BioTeke, Beijing, China). The final real-time qPCR reaction mixture of volume 20 µL consisted of 10 µL of SYBR GREEN Master Mix, 0.5 µL of each primers (*eIF5A2*, forward: 5'-AAGATGGTTACCTTTCCCTG-3', reverse: 5'-TACAGCATATTCTTCACTCATTG-3'; β -actin, forward: 5'-CTTAGTTGCGTTACACCCTTTCTTG-3', reverse: 5'-CTGTACCTTCACCGTTCCAGTTT-3'), 1 µL of the cDNA template, and 8 µL of Rnase-free H₂O. Thermal cycling parameters for the amplification were set up as the following: a denaturation step at 95°C for 10 minutes, followed by 40 cycles at 95°C for 10 seconds, 60°C for 20 seconds, and 72°C for 30 seconds. Relative expression level of targeted gene was

calculated with Exicycler 96 (BIONEER, South Korea) according to the expression of $2^{-\Delta\Delta\text{Ct}}$.

Western Blotting

Protein product of different samples was extracted using Whole Protein Extraction Kit according to the manufacturer's instruction (WLA019; Wanleibio, China), and β -actin was used as reference protein. The concentration of the extracted protein samples was determined according to the BCA method. For Western blotting assay, 40 µg protein in 20 µL solution was subjected to a 13% sodium dodecyl sulfate polyacrylamide gel electrophoresis and subsequently transferred onto polyvinylidene difluoride sheets. Then, the membranes were washed in TTBS for 5 minutes and incubated with 5% skim milk powder solution for 1 hour. Primary antibody against eIF5A2 (1:1000) for tissues, eIF5A2 (1:5000) for cells, RhoA (1:5000), ROCK I (1:5000), ROCK II (1:5000), or β -actin (1:1000) was added into the solution and incubated with the membranes at 4°C overnight. At the second day, the membranes were washed with TTBS for 4 times and incubated with secondary immunoglobulin G-horseradish peroxidase antibodies (1:5000) for 45 minutes at 37°C . After the final 6 washes with TTBS, the blots were developed using Beyo ECL Plus (Wanlei, Shenyang, China) reagent, and the results were observed in the Gel Imaging System (Liuyi, Beijing, China). The relative expression levels of eIF5A2 in different groups were calculated with Gel-Pro-Analyzer (Media Cybernetics, Rockville, Maryland).

MTT Assay

The cell viabilities of HeLa cells in different groups were measured by MTT assay; briefly, 50 µL exponentially growing cells (3×10^3 cells/mL) was seeded into 1 well of a 96-well plate and cultured for 120 hours. Each group was represented by 25 replicates, and the cell viabilities of 5 randomly selected wells at 24, 48, 72, 96, and 120 hours were determined, respectively. For each well, 5 mg/mL MTT was added, and after incubating for additional 4 hours at 37°C , 200 µL dimethyl sulfoxide was added. The cell viability was determined according to the optical density (OD) value at 490 nm recorded with a microplate reader.

Flow Cytometry

HeLa cells in different groups were cultured in DMEM medium supplemented with 10% FBS at 37°C and collected by centrifugation at 1500 rpm for 5 minutes. Flow cytometry was conducted to determine the effect of *eIF5A2* knockdown on the cell cycle distribution of HeLa cells; briefly, cells were first fixed with 70% alcohol at 4°C for 2 hours and collected by being subjected to centrifugation at 2000 rpm for 5 minutes. After administration with 100 µL RNase A for 30 minutes at 37°C , 500 µL propidium iodide-fluorescein isothiocyanate was added to different samples to stain DNA in dark at 4°C for 30 minutes. After additional 20-minute incubation at room

temperature, the DNA contents of the cells were analyzed using a flow cytometer (Accuri C6; BD, Mountain View, California).

Colony Formation Assay

The anchorage-independent growth capability of HeLa cells in different groups was measured by colony formation assay; cells (600/plate) were seeded into DMEM medium containing 3% methylcellulose. After 2 weeks in culture, the colonies were fixed with paraformaldehyde and stained with Wright-Giemsa stain for 5 minutes. The number of colonies was recorded under a microscope, and colony formation rate was equal to ([colony number/inoculation cell number] $\times$ 100%).

Scratch Assay

Cells (2×10^4 /well) were seeded into a 24-well plate, and reference points were marked to guarantee the same area of image acquisition with a 200 μ L pipette tip. Then, the plates were incubated at 37°C in an atmosphere of 5% CO₂ for 24 hours to stimulate the formation of confluent monolayer. Afterward, the cell layers were scratched to form a cell-free straight line and washed with PBS to remove debris. The migration of cells in the “scratch” was recorded at the matching reference points. For each well, 3 images (0, 12, and 24 hours) were taken with a phase-contrast microscope, and the distances between the 2 edges of the scratch were measured at the reference points. Wound healing was defined as the percentage of the starting distance between the 2 edges of each wound, and data were analyzed by ImageJ (version 1.45s) software (US National Institutes of Health).

Transwell Experiment

The transwell experiment that evaluated the migration ability of HeLa cells in different groups was performed; 200 μ L incubation (with 1 mmol/L MgCl₂) medium containing 1×10^4 cells was seeded into the upper transwell chambers (Corning star, Cambridge, Massachusetts). Then, cells were incubated at 37°C for 24 hours to allow the migration through the porous membrane. Upon completion of the culture, cells remaining at the upper surface of the chamber were completely removed. The lower surfaces of the membranes were fixed with 4% paraformaldehyde for 20 minutes and stained in a solution containing 0.5% (wt/vol) crystal violet for 5 minutes. After being washed using ddH₂O, cell numbers in different treatments were determined using an inverted phase contrast microscope (AE31; Motic, Xiamen, China).

Detection of Association Between eIF5A2 and RhoA/ROCK Signaling Transduction Pathway

The effect of *eIF5A* knockdown on the expression of RhoA, ROCK I, and ROCK II was quantified using Western blotting assay as described above. To further elucidate the interaction between eIF5A2 and RhoA/ROCK pathway, an expression

vector encode RhoA was constructed. Four groups were set up for subsequent analyses: (1) NC group, HeLa cells transfected with pGCsi-H1-NC plasmid; (2) sh*eIF5A2* group, HeLa cells transfected with pGCsi-H1-eIF5A2 plasmid; (3) vector group, *eIF5A2* knockdown cells transfected with blank vector; and (4) RhoA group, *eIF5A2* knockdown cells transfected with RhoA expression vector. Each treatment was represented by at least 3 replicates. The transfection efficiency of RhoA expression vector was measured by Western blotting assay and shown in Supplemental File 2. The viabilities of cells in different groups after 0, 24, and 48 hours of incubation were determined using MTT assay as described above. The migration ability of cells under different treatments was also determined using scratch assay as described above.

Statistical Analysis

All the data were expressed in the form of mean $\pm$ standard deviation. Student *t* test, analysis of variance, and post hoc tests were performed with a significant level of .05 using GraphPad Prism 6 (GraphPad Software, San Diego, California).

Results

Expression of eIF5A2 in CC Samples Was Upregulated

To investigate the status of gene expression of eIF5A2 in CC, the messenger RNA (mRNA) and protein production in 15 pairs of primary CC specimen and adjacent normal cervical tissues were detected. All the CC samples showed upregulated *eIF5A2* mRNA expression when compared with their corresponding paratumor tissues, and the average difference between CC and paratumor samples was statistically significant ($P < .05$; Figure 1A). A total of 9 of the 15 CC samples showed upregulated expression of eIF5A2 protein product compared with paired paratumor samples, and the average difference was statistically significant ($P < .05$; Figure 1B).

Knockdown of eIF5A2 Inhibited the Tumor Growth In Vivo

As shown in Figure 2A, solid tumors could be detected since the ninth day of the assay. In each group, the volumes of the tumors kept increasing until the completion of the assay. However, for recording points since the 18th day, the average volume in the sh*eIF5A2* group was significantly lower than that in the control group or in the NC group ($P < .05$). The morphology of the solid tumors in different groups is shown in Figure 2B; evident differences among the volumes of tumors in different groups could be observed with naked eyes. Validated by qPCR, the expression level of *shIF5A2* mRNA in the sh*eIF5A2* group was also significantly lower than that in the control group or in the NC group ($P < .05$; Supplemental File 3). Taken together, the results clearly revealed the inhibition effect of *eIF5A2* knockdown on the development of CC *in vivo*.

Figure 1. The expression of eIF5A2 is upregulated in clinical cervical carcinoma (CC) samples. A, Quantitative analysis result of real-time quantitative polymerase chain reaction (qPCR) detection of clinical samples. B, Quantitative analysis result and representative images of Western blotting assay of clinical samples.

Figure 2. Knockdown of *eIF5A2* inhibited the growth of cervical carcinoma *in vivo*. A, Quantitative analysis results of growth curve of solid tumors in different groups; tumor volumes at the last 4 recording time points were significantly inhibited by knockdown of *eIF5A2*. B, Representative images of the morphology of solid tumors in different groups. *Significantly different from the control group, $P < .05$. #Significantly different from the NC group, $P < .05$. NC, negative control.

Figure 3. Knockdown of *eIF5A2* attenuated cell viability and anchorage-independent growth capability in HeLa cells. A, The results of MTT assay; cell viabilities at the last 4 time points were significantly inhibited by the inhibition of *eIF5A2*. B, The results of colony formation assay; cell numbers in shEIF5A2 cells were significantly reduced compared with the control or NC group. *Significantly different from the control group, $P < .05$. #Significantly different from the NC group, $P < .05$. NC, negative control; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide.

Knockdown of *eIF5A2* Attenuated Viability and Anchorage-Independent Growth Ability of HeLa Cells

The viability of cells that undergone different treatments was quantified using MTT assay. As shown in Figure 3A, compared with the NC group, transfection of *eIF5A2*-specific shRNA decreased the OD₄₅₀ value in the shEIF5A2 group since the 48th hour of the assay, and the differences between the shEIF5A2 group and the control or NC group were statistically

Figure 4. Knockdown of *eIF5A2*-induced G1 phase arrest in cell cycle distribution. *Significantly different from the control group, $P < .05$. #Significantly different from the NC group, $P < .05$. NC, negative control.

significant for the last 4 time points ($P < .05$; Figure 3A). Additionally, the anchorage-independent growth ability of HeLa cells was inhibited by *eIF5A2* knockdown as well; the colony formation rate in shEIF5A2 was $26.9\% \pm 3.3\%$, which was significantly different from those in the control or NC groups ($49.4\% \pm 5.1\%$ and $46.9\% \pm 4.7\%$; $P < .05$; Figure 3B).

Knockdown of *eIF5A2*-Induced Cell Cycle Arrest in HeLa Cells

Cell cycle distribution of different groups was analyzed by flow cytometry. Post knockdown of *eIF5A2*, cells in the shEIF5A2 group showed G1 phase arrest with cell percentage in G1 phase increasing to $75.0\% \pm 5.5\%$, which was significantly different from the results of the other 2 groups ($55.3\% \pm 3.4\%$ for the control group and $55.7\% \pm 4.4\%$ for the NC group; $P < .05$; Figure 4). The inhibition of *eIF5A2* in HeLa cells also resulted in a concomitant decrease in the fraction of S phase, revealing that downregulation of *eIF5A2* could halt the proliferation of HeLa cells via cell cycle arrest at the G1 phase.

Knockdown of *eIF5A2* Decreased the Cell Migration Ability of HeLa Cells

The function of *eIF5A2* in the migration of HeLa cells was determined by 2 methods. First, a scratch assay was performed, and the results indicated that the cell mobility was inhibited by knockdown of *eIF5A2*; as illustrated in the representative

Figure 5. Knockdown of *eIF5A2* decreased the cell migration ability in HeLa cells. A, Representative images and quantitative analysis results of scratch assay; wound healing rate of the shIF5A2 group was significantly lower than those in the control and NC groups. B, Representative images and quantitative analysis results of transwell experiment; cell numbers moving through the porous member of the shIF5A2 group were significantly lower than those in the control and NC groups. *Significantly different from the control group, $P < .05$. #Significantly different from the NC group, $P < .05$. NC, negative control.

images and quantitative analyses, cells transfected with *eIF5A2* shRNA showed a delayed process of wound closure compared with cells in the other 2 groups, representing markedly inhibited migrational movement (Figure 5A). Additionally, in transwell experiment, the number of cells migrating through the porous membrane in the shIF5A2 group (43 ± 4) was significantly lower than those in the other 2 groups (70 ± 6 for the control group and 64 ± 6 in the NC group; $P < .05$; Figure 5B), indicating the inhibition on the cell mobility due to the inhibition of *eIF5A2*.

Knockdown of *eIF5A2* Exerted Its Function in HeLa Cells Through an RhoA/ROCK Pathway-Dependent Manner

To explore the mechanism that drove *eIF5A2* knockdown on the biological alterations in HeLa cells, the production of RhoA, ROCK I, and ROCK II was quantified with Western blotting assay. It was found that inhibition of *eIF5A2* suppressed the expression of all the 3 proteins (Figure 6A); based on the quantitative analysis, the difference between the *eIF5A2* group and the control or NC group was statistically significant ($P < .05$). The result of Western blotting assay demonstrated that *eIF5A2* might play a part in the carcinogenesis of CC through an RhoA/ROCK-dependent manner. To verify this hypothesis, *eIF5A2* knockdown HeLa cells were subsequently transfected with an RhoA expression vector. It was found after upregulation of the expression of RhoA, the inhibition in viability and mobility of HeLa cells due to *eIF5A2* knockdown

was dramatically reversed (Figure 6B and C), representing a key role of RhoA downregulation in the effect of *eIF5A2* knockdown on CC cells.

Discussion

Eukaryotic initiation factor 5A is one of the only 2 isoforms of *eIF5A* family and localized at a chromosomal region. The factor is confirmed as an oncogenic protein and often noted for chromosomal instability in many types of cancers, including colon, ovarian, and bladder cancer.^{8,12,13} These studies focusing on *eIF5A2* have provided a better understanding of the molecular events governing the pathogenesis in cancers. But regarding CC, although previous study reported that the inhibition of *eIF5A1* in CC cells could contribute to the suppression of cancer-related genes and cancer cell proliferation, no further study on the function of *eIF5A2* in this cancer was performed.⁷ Targeting the function of *eIF5A2* in CC, the current study provided the first evidence that *eIF5A2* played a determinant role in the proliferation and metastasis of CC cells. Moreover, *eIF5A2* mediated these processes through an RhoA/ROCK-dependent manner.

The expression status of *eIF5A2* was first validated in clinical CC samples with qPCR and Western blotting assay. It was found that in majority of paired samples, the levels of *eIF5A2* in cancer tissues were significantly higher than those in paratumor tissues, both at mRNA and protein levels. These results confirmed the potential of *eIF5A2* as an important biomarker

Figure 6. Eukaryotic initiation factor 5A2 determined the proliferation and mobility of cervical carcinoma (CC) cells through an RhoA/Rho-associated kinase (ROCK)-dependent manner. A, Quantitative analysis results and representative images of Western blotting assay; the expression of RhoA, ROCK I, and ROCK II was downregulated by knockdown of eIF5A2. B, Results of MTT assay; overexpression of RhoA attenuated the negative effect of *eIF5A2* knockdown. C, Results of scratch assay; overexpression of RhoA attenuated the negative effect of *eIF5A2* knockdown. *Significantly different from the control group, $P < .05$. #Significantly different from the NC group, $P < .05$. ^aSignificantly different from the NC group, $P < .05$. ^bSignificantly different from the shIF5A2 group, $P < .05$. ^cSignificantly different from the vector group, $P < .05$. NC, negative control; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide.

for the prognosis and a therapeutic target for types of human tumors.¹⁴ Afterward, the overexpression of eIF5A2 in human CC cell line HeLa was inhibited by transfection of a specific shRNA plasmid. Then, a series of *in vitro* assays were conducted to characterize the role of eIF5A2 in regulating the CC cell proliferation, cell cycle distribution, and mobility. And the results showed that the suppression of eIF5A2 could significantly attenuate the cell viability, induce G1 cell cycle arrest, and impair cell mobility. All these findings strongly supported the hypothesis that the overexpression of eIF5A2 played a critical role in the development and metastasis of CC, and interference of the expression of this factor could serve as a promising therapy antagonizing CC.

Previous study based on hepatocellular carcinoma inferred that eIF5A2 was able to activate RhoA/ROCK pathway to stimulate the formation of stress fiber and lamellipodia.¹⁰ Rho is capable to convey signals from extracellular stimuli to the actin cytoskeleton and the nucleus and to regulate cell migration and malignant transformation.¹⁵ As the prototype of Rho, RhoA protein is a crucial mediator transducing the upstream signaling evoked by various factors to its downstream-targeted ROCKs.¹⁶⁻¹⁸ Then, responses in the pathway initiated by RhoA

are subsequently mediated by ROCKs and lead to dynamic reorganization of cytoskeletal proteins, that is, formation of stress fiber and focal adhesions.¹⁹ Rearrangement of actin cytoskeleton is central to the determination of migration of cancer cells, which will further facilitate the metastasis processes. In bladder cancer, the expressions of RhoA and ROCKs were positively correlated with tumor progression; higher levels of RhoA and ROCK I/ROCK II were associated not only with higher stages of disease but also with poorer survival and lymph node metastasis.¹⁵ Upregulation of RhoA and ROCK II by vascular endothelial growth factor C was also proved to promote the activation of moesin protein and metastasis of CC cells.²⁰ Although the exact function of ROCK I and ROCK II in mediating RhoA signaling remains unclear, this pathway may represent a promising molecular target for the prevention of CC invasion and metastasis.

The above findings could be partly elucidated by the data derived from the current study. It was found that similar to the result of previous studies,¹⁰ gene silencing of *eIF5A* by shRNA reduced the levels of RhoA, ROCK I, and ROCK II in HeLa cells. And the downregulation of the 3 indicators was synchronized with the decrease in cell mobility of HeLa cells.

Additionally, post upregulation of RhoA expression by an expression vector, the inhibition in cell viability and mobility by *eIF5A2* knockdown was dramatically reversed, which further demonstrate that the antagonizing effect of *eIF5A2* blockade against CC exclusively functioned through the downregulation of RhoA. However, it should be aware that our data were merely obtained from *in vitro* experiments, and only 3 molecules involved in the RhoA/ROCK pathway were detected. The downstream effectors of RhoA/ROCK signaling associated with the function of *eIF5A2* in CC and whether our findings exist *in vivo* still need to be further explored.

Taken together, the current study demonstrated the potential of *eIF5A2* as a promising prognosis predictor and therapeutic target for CC treatment. Blocking modification of *eIF5A2* resulted in a significant decrease in the cell viability and mobility in CC cells. And our data also inferred that the factor might function through an RhoA/ROCK-dependent manner. Genes and proteins regulating this signaling transduction pathway are valuable for further investigation to facilitate the development of treatment strategies for patients with advanced CC.

Declaration of Conflicting Interests

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Supplemental Material

Supplementary material for this article is available online.

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