HPV16 E6/E7 promote the translocation and glucose uptake of GLUT1 by PI3K/AKT pathway via relieving miR-451 inhibitory effect on CAB39 in lung cancer cells

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Abstract

Background: HPV16 E6/E7 proteins are the main oncogenes and only long-term persistent infection causes lung cancer. Our previous studies have shown that HPV16 E6/E7 protein up-regulates the expression of GLUT1 in lung cancer cells. However, whether E6 and E7 protein can promote the glucose uptake of GLUT1 and its molecular mechanism are unclear.

Methods: The regulatory relationships of E6 or E7, miR-451, CAB39, PI3K/AKT, and GLUT1 were detected by double directional genetic manipulations in lung cancer cell lines. Immunofluorescence and flow cytometry were used to detect the effect of CAB39 on promoting the translocation to the plasma membrane of GLUT1. Flow cytometry and confocal microscopy were performed to detect the glucose uptake levels of GLUT1.

Results: The overexpression both E6 and E7 proteins significantly down-regulated the expression level of miR-451, and the loss of miR-451 further up-regulated the expression of its target gene CAB39 at both protein and mRNA levels. Subsequently, CAB39 up-regulated the expression of GLUT1 at both protein and mRNA levels. Our results demonstrated that HPV16 E6/E7 up-regulated the expression and activation of GLUT1 through the HPV–miR-451–CAB39–GLUT1 axis. More interestingly, we found that CAB39 prompted GLUT1 translocation to the plasma membrane and glucose uptake, and this promotion depended on the PI3K/AKT pathway.

Conclusion: Our findings provide new evidence to support the critical roles of miR-451 and CAB39 in the pathogenesis of HPV-related lung cancer.

Keywords: CAB39, GLUT1, HPV16, lung cancer, miR-451, PI3K/AKT

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It is well known that the cancer cells use the Warburg effect to consume more glucose to obtain energy by aerobic glycolysis. During this process, activated GLUT1, the main glucose transporter, plays a critical role. GLUT1 is up-regulated on a variety of tumor cell plasma membranes, including lung cancer cells. The key role that GLUT1 plays in the Warburg effect is not to increase the expression of GLUT1 in the cytoplasm, but to be activated. Mechanism studies have shown that the activation of GLUT1 is mainly through stimulating to increase the translocation of GLUT1 from the cytoplasm to the plasma membrane, thus promoting the glucose uptake of GLUT1.

Since Syrjänen first hypothesized that human papillomavirus (HPV) infection might play an important role in the occurrence of lung cancer in 1979, an increasing number of studies have shown that HPV16 E6/E7 proteins were the main oncogenes, and only long-term persistent infection could cause lung cancer. Our previous studies demonstrated that HPV16 E6/E7 proteins up-regulated GLUT1 at both protein and mRNA levels in four well-established lung cancer cell lines.

Thus, the investigation of the relationship among HPV16 E6/E7, miR-451, and GLUT1 is our main aim in the current study. Using double directional genetic manipulations in the well-established lung cancer cell lines, we show that both E6 and E7 proteins significantly down-regulate the expression level of miR-451, and the loss of miR-451 further up-regulates the expression of its target gene, calcium binding protein 39 (CAB39), at both the protein and mRNA levels. Subsequently, CAB39 up-regulates the expression of GLUT1 at both protein and mRNA levels. Our results demonstrate that HPV16 E6/E7 proteins up-regulate the expression of GLUT1 through the HPV–miR-451–CAB39–GLUT1 axis. More interestingly, we found that CAB39 prompted GLUT1 translocation to the plasma membrane and glucose uptake. We further confirmed that CAB39 promoted GLUT1 through PI3K/AKT pathways.

**Materials and methods**

The study was conducted according to the guidelines of the institutional review boards at the First Affiliated Hospital of China Medical University; we had obtained an internal review board approval for this study.

**Cell culture**

Three human non-small cell lung carcinoma (NSCLC) cell lines H460, A549, LK2, and the normal human bronchial epithelial (HBE) cell line were used in this study. HBE was used as a control to determine the expression levels of miR-451 and CAB39. HBE, H460, and A549 cell lines were purchased from the cell bank of the Chinese Academy of Science (Shanghai, China). HBE was cultured in Hyclone (Logan, UT, USA) minimum Eagle’s medium (MEM) supplemented with 10% fetal bovine serum (FBS; Cellmax, Beijing, China). H460 and A549 were cultured in Hyclone RPMI 1640 medium supplemented with 10% FBS. The LK2 cell line was obtained from Dr. Hiroshi Kijima (Department of Pathology and Bioscience, Hirosaki University Graduate School of Medicine, Japan) and cultured in Hyclone Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS. Cells were cultured in a humidified incubator at 37°C with 5% CO2.

**Plasmid construction and small-interfering RNA**

The plasmids for pEGFP-N1-HPV16 E6, pEGFP-N1-HPV16 E7, and pEGFP-N1 were kindly provided by Professor Xudong Tang (Institute of Biochemistry and Molecular Biology, Guangdong Medical College, China). The mimics for mirRNA-451-on or mirRNA-mimic were purchased from GenePharma (Shanghai, China). The plasmids pCMV3-C-Myc-CAB39 and pCMV3-C-Myc were purchased from Sino Biological (Sino Biological, Inc., USA). Small-interfering RNA (siRNA) was performed to silence the expression of specific genes. The siRNAs against HPV16 E6 and HPV16 E7 were purchased from RIBOBIO (Guangzhou, China) and the scrambled siRNA (sc-37007) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) as a nonspecific siRNA control. The CAB39 siRNA and scrambled siRNA were purchased from RIBOBIO (Guangzhou, China).

To inhibit PI3K/AKT signaling, cells were treated with 10 μM Miltefosine (ApexBio, USA), an inhibitor that blocks the PI3K/AKT pathway. Miltefosine was dissolved in dimethyl sulfoxide...
(DMSO) and added 12 h after a 36-h transfection, with the same volume of DMSO added to control cells.

**Transfection and interference**
The transfection and interference have been described previously.16

**Quantitative real-time PCR assays**
Total RNA was extracted from cells using RNAiso Plus (TaKaRa, Japan) according to the manufacturer’s instructions. Total 600 ng RNA was subjected to reverse transcription reaction to obtain cDNAs by using the Prime Script TM RT reagent Kit (TaKaRa, Japan) as follow: 37°C for 15 min, 85°C for 5 s, and the quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) was performed on 7900HT Fast Real-Time PCR System (Applied Biosystems) using SYBR® Premix Ex Taq II (TaKaRa, Japan) as follows: 95°C for 2 min, 40 cycles of 95°C for 15 s, 60°C for 60 s. A total of 3000 ng RNA miR-451 and U6 were subjected to reverse transcription reaction to obtain cDNAs by using the Hairpin-itTM miRNAs qPCR Quantitation Kit (GenePharma, China) as follows: 25°C for 30 min, 42°C for 30 min, 85°C for 5 min. The qRT-PCR was performed using the Hairpin-itTM miRNAs qPCR Quantitation Kit as follows: 95°C for 3 min, 40 cycles of 95°C for 12 s, 62°C for 40 s. A dissociation procedure was performed to generate a melting curve for confirmation of amplification specificity. GAPDH and U6 were used as the reference gene. The relative levels of gene expression were represented as \( \Delta \Delta Ct = Ct_{\text{target}} - Ct_{\text{reference}} \), and the fold change of gene expression was calculated by the \( 2^{-\Delta \Delta Ct} \) method. Experiments were repeated in triplicate. The detailed information of the primers is given in Table 1.

| Gene | Forward | Reverse | Sequence (5'–3') | Size (bp) | Melting (°C) | mRNA |
|------|---------|---------|------------------|----------|-------------|------|
| E6   | 270     | GTATGGAACAAACTAGACGCAA | 79       | 54.65     | KX545363    |
|      | 349     | GTGCTTTAGAACTAATACCCA | 53.55    |           |             |
| E7   | 482     | GCATGGAGATAACCTACATTG | 273      | 51        | KX545363    |
|      | 754     | TGTTTCCTGAAACAGATGG | 58       |           |             |
| CAB39| 1211    | TGAACCTGCTGCCAGGACAAAA | 88       | 60        | NM_016289.4 |
|      | 1298    | TGCGTCTGTAGGATTGCTA | 60       |           |             |
| GLUT1| 1071    | CTGGCATCAACCGCTGCTTC | 167      | 60        | NM_006516.3 |
|      | 1237    | GCCTATGAGGGTCAGGTC | 60       |           |             |
| GAPDH| 50      | TTCTTTTGGCGAGGCGACGAG | 71       | 51.06     | XM_01902318 |
|      | 120     | CAGCGCCCAATACGACCAAA | 51.06    |           | 8.1         |
| miR-451 |       | CCTCGGAAACGTGACGATT | 62       |           |             |
|      |         | TATCCCTTGTGCGATCCCCAC | 62       |           |             |
| U6   |         | CGCTTCGAGACACATATAC | 62       |           |             |
|      |         | TTCAGGAATTTCGCACTC | 62       |           |             |

mRNA, messenger RNA; miR, micro RNA; qRT-PCR, quantitative real-time reverse transcriptase polymerase chain reaction.
Western blot analysis

Western blot analysis was performed to quantify the amount of target protein. The Western blot has been described previously.16 HPV16 E6 (1:700, Abcam, Boston, MA, USA), HPV16 E7 (1:200, Bioss, Beijing, China), CAB39 (1:1000; Cell Signaling Technology, Beverly, MA, USA), PI3K(P85α) (1:1000; Proteintech, Wuhan, China), P-AKT (ser473) (1:1000; Proteintech), AKT (1:1000; Proteintech), GLUT1 (1:500; Wanleibio, China), and GAPDH (1:15000, Proteintech). After the membranes were further incubated with appropriate horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG (1:5000; Proteintech) at 37°C for 2 h, the immunosignal was detected by using ECL Western blot kit (advansta, USA). The bands were analyzed with BioImaging systems (UVP Inc., Upland, CA, USA).

Immunofluorescence

Cells with 90% confluent were seeded into a six-well culture plate. After gene transfection or siRNA interference was performed, the cells were cultured for another 24 h. Then the cells with a concentration of 5 × 10⁴ cell/ml (500 μl/well of the cell suspension) were seeded into a 24-well culture plate. Each bottom of the 24 wells were pre-inserted a 15 mm round cover glass slide (NEST Biotechnology Co., China) and the cells were cultured for 24 h to form a confluent monolayer. The cells were washed with ice-cold PBS and fixed with 4% formaldehyde (Solarbio, Beijing, China) for about 20 min. After the permeability treatment with PBS containing 0.1% Triton X-100 (Solarbio, Beijing, China) for 15 min and blocked with 3% bovine serum albumin for 1 h at 37°C. Subsequently, the coverslips were incubated with CAB39 (1:50; Cell Signaling, USA) or GLUT1 (1:50; Proteintech) antibody at 4°C overnight. After incubation with CoraLite488-conjugated Affinipure Goat Anti-Rabbit IgG (H+L) secondary antibody (SA00013-2, PTG, Beijing, China) for 1 h, the cover slips were mounted in Prolong Gold antifade reagent with DAPI (Beyotime, Shanghai, China) for 5 min, and coverslipped. Images were obtained on a confocal microscope (Carl Zeiss, Germany).

Glucose uptake assay

Cells (2–5 × 10⁴ cells/well) were seeded 1 day before starting the assay. After 12 h, regular culture medium (10% FBS) were removed and cells were treated with CAB39 transfected or un-transfected in 400 μl cell culture medium with 0.5% FBS and incubated at 37°C with 5% CO₂ for 3 h. Then 4 μl of fluorescent glucose analog 2-NBDG (2-N-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-D-glucose; BioVision, CA, USA) was added to each sample and incubated at 37°C with 5% CO₂ for 30 min. After incubation, the cells from the plate were kept on ice and washed once with 1 ml ice-cold 1X Analysis Buffer. Two methods were adopted for measuring the levels of glucose uptake. One analysis was performed using flow cytometry. Briefly, the cells were collected from the plates and the cell pellet was resuspended in 400 μl of 1X Analysis Buffer and analyzed on a flow cytometer (488 nm excitation laser; BD Accuri™ C6 Plus personal flow cytometer, USA). For flow acquisition and analysis mounting, the main cell groups were selected in the Forward scatter (FSC) versus Side scatter (SSC) plot to exclude dead cells and cellular debris. Within the main cell population, mean fluorescence intensity in FL1 was quantified and compared between cells treated with CAB39 transfected and untreated control cells. The other analysis was performed by using confocal microscopy to perform the image analysis. Briefly, the cells seeded into a 24-well culture plate were washed with 200 μl of fresh 1X Analysis Buffer and the images were acquired immediately under a confocal microscope using a blue excitation fluorescence filter (excitation range 420–495 nm). The fluorescence intensity was kept at a minimum to minimize photo-bleaching. Images were taken randomly from at least five different fields.

Statistical analysis

SPSS 22.0 software was utilized for statistical analyses in this study. Each assay was performed at least three times. The data were expressed as mean ± SD and the significance of differences in multiple comparisons was determined by Student’s t-test. p < 0.05 was considered to be statistically significant.

Results

The screening of lung cancer cell lines

Based on our previous results, H460 cells were low E6 and E7 expression cell lines, and A549 and LK2 cells were high E6 and E7 expression
cell lines. To investigate the roles of miR-451 and CAB39 on regulating GLUT1 translocation to the plasma membrane in lung cancer cell, several lung cancer cell lines were tested and three cell lines, A549, LK2, and H460, were selected. The HBE cell line was selected to be used as a control for the expression levels of miR-451 and CAB39; higher than HBE was considered as high expression and lower than HBE was considered as low expression. The expression level of miR-451 was high in LK2, but low in A549 and H460 (Supplemental Figure A). The expression level of CAB39 was high in A549, medium in LK2, and low in H460 (Supplemental Figure B). Low expression level of GLUT1 on the plasma membrane was observed in all three cell lines (Supplemental Figure C). Low glucose uptake (average fluorescence intensity per cell) happened in all three cell lines (Supplemental Figure D). Further assays were designed and performed based on these results.

**HPV16 E6/E7 down-regulated the expression of miR-451 but up-regulated the expression of CAB39, PI3K (P85α), P-AKT (ser473), and GLUT1**

The pEGFP-N1-E6 or E7 vectors were transiently transfected into the low expression H460 cell line. Overexpression of E6 or E7 significantly down-regulated the expression of miR-451, but up-regulated the expression of CAB39, PI3K (P85α), and P-AKT (ser473) at protein levels only, as well as up-regulated the expression of GLUT1 at both protein and mRNA levels. The expression of AKT showed minimal or no change. The results are presented in Figure 1A. On the other hand, the inhibition of both E6 and E7 received the opposite results in A549 and LK2 cells, and they are presented in Figure 1B and C.

**miR-451 down-regulated the expression of CAB39, PI3K (P85α), P-AKT (ser473), and GLUT1**

The Hsa-miR-451 was transiently transfected into the low expression H460 and A549 cell lines. The overexpression of miR-451 significantly down-regulated the expression of CAB39 and GLUT1 at both protein and mRNA levels, and the expression of PI3K (P85α) and P-AKT (ser473) at the protein level only. The expression of AKT showed minimal or no change. The results were presented in Figure 2A and B. The converse results were achieved when we inhibited the expression of miR-451 in the high expression LK2 cells and the results were presented in Figure 2C.

**CAB39 up-regulated the expression of PI3K (P85α), P-AKT (ser473), and GLUT1**

The pCMV3-C-Myc-CAB39 vector was transiently transfected into the H460 and LK2 cell lines. The results showed that overexpression of CAB39 significantly up-regulated the expression of GLUT1 at both protein and mRNA levels, and the expression of PI3K (P85α) and P-AKT (ser473) at the protein level only whereas the expression of AKT showed minimal or no change (Figure 3A and B). The opposite results of inhibitory effect of CAB39 in A549 and LK2 cells were presented in Figure 3C and D.

**CAB39 promoted the activation of GLUT1 and the promotion was dependent on the PI3K/AKT pathway**

The overexpression of CAB39 significantly promoted both the plasma membrane translocation and the glucose uptake of GLUT1 in the H460 and LK2 cells, the results are presented in Figure 4.

The image analysis data showed that GLUT1 expression could only be detected on the plasma membrane transfected with CAB39, but not on the plasma membrane transfected with empty vectors. Compared with the plasma membrane transfected with empty vector, the percentages of GLUT1 expression on the plasma membrane of transfected CAB39 were significantly higher in H460 (45.5%) and LK2 (67.1%) cells. The results are presented in Figure 4A and B.

The flow cytometry data showed that the glucose mean density was 4.3 times higher in H460 cells transfected with CAB39 than that transfected with empty vectors ($p \leq 0.01$), and 3.0 times higher in LK2 cells transfected with CAB39 than that transfected with empty vectors ($p \leq 0.01$), the results are presented in Figure 4C and the image by confocal microscope analysis are presented in Figure 4D.

To further verify whether the promotion of CAB39 was dependent on the PI3K/AKT pathway, we used a specific PI3K/AKT blocker, Miltefosine, to inhibit the PI3K/AKT pathway
in CAB39 transfected H460 and LK2 cell lines. The results showed that the promotion effects of CAB39 on GLUT1 protein expression, plasma membrane translocation, and glucose uptake were reversed. The results are presented in Figure 5.

Figure 1. HPV16 E6/E7 down-regulated the expression of miR-451 but up-regulated the expression of CAB39, PI3K [P85α], P-AKT [ser473], and GLUT1. Proteins of E6, E7, CAB39, PI3K [P85α], P-AKT [ser473], AKT, and GLUT1 were demonstrated by Western blotting and the mRNAs of E6, E7, miR-451, CAB39, and GLUT1 were demonstrated by qRT-PCR in H460 or A549 or LK2 cells.

Mock, mock transfection or mock siRNA; NS, nonspecific siRNA; Vector, empty vector; qRT-PCR, quantitative real-time reverse transcriptase polymerase chain reaction.

*p < 0.05; **p < 0.01.
Figure 2. miR-451 down-regulated the expression of CAB39, PI3K [P85α], P-AKT [ser473], and GLUT1. Proteins of CAB39, PI3K [P85α], P-AKT [ser473], AKT, and GLUT1 were demonstrated by Western blotting and the mRNAs of miR-451, CAB39, and GLUT1 were demonstrated by qRT-PCR in H460 or A549 or LK2 cells. Mock, mock transfection or mock siRNA; NC, negative control; NS, nonspecific siRNA; Mimics, Hsa-miR-451 mimics; Inhibitor, miR-451 inhibitor; qRT-PCR, quantitative real-time reverse transcriptase polymerase chain reaction. *p < 0.05; **p < 0.01.

Discussion
In our previous studies, we found that HPV16 E6/E7 proteins up-regulated the expression of GLUT1 at protein and mRNA levels, but whether they promoted the membrane localization and the glucose uptake of GLUT1 was...
Figure 3. CAB39 up-regulated the expression of PI3K (P85α), P-AKT (ser473), and GLUT1. Proteins of CAB39, PI3K (P85α), P-AKT (ser473), AKT, and GLUT1 were demonstrated by Western blotting and the mRNAs of CAB39 and GLUT1 were demonstrated by qRT-PCR in H460 or A549 or LK2 cells.

Mock, mock transfection or mock siRNA; NS, nonspecific siRNA; Vector, empty vector; qRT-PCR, quantitative real-time reverse transcriptase polymerase chain reaction.

*p < 0.05; **p < 0.01).

unclear. Both the membrane localization and the glucose uptake of GLUT1 play important roles in the Warburg effect. In this study, we have provided evidence that the overexpression of both E6 and E7 in HPV16 down-regulated the expression level of miR-451 in lung cancer cells.
The image analysis results showed that the expression of GLUT1 on membrane was detected in cells transfected with cab39. Compared with un-transfected cells, the expression rates of GLUT1 in H460 cells (A) and LK2 cells (B) were 45.5% and 67.1%, respectively. Efficiency was measured by Western blotting. (C) The results of flow cytometry showed that the average density of glucose in H460 cells transfected with cab39 increased by 3.3 times ($p < 0.01$), and that in LK2 cells transfected with cab39 increased by 2.0 times ($p < 0.01$). (D) After 30 min of culture with fluorescent glucose analog 2-NBDG, clear images were obtained by confocal microscopy.

Vector, empty vector.

However, because the carcinogenesis mechanism of E6 and E7 proteins is not the same, the regulation pathways of miR-451 by both proteins may not be the same. The detailed differences need to be further studied in the future. The knockdown of miR-451 up-regulated the expression of...
Figure 5. (Continued)
CAB39 at both protein and mRNA levels, subsequently CAB39 up-regulated the expression of GLUT1 at both protein and mRNA levels. The overexpression of CAB39 also promoted the membrane translocation and the glucose uptake of GLUT1. Thus, we demonstrated that miR-451 acted as a safeguard against HPV-stimulated aerobic glycolysis and tumorigenesis by inhibiting its two down-stream effectors CAB39 and GLUT1. Our results provide new molecular mechanisms in HPV16 E6/E7 that promote both the membrane translocation and the glucose uptake of GLUT1 by relieving miR-451 inhibitory effect on CAB39 in lung cancer cells.

The expression level of miR-451 was low in NSCLC tissues by miRNA chips, and the expression levels was related with tumor differentiation, pathologic stage, and lymph node metastasis. The overexpression of miR-451 significantly inhibited both the proliferation and the migration of NSCLC cells in vitro. Our conclusion was consistent with previous in vitro studies.

Based on this evidence, we further explored the regulatory effect of CAB39 on the activation of GLUT1. We found that the transfection of CAB39 prompted GLUT1 translocation to the plasma membrane. To further confirm whether CAB39 promoted GLUT1 plasma membrane translocation was dependent on the PI3K/AKT pathway, we used a specific inhibitor, Miltefosine, to inhibit the PI3K/AKT pathway in the cells transfected with CAB39 and the results showed that the effects of CAB39 on the GLUT1 protein expression, plasma membrane translocation, and glucose uptake were reversed. Our results demonstrate for the first time that the activation of CAB39 was one of the key steps for GLUT1 translocation to the plasma membrane and glucose uptake.

In conclusion, we have demonstrated that HPV16 E6/E7 proteins inhibited the expression of miR-451 in lung cancer cells, the down-regulation of miR-451 further up-regulated the expression of CAB39, and subsequently up-regulated GLUT1. Our results proposed an HPV–miR-451–CAB39–GLUT1 axis for the tumorigenesis of lung cancer. Furthermore, we found CAB39 played a decisive role in GLUT1 translocation to the plasma membrane and glucose uptake. Our findings provide new evidence to support the critical role of CAB39 in the pathogenesis of HPV-related
lungs, and suggest novel therapeutic targets.

**Authors’ contributions**  
Hong-Miao Wang and Guang-Ping Wu contributed to the study design and manuscript writing. Hong-Miao Wang, Ying-Jie Lu, Ling He, and Na-Jin Gu analyzed the data and provided statistical support. Shi-Yu Wang contributed to language editing in this article. All authors made substantial contributions to interpretation of results, were involved in drafting the manuscript and/or revising it critically for important intellectual content, approved the final version for submission, and agree to be accountable for all aspects of the work.

**Conflict of interest statement**  
The authors declare that there is no conflict of interest.

**Ethical approval**  
Ethical approval was obtained for the experimental procedures by the Ethics Committee of the First Hospital of China Medical University (APPROVAL NUMBER/2016-125), Shenyang, China.

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This article does not contain any studies with human or animal subjects.

**Supplemental material**  
Supplemental material for this article is available online.

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