The Correlation of CIP4 Expression with Glioma Cell Migration and Invasion

ความสัมพันธ์ระหว่างการแสดงออกของโปรตีนซิพ 4 กับการแพร่กระจายและการรุกรานเซลล์ข้างเคียงในเซลล์มะเร็งสมองชนิดเซลล์เกลีย

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ABSTRACT

Gliomas are the most common and aggressive brain tumors that derived from glial cells. The causes and risk factors of these tumors are still unknown. The factors that regulate cancer cell migration and invasion are important for controlling behavior of the cancer cells. CIP4 is a protein that has been shown to regulate actin dynamic and membrane remodeling, the processes required for several cellular activities including endocytosis, cell migration and invasion. A recent study showed that CIP4 promotes metastasis in breast cancer and that the high levels of CIP4 correlates with the strongly invasive breast cancer cell line. However, the expression of CIP4 in brain tumor is unknown. Here we determined the correlation between CIP4 expression level and the phenotypes of glioma cells by using two types of glioma cell line (C6 and ASK). This study shows that CIP4 is highly expressed in C6 cells correlated with its high rates of migration and invasion suggesting that CIP4 might play a role in glioma cell migration and invasion.

บทคัดย่อ

เซลล์มะเร็งสมองที่เจริญมาจากเซลล์เกลียเป็นมะเร็งสมองที่พบบ่อยและมีความรุนแรง สาเหตุและปัจจัยเสี่ยงของเนื้องอกเหล่านี้ยังไม่ทราบแน่ชัด ปัจจัยที่ควบคุมการแพร่กระจายและการรุกรานเซลล์ข้างเคียงมีความสำคัญต่อการควบคุมพฤติกรรมของเซลล์มะเร็ง โปรตีนซิพ 4 ทำหน้าที่ควบคุมการเปลี่ยนแปลงการเรียงตัวของโปรตีนแอกทินและลักษณะของเยื่อหุ้มเซลล์ ซึ่งมีความเกี่ยวข้องกับกลไกการทำงานของเซลล์หลายด้าน ได้แก่ การนำสารเข้าสู่เซลล์ การแพร่กระจาย และการรุกรานเซลล์ข้างเคียง ในการศึกษาปัจจุบันพบว่าโปรตีนซิพ 4 กระตุ้นให้เซลล์มะเร็งเต้านมมีการกระจายตัว และการแสดงออกของโปรตีนซิพ 4 ยังสัมพันธ์กับการรุกรานของเซลล์มะเร็งเต้านมอีกด้วย แต่อย่างไรก็ตามการแสดงออกของโปรตีนซิพ 4 ในเซลล์มะเร็งสมองยังไม่มีการศึกษา การศึกษานี้จึงมีวัตถุประสงค์หาความสัมพันธ์ระหว่างการแสดงออกของโปรตีนซิพ 4 ต่อการแพร่กระจายและการรุกรานเซลล์ข้างเคียงของเซลล์มะเร็งสมองชนิดเซลล์เกลีย 2 ชนิด (ซี6 และ เอเอสเค) ผลการศึกษาแสดงให้เห็นว่าในเซลล์มะเร็งสมองชนิด ซี6 มีการแสดงออกของโปรตีนซิพ 4 เป็นจำนวนมาก ซึ่งมีความสัมพันธ์กับอัตราการแพร่กระจายและการรุกรานเซลล์ข้างเคียง แสดงให้เห็นว่าโปรตีนซิพ 4 อาจจะมีบทบาทสำคัญต่อการแพร่กระจายและการรุกรานเซลล์ข้างเคียงในเซลล์มะเร็งสมองชนิดเซลล์เกลีย

Keywords: Cdc42 interacting protein 4 (CIP4), Cell migration, Cell invasion
คำสำคัญ: โปรตีนซิพ4 การแพร่กระจาย การรุกรานเซลล์ข้างเคียง

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Introduction

Gliomas are the most common and aggressive brain tumors. The median survival time of patients with gliomas does not exceed 15 months. Gliomas are groups of brain tumors that derived from glial cells which are the most abundant cells in the brain. Gliomas are mostly found in the anterior cerebral hemisphere. (Louis et al., 2007; Schwartzbaum et al., 2006) The World Health Organization (WHO) defines gliomas by cell type, location and grade. They can be categorized into four classes (Lassman, 2004): i) Grade I tumors, or pilocyticastrocytomas; ii) Grade II tumors, also called low-grade astrocytomas; iii) Grade III tumors, or anaplastic astrocytomas; and iv) Grade IV tumors, also known as glioblastoma multiforme (GBM) (Louis et al., 2007). The characteristics of tumor grade IV are high rate of proliferation, migration and metastasis. Patients with grade IV gliomas demonstrate high severity of disease and rapid cancer progression. Increasing in cell migration and invasion are hallmarks of cancer progression (Hanahan, Weinberg, 2011). Therefore factors that regulate cell migration and invasion are important for controlling the aggressiveness of the cancer cells. Membrane remodeling and actin dynamic are crucial processes required for cell migration and invasion (Najm, El-Sibai, 2014). These two processes require tight regulations from several molecules in the cells.

The Cdc42-interacting protein 4 (CIP4) is a member of the F-BAR proteins family, which are proteins play a role in regulation of several cellular activities including cell invasion, endocytosis, phagocytosis and protrusion. CIP4 is composed of three important domains, F-BAR domain, HR1 domain and SH3 domain. CIP4 protein can interacts with the negative charged membrane phospholipids through its F-BAR domain. Moreover, it's binds to various actin-associated proteins through its SH3 domain (Aspenstrom, 2009). Therefore, CIP4 acts as a linker between membrane and actin filaments and can regulate both membrane and actin dynamics. A previous study showed that CIP4 regulates the formation of invadopodia, a structure that is important for cancer cell invasion (Hu et al., 2011). In addition, a high level of CIP4 has been shown to correlates with the invasiveness of breast cancer cells (Pichot et al., 2010) and promotes metastasis in breast cancer (Cerqueira et al., 2015). However, the level of CIP4 and its roles in migration and invasion in another cancer cell types, especially brain tumor, is unknown.

In this study, we determined the correlation between CIP4 expression level and migration as well as invasion of glioma cells by comparing the expression of CIP4 in two types of glioma cell line and determine whether the level of CIP4 is correlated with the migration and invasion abilities of the cells.

Objective of the study

The aim of this study is to determine the correlation between CIP4 expression and phenotypes of two different glioma cells.

Research Methodology

Cell culture and cell lysate

Two rat glioma cells lines, C6 and ASK cells were used in this study. C6 cells were cultured in DMEM-F12 (Gibco, life technologies) containing 5% of fetal bovine serum (FBS) (Thermo scientific or Hiclone, life sciences) plus 5% of horse serum. ASK cells were cultured in MEM (Gibco, life technologies) with 10% fetal bovine serum. Both cell lines were

plated in 25 cm^2 flasks, until 80-90% confluence. The cells were collected by scrapping and centrifuge at 3,000 RPM at 4°C for 5 minutes. The cell pellet were then lysed by using RIPA buffer and centrifuged at 12,000 RPM at 4°C for 10 minutes. Finally, supernatant were collected and measured for protein concentration by using by BCA (Bicinchoninic acid) method.

Immunoblotting analysis

CIP4 proteins level was measured by using western blot analysis with antibody for CIP4. Beta-actin was measured as a loading control. Twenty microgram of protein samples were mixed with 5X sample buffer, heated at 95°C and loaded into the gel. The proteins were then transferred to the PVDF membrane. The membranes were incubated with 5% nonfat dry milk (Bio-Rad Laboratories; Hercules, CA, USA) in TBST for 1 hour at room temperature and incubated with primary antibodies against beta-actin, and CIP4 with 5% BSA in TBST at 4°C overnight. Membranes were incubated with secondary antibodies (HRP goat anti-mouse) in 5% nonfat dry milk in TBST for 1 hour at room temperature. Last, signal was developed with Luminata crescendo Western HRP substrate (Milliphore Billerica, MA, USA) and detected with hyperfilm (Amersham; Little Chalfont, Buckinghamshire, UK). The intensity of the bands was analyzed by ImageJ program.

Migration and Invasion assays

Cell migration was performed by using wound healing assay. Both cells were plated at the density of 200,000 cells/well. After 24 hours, the cells were scratched with sterile pipette tip to generate wound. The cells were imaged at 0 and 12 hours after the scratching using inverted microscope (10x objective lens) (Dutta et al., 2015). The distances of the cell migration were measured using ImageJ program.

Cell invasion assay was performed in transwell plates (8-µm pore size, 6.5-mm diameter; Corning Life Sciences, Lowell, MA). This transwells were pre-coated with Matrix gel Basement Membrane Matrix (1 mg/ml; BD Biosciences, Franklin Lakes, NJ) for 12 hours. The 50,000 cells of both C6 and ASK cells were plated on the upper chamber system and cultured with serum-free media. Bottom wells in the system were filled with 750 µl of complete MEM or DMEM-F12 media. Cells were allowed to invade for 12 hours thought Matrix gel. After 12 hours, media in the upper chamber were removed. Cells were fixed with 25% Methanol for 20 minutes. Then, cells in the upper side of chamber were scrubbed. Cells that had invaded through Matrigel matrix membrane were stained with crystal violet for overnight. The chambers were washed with water for 3-5 times and let dried for 1 hour. The number of invaded cells were imaged under inverted microscope (10x objective lens) and counted in ImageJ program (Dutta et al., 2015).

Results

1. CIP4 expression level in C6 cells is higher than in ASK cells. To determine the expression of CIP4 in both two glioma cell lines, the level of CIP4 in lysate of both cell lines were measured by using western blotting analysis with CIP4 and actin antibodies. The results demonstrate that the expression level of CIP4 protein in C6 cells was significantly higher than that in ASK cells (Fig1. A-B).

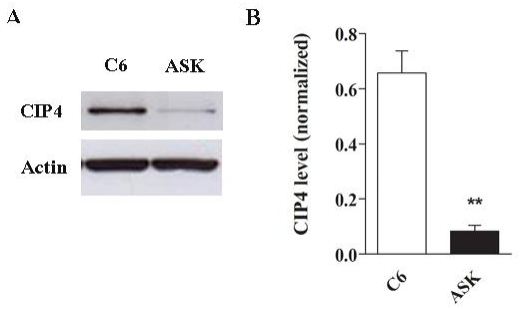

Figure 1 The expression level of CIP4 in C6 is higher than ASK. Western blot analysis (A) and bar graph (B) of CIP4 expression level in C6 and ASK cells. The expression levels of CIP4 in C6 was significant higher than that in ASK cells. The CIP4 levels shown in the graph were normalized to actin. **p<0.005 (Student t-test).

2. The migration rate of C6 cells is higher than ASK cells. To determine the migration rate of both two glioma cells wound healing assay was used. Cells were scratched after 12 hours of plating. The cells were imaged at 0 and 12 hours after scratching. The distances that the cell migrated were measured. The results showed that after 12 hours the distance that the C6 cells migrated was significantly longer than that that for the ASK cells (Fig2. A-B) suggesting that the migration rate of the C6 cells is higher than the ASK cells.

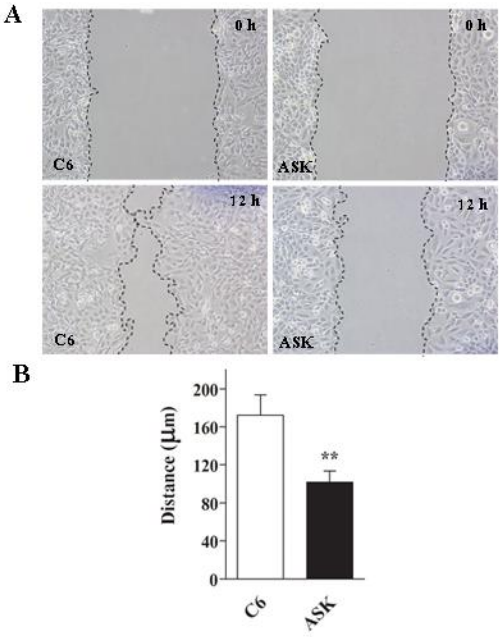

Figure 2 Migration rate of the C6 cells is higher than that of the ASK cells. C6 and ASK cells were seeded in 12 wells plate, wounded and allowed to recover for 12 hours. The black dash lines demonstrate the border of the wound. Images are representative of multiple independent experiments. The average distance that the cell migrated was quantified at t_0 and t_{12} h after wounding. **p<0.01 (Student t-test).

3. The invasion rate of C6 cells is higher than that of ASK cells. To determine the invasion rate of both glioma cells, the invasion assay was performed. The numbers of cells that can invade thought the Matrix gel coated on the upper chamber were determined. C6 and ASK cells were plated onto the upper chamber of the transwell. Cells were then allowed to invade for 12 hours thought Matrix gel. After 12 hours, cells were fixed, the upper chamber were scrubbed to remove non-invaded cells remained in the upper chamber. The chambers were then stained with crystal violet overnight to label the cells

that were invaded. The results showed that the percentage of C6 cell invasion was significantly higher than that of ASK cells (Fig3. A-B). This result suggests that the invasion rate of the C6 cells is higher than that of the ASK cells, indicating that C6 cells are more invasive than ASK cells.

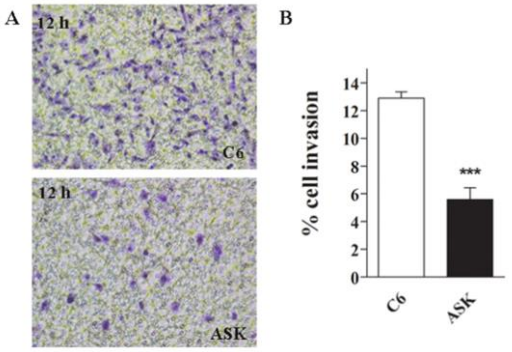

Figure 3 Invasion rate of C6 cells is higher than that of ASK cells. Images of the C6 and ASK cells that were invaded through the Matrix gel after 12 hours of plating. Graph showing the percentage of cell invasion calculated from the total number of cells that were invaded divide by the total number of the cells that were plated. ***p<0.001 (Student t-test).

Discussion and Conclusion

We demonstrate here that CIP4 protein is expressed in both glioma cells. However, the expression level of CIP4 is significantly higher in C6 cells as compared to ASK cells. Interestingly, the migration and invasion rates of C6 cells were also higher than ASK cells. These phenotypes are correlated with the pattern of CIP4 expression. These results suggest that CIP4 might play a role in cell migration and invasion of glioma cells. The high expression of CIP4 protein might be related to the aggressiveness of the glioma. These results are similar with the previous study showing that the expression of CIP4 was highly expressed in more aggressive breast cancer cells (Pichot et al., 2010) and that CIP4 promotes metastasis in breast cancer (Cerqueira et al., 2015). The regulation of cancer cell motility such as cell migration and invasion require tight regulations of actin dynamics and membrane remodeling. Several studies reported that CIP4 can regulate both membrane remodeling and actin dynamics (Saengsawang et al., 2012; Saengsawang et al., 2013). Thus, CIP4 might also play important roles in glioma cell migration and invasion through modulation of these processes.

In summary, this study is the first to show the positive correlation between CIP4 protein expression and the ability of the glioma cells to migrate and invade. Further studies are required to verify that the CIP4 is sufficient and necessary to promote glioma cell migration and invasion by overexpression and knockdown of CIP4 protein, respectively.

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